Protection against UVB deleterious skin effects in a mouse model: effect of a topical emulsion containing *Cordia verbenacea* extract

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Abstract

Cordia verbenacea DC (Boraginaceae) is a flowering shrub found along the Brazilian Atlantic Forest, Brazilian coast, and low areas of the Amazon. The crude extract of its leaves is widely used in Brazilian folk medicine as an anti-inflammatory, both topically and orally. The aim of this study is to evaluate the activity of *C. verbenacea* ethanolic leaves extract (CVE) against UVB-triggered cutaneous inflammation and oxidative damage in hairless mice. CVE treatment recovered cutaneous antioxidant capacity demonstrated by scavenging ABTS⁺ free radical and iron-reducing antioxidant potential evaluated by FRAP. CVE also controlled the following UV-triggered events in the skin: reduced glutathione (GSH) depletion, catalase activity decrease, and superoxide anion (O^{•−}) build-up. Furthermore, mice treated with CVE exhibited less inflammation, shown by the reduction in COX-2 expression, TNF- α , IL-1 β , IL-6, edema, and neutrophil infiltration. CVE also regulated epidermal thickening and *sunburn cells*, reduced dermal mast cells, and preserved collagen integrity. The best results were obtained using 5% CVE-added emulsion. The present data demonstrate that topical administration of CVE presents photochemoprotective activity in a mouse model of UVB inflammation and oxidative stress. Because of the intricate network linking inflammation, oxidative stress, and skin cancer, these results also indicate the importance of further studies elucidating a possible role of *C. verbenacea* in the prevention of UVB-induced skin cancer and evaluating a potential synergy between CVE and sunscreens in topical products against UVB damaging effects to the skin.

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Graphic abstract

Effect of topical treatment with *Cordia verbenacea* extract-enriched emulsion against UVB damage in hairless mice

Keywords COX-2 · GSH · Inflammation · Oxidative stress · Radical scavenger · Sunburn cells

1 Introduction

Brazil is number one among the world's seventeen megadiverse countries [1]. Brazilian flora hosts 45,000 higher plant species (20 to 25% of all world species), many of which with medical and pharmaceutical applications, encompassing seven biomes, including the Amazon and the Atlantic Forest. This immense biodiversity associated with a plural cultural heritage translates into a very rich and rooted plant-based medicine [2].

Cordia verbenacea (Boraginaceae) is a flowering shrub, popularly known as “Erva baleeira,” distributed along the Brazilian Atlantic Forest, Brazilian coast, and low areas of the Amazon. The plant's crude extract, obtained from the aerial parts, has been widely recognized in folk medicine as an anti-inflammatory, both topical and oral. The most popular extractive forms are hydroethanolic extracts (tinctures), decocts, and infuses. Pharmacological studies have demonstrated anti-inflammatory, antioxidant, anti-ulcerogenic, analgesic, and antimicrobial activities [3–6].

Inflammation is the primary acute cutaneous reaction to intense UVB exposure. High energetic UVB radiation

incites keratinocytes to produce mediators, such as prostaglandins (PGE2) and cytokines, causing vasodilation, vase permeation, and infiltration of immune cells [7, 8]. UVB exposure associated with an oxygen-rich environment and inflammatory activity stimulates excessive ROS production, which overpowers the tissue's antioxidant capacity leading to a condition known as oxidative stress. When lipid peroxidation is engaged, a chain that involves the production of several lipid peroxides leads to cell membrane destruction and intensification of inflammation [9]. ROS are also recognized as triggers for cutaneous autoimmune diseases, phototoxicity, photosensitivity, skin aging, and cancer [10]. Furthermore, multiple pieces of evidence suggest that UVB radiation is responsible for initiating and promoting the most frequently diagnosed cutaneous malignancy, non-melanoma skin cancer (NMSC) [11]. Therefore, approaches aimed at counteracting UVB-induced inflammation and ROS production may also help prevent NMSC.

Plant-derived extracts that are rich in antioxidants and anti-inflammatories offer a broad photochemoprotective activity. They carry molecules that can protect the cell against direct UVB/ROS-mediated damage and regulate

signaling pathways involved in inflammation and oxidative stress [12]. Polyphenols and terpenes are the most common phytochemicals found in the crude ethanolic extract from *C. verbenacea* leaves. Some of these molecules are already recognized for their antioxidant, anti-inflammatory, and anti-tumoral properties [13]. If used in synergy with sunscreens, these plant materials may improve the sun-protective performance by adding intrinsic anti-inflammatory and radical-scavenging activities, which cannot be expected from the typical commercial sunscreens alone [14, 15].

Although *C. verbenacea* anti-inflammatory activity and in vitro antioxidant capacity have been well reported, the effect of a topical formulation containing the ethanolic extract obtained from *C. verbenacea* leaves (CVE) against UVB-induced inflammation and oxidative stress has not been demonstrated yet. With this aim, CVE was mixed to a suitable ointment base and applied topically in hairless mice exposed to acute UVB radiation. The skin collected from these mice was then tested for inflammatory activity and oxidative imbalance. The results were compared to UVB exposed and non-exposed control groups.

2 Materials and methods

2.1 Chemicals

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS+); 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), bisacrylamide, hexadecyl trimethyl ammonium bromide (HTAB), brilliant blue stain, phenanthroline, phenylmethylsulfonyl fluoride, reduced glutathione (GSH), nitroblue tetrazolium (NBT), *o*-dianisidine dihydrochloride, and Trolox were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tris from Amresco (Solon, OH, USA). Enzyme-Linked Immunosorbent Assay (ELISA) kits from eBioscience (San Diego, CA, USA). Acrylamide sodium dodecyl sulfate (SDS), SuperscriptIII, Oligo(dT)12–18 primers, Platinum SYBRGreen 1, and primers from Invitrogen (Carlsbad, CA, USA). Galena (Campinas, São Paulo, Brazil) provided the excipients used

to prepare the emulsion. All the remaining reagents were of pharmaceutical grade.

2.2 Plant material and extract

Cordia verbenacea dried leaves and stems were donated by the company Santosflora®, lot number 1507080477. The plant's name was checked with The Plant List (TPL) database (<http://www.theplantlist.org>), and the website was accessed on November 18, 2020.

The material was identified comparing to data from the Brazilian Pharmacopeia 4th and 5th editions [16]. The results were presented in the analysis certificate number 430/15 (Table 1).

The 70°GL ethanolic extract was prepared according to Matos et al. [17], and the extract's dry weight was evaluated by gravimetry according to Brazilian Pharmacopeia 5th edition [16].

2.3 Topical emulsion preparation

Phase A (oil phase) and phase B (water phase) (Table 2) were heated separately at 70 °C. After the fusion of the solids, phase B was poured over phase A, and the two phases were mixed under constant agitation until the temperature reached 45 °C. Phase C (preservative) and phase D (humectant) were then added and mixed (Table 2). CVE (dry weight = 633.6 mg/g) was incorporated to the emulsion at

Table 2 Ingredients of the emulsion containing *C. verbenacea*

Raw material	%
(A) Polawax® ^a (self-emulsifying wax)	10
Caprylic/capric triglyceride (emollient)	5
(B) Purified water to complete	100
(C) Phenonip® ^b (preservative)	0.4
(D) Propylene glycol (humectant and solvent)	6

^aPolawax®: nonionic self-emulsifying wax (cetoestearyl alcohol + sorbitan monostearate – 20 OE)

^bPhenonip®: mixture of phenoxyethanol and parabens

Table 1 *C. verbenacea* plant material quality certificate 430/15

Analysis	Specifications	Results	Reference
Identification	Leaves and stems	Passed	Brazilian Pharmacopeia 5th edition, V.I
Color	Green	Passed	Brazilian Pharmacopeia 5th edition, V.I
Organoleptic identification (odor and taste)	Characteristic	Passed	Brazilian Pharmacopeia 4th edition, V.I
Moisture content (%/weight)	≤ 20%	13.21%	Brazilian Pharmacopeia 5th edition, V.I
Total ash (%/weight)	≤ 17%	8.69%	Brazilian Pharmacopeia 5th edition, V.I
Foreign matter (%/weight)	≤ 5%	Not found	Brazilian Pharmacopeia 5th edition, V.I
Flavonoid content (%/weight)	≥ 0.1%	0.1%	Brazilian Pharmacopeia 5th edition, V.II

room temperature (RT) to reach the final concentration of 1 or 5% (w/w). An emulsion control with the same formulation but without CVE was also produced.

2.4 Evaluation of in vivo efficacy of CVE added formulation

2.4.1 Animals

The study was conducted using female, 20–30 g hairless mice (HRS/J), randomly distributed into five groups of 5 animals each, labeled as follows:

- Naive: not exposed to UVB radiation or treated with CVE-added emulsion;
- UVB stimulation: not treated with CVE-added emulsion but exposed to UVB radiation;
- Vehicle: treated only with the emulsion without CVE and exposed to UVB radiation;
- CVE 1%: treated with 1% CVE-added emulsion and exposed to UVB radiation;
- CVE 5%: treated with 5% CVE-added emulsion and exposed to UVB radiation.

The institution commission on animal welfare, named “Animal Ethics Committee of the Londrina State University” (CEUA-UEL), approved this project under document n° 030/201/ process n° 1818.2015.2400.

2.4.2 Experimental protocol

2.4.2.1 Treatments and radiation Treated groups received 0.5 g of emulsion (vehicle only or CVE-added emulsion), applied topically with a brush, every 6 h, starting 12 h before the radiation session and ending right after its end, adding to a total of 4 treatments, three before UVB stimulation and one after. All groups were radiated simultaneously, receiving 4.14 J/cm² UVB. This dose was selected based on a dose–response curve performed during standardization experiments in which the timepoints of all parameters were determined. The source of UVB radiation was a Philips TL/12 RS 40 W (Medical-Holland) lamp with peak emission at 313 nm and radiation of 0.209 mW/cm², which was set 20 cm from the mice dorsal area during the irradiation session. A radiometer was used to check the radiation intensity [18, 19].

Two concentrations of CVE in the emulsion (1 and 5%) were evaluated, and the following assays were conducted to achieve a dose-dependent result: edema and MPO activity, FRAP, ABTS radical scavenging capacity, and GSH levels. For all the remaining assays, treatments were conducted with the best performing dose.

2.4.2.2 Euthanasia procedures and sample collection

- Mice were terminally anesthetized with isoflurane 5% 12 h after radiation session to test edema; GSH level; FRAP capacity; ABTS scavenging capacity; neutrophil infiltration (as per myeloperoxidase [MPO] activity); metalloproteinase-9 (MMP-9) activity; and histology analysis for epidermal thickness, presence of *sunburn* keratinocytes, dermal mast cells infiltration, and collagen degradation.
- Mice were anesthetized with isoflurane 5% and decapitated 4 h after the radiation session to determine cutaneous COX-2 expression and cytokines levels.
- Finally, the animals were anesthetized with isoflurane 5% and decapitated 2 h after the radiation session to determine catalase activity and O₂^{•−} level.

For edema determination, standard size skin pieces were weighed immediately after the dissection. Samples for histology were kept in formol 10% at RT until analysis, and samples for the remaining tests were kept until analysis (− 70 °C).

2.4.3 Skin edema.

Edema was assessed by the increased weight of mice dorsal skin (expressed in mg) that underwent UVB exposure. Standard-sized skin circles from each group were cut and weighed. The CVE-added emulsion treated group results were compared to naive, vehicle, and UVB-stimulation [20–22].

2.4.4 Myeloperoxidase (MPO) and neutrophil infiltration

Neutrophil infiltration was determined colorimetrically by MPO activity, which is abundant in neutrophils and promotes the oxidation of *o*-dianisidine to a colored compound. The colorimetric assay was performed according to Casagrande et al. [24]. The skin pieces were crushed and homogenized in 50 mM phosphate buffer (pH 6.0) with 0.5% hexadecyltrimethylammonium bromide (HTAB). The results were reported as number of neutrophils/mg of skin [23, 24].

2.4.5 Cytokine levels

IL-1β, IL-6, and TNF-α levels were evaluated by sandwich Enzyme-Linked Immunosorbent Assay (ELISA), using commercial kits (eBioscience), and following the instructions from the manufacturer. Skin samples were crushed, homogenized in saline solution, and centrifuged (2000g, 15 min., 4 °C), and the tests were conducted using the supernatants after centrifugation. Readings were taken in a spectrophotometric microplate reader at 490 nm. The results

were calculated using standard curves for each cytokine and reported in picograms (pg)/mg of skin [25].

2.4.6 Metalloproteinase-9 (MMP-9) activity

MMP-9 proteolytic activity was assessed by zymography in polyacrylamide gel with sodium dodecyl sulfate (SDS) and gelatin. The assay is based on the degree of gelatin degradation by MMP-9, which is detected by discoloration of the enzyme's band against the Coomassie Blue dark-stained gel [26]. Proteolytic activity was quantitatively analyzed by comparing data from naive, UVB, vehicle, and CVE-added emulsion groups using ImageJ® software (NIH, Bethesda, MD, USA) [24].

2.4.7 Ferric Reduction Antioxidant Power (FRAP) assay

Skin samples were collected, crushed, and homogenized in a 1.15% KCl solution. After centrifugation (1000g, 4 °C, 10 min.), a supernatant aliquot was combined with FRAP reagent, and the readings were achieved at 595 nm. The results were calculated using a Trolox standard curve (0.01–20 nmol) and reported as nmol Trolox equivalent per mg of skin [22].

2.4.8 ABTS scavenging capacity assay

Radical ABTS^{•+} scavenging activity assay was performed based on the measure of discoloration of the ABTS solution [27] by supernatant aliquots obtained from skin samples collected, crushed, homogenized in a 1.15% KCl solution, and centrifuged (1000g, 4 °C, 10 min.). The readings were taken at 730 nm and calculated using a Trolox standard curve (0.01–20 nmol). The results were reported as nmol Trolox equivalent per mg of skin [28].

2.4.9 Catalase assay

Catalase activity assay uses UV spectrophotometry (240 nm) for monitoring the reduction in the substrate (H₂O₂) along with the rise of oxygen, which is perceived by a decay in UV absorbance [29]. Skin samples were collected, crushed, and homogenized in buffer Tris–HCl 1 M with EDTA 5 mM (pH 8.0). The enzyme activity was assessed by measuring the variation between the initial reading (time 0) and 30 s after the reaction started. Catalase activity was reported as units of enzyme/mg of skin/minute [30].

2.4.10 GSH assay

GSH assay is based on the colorimetric detection of the yellow compound 5-mercapto-2-nitrobenzoic acid derived from 5',5'-dithiobis-(2-nitrobenzoic acid) (DTNB) breakage

of disulfide bond by the glutathione's sulfhydryl group. Skin samples were collected, crushed, and homogenized in buffer Tris–HCl 1 M with EDTA 5 mM (pH 8.0). GSH levels were determined as formerly described [22]. Results were analyzed using a calibration curve (standard GSH 5–150 μM) and reported as μM GSH/mg of skin.

2.4.11 Superoxide anion (O₂^{•−}) production

The skin pieces were collected, crushed, homogenized in 0.02 M EDTA and centrifuged (2000g, 4 °C, 20 s.). A supernatant aliquot was incubated with NBT. O₂^{•−} was detected by the reduction of NBT to a purple-colored compound known as formazan, which causes a rise of the optical density (OD) at 620 nm. O₂^{•−} production was reported as OD/10 mg of skin [25].

2.4.12 COX-2 expression

COX-2 relative mRNA expression was determined using the comparative 2-(ΔΔC_q) method by reverse transcriptase (RT) and quantitative polymerase chain reaction (qPCR) using GoTaq12-Step RT-qPCR System (Promega) on a Step One Plus TM Real-Time PCR System (Applied Biosystems1). The expression of β-actin mRNA was performed to control tissue integrity in all samples [31]. The primers used were:

β-actin, sense 5'-CGGTTCCGATGCCCTGAGGCT CTT-3', antisense 5'-CGTCACACTTCATGATGG AATTGA-3';
COX-2, sense: 5' AACCGCATTGCCTCTGAAT 3', antisense: 5' CATGTTCCAGGAGGATGGAG 3'

2.4.13 Skin histologic evaluation

Skin samples were collected in formaldehyde 10% solution and prepared following standard histological protocols (fixed in paraformaldehyde 4%, dehydrated in ascending ethanol concentrations, cleared in xylene, embedded in paraffin), and were cut into 5 μm thickness pieces.

The specimens were stained with Hematoxylin and Eosin stain (H&E) to determine epidermal thickness [32] and number of sunburn cells [33]. To evaluate the number of mast cells in the dermis, the specimens were stained with Toluidine Blue. Cell counting was performed using the software Infinity Analyze (Lumenera R Software). The specimens were stained with Masson's Trichrome to evaluate collagen fibers density. The blue color intensity in the dermal area of the skin was proportional to collagen density. The comparison between groups was performed with the aid of Image J software (NIH) [34].

2.4.14 Statistical analysis

Statistical analysis was conducted using GraphPad Prism 7 software (GraphPad Software Inc., San Diego, USA). Data were analyzed by One-way analysis of variance (ANOVA) followed by Tukey multiple comparisons test considering $p < 0.05$. All assays were conducted twice with five animals per group in every assay. Results are mean $\pm$ standard error (SEM).

3 Results

3.1 Preliminary anti-inflammatory assessment: edema and MPO activity

Edema and neutrophil infiltration are primary signs of the acute inflammatory state promoted by UVB exposure. Edema was assessed by the increase in skin weight and neutrophil infiltration by myeloperoxidase activity. Samples from UVB-stimulation mice weighed significantly more than the naive, but treatment with 5% CVE-added emulsion prevented this effect and maintained skin weight at basal levels. Animals treated only with the vehicle and with 1% CVE-added emulsion did not present the same protective effect (Fig. 1A). Similar results were found for neutrophil infiltration. MPO activity was significantly increased in the UVB-stimulation group compared with the naive group. Treatment with the vehicle and with 1% CVE-added emulsion did not control neutrophil infiltration, but treatment with 5% CVE-added emulsion maintained MPO at basal levels (Fig. 1B).

3.2 COX expression and cytokine levels

The results from edema and MPO assays indicate that 5% CVE is the concentration of choice to deeper investigate

CVE's anti-inflammatory mechanism by assessing the impact of the treatment on UVB-induced COX expression and overproduction of cytokines. UVB stimulates keratinocytes to produce prostaglandin E_2 (PGE_2) by promoting lipid peroxidation and phospholipase A_2 (PLA_2) expression, causing an increase in arachidonic acid and, consequently, cyclooxygenase-2 (COX-2) induction [35, 36]. Results from the qPCR assay demonstrated that UVB exposure considerably raised COX-2 expression, while treatment with 5% CVE-added emulsion kept COX-2 expression close to the basal level (Fig. 2A). Topical treatment with 5% CVE-added emulsion also inhibited UVB-triggered overproduction of $TNF-\alpha$, $IL-1\beta$, and $IL-6$ by keratinocytes and inflammatory cells (Fig. 2B–D).

3.3 Dermal mast cell infiltration

Although mast cells are found regularly in the dermis, the microscopic examination of toluidine-stained skin samples demonstrated that UVB increased the number of non-degranulated and degranulated mast cells in the dermis of UVB-stimulation mice. Topical treatment with 5% CVE avoided the rise in mast cell count, keeping it equal to the naive group and substantially different from the UVB-stimulation group. Once again, vehicle-treated mice did not present any significant decrease in mast cells compared to UVB-stimulation mice (Fig. 3A, B).

3.4 Preliminary testing for CVE antioxidant capacity: ferric reduction antioxidant potential (FRAP), $ABTS^{+}$ scavenging capacity, and GSH level

UVB exposure triggers intense production of ROS with depletion of the antioxidant activity in the skin. FRAP and $ABTS^{+}$ cutaneous scavenging capacity decreased by the

Fig. 1 Effect of 1 and 5% CVE-added-emulsion treatments on edema and neutrophil infiltration in skin exposed to 4.14 J/cm² UVB radiation. Samples were collected 12 h after the end of the UVB exposure for edema (A) and neutrophil infiltration (expressed by the activity of MPO) (B). Bars represent means $\pm$ SEM of two experiments ($n=5$). One-way ANOVA analysis and Tukey's *posthoc* test (Prism software) with * $p < 0.05$ versus naive (green bars); # $p < 0.05$ versus UVB-stimulation controls (red bars)

Fig. 2 Effect of 5% CVE-added emulsion on mRNA expression of COX-2 assessed by quantitative polymerase chain reaction (qPCR) and cytokines by ELISA in skin exposed to 4.14 J/cm² UVB radiation. Samples were collected 4 h after the end of the UVB exposure for mRNA expression of COX-2 (A) and levels of cytokines: TNF α (B), IL-1 β (C), and IL-6 (D). Bars represent means $\pm$ SEM of two experiments ($n=5$). One-way ANOVA analysis and Tukey's as *posthoc* test (Prism software) with * $p < 0.05$ versus naive (green bars); # $p < 0.05$ versus UVB-stimulation controls (red bars)

UVB exposure (Fig. 4A, B). The radiation also induced an acute decrease in levels of the natural antioxidant GSH (Fig. 4C). Topical treatment with 5% CVE-added emulsion improves skin antioxidant capacity of irradiated mice by increasing FRAP, ABTS⁺ scavenging capacity, and GSH levels (Fig. 4A–C). Treatment with the vehicle only and with the emulsion added of 1% CVE did not show any significant effect.

3.5 Superoxide anion production and catalase activity

The results from FRAP, ABTS, and GSH indicate that 5% CVE was the dose of choice to further evaluate CVE's

antioxidant capacity by catalase activity and superoxide anion inhibition ($O_2^{\cdot-}$) tests. UVB-stimulation group presented much higher levels of $O_2^{\cdot-}$, in comparison to the naive group (Fig. 5A). In contrast, the UVB-stimulation group demonstrated a significant decrease in catalase activity in comparison to the naive group (Fig. 5B). In both cases, the treatment with 5% CVE-added emulsion demonstrated a protective effect, since samples from the CVE group showed levels of $O_2^{\cdot-}$ significantly lower than the UVB-stimulation group with catalase activity analogous to the naive group. The results from mice treated only with vehicle were not significantly different from the UVB-stimulation group both in $O_2^{\cdot-}$ levels and catalase activity (Fig. 5A, B).

Fig. 3 Effect of 5% CVE-added emulsion in the number of mast cells by 4.14 J/cm² UVB radiation in the dermis. Skin samples were collected 12 h after the end of the UVB exposure. Specimens were stained with blue toluidine. Number of mast cells (A) and images of blue toluidine-stained mast cells in all four groups (B). Bars represent

means $\pm$ SEM of two experiments ($n=5$). One-way ANOVA analysis and Tukey's as *posthoc* test (Prism software) with * $p<0.05$ versus naive (green bar); # $p<0.05$ versus UVB-stimulation controls (red bars)

Fig. 4 Effect of CVE-added emulsion on the antioxidant capacity of the skin exposed to 4.14 J/cm² UVB radiation. For the determination of total antioxidant capacity [FRAP (A), ABTS (B)], and GSH levels (C), samples were collected 12 h after the end of the UVB exposure.

Bars represent means $\pm$ SEM of two experiments ($n=5$). One-way ANOVA analysis and Tukey's as *posthoc* test (Prism software) with * $p<0.05$ versus naive (green bars); # $p<0.05$ versus UVB-stimulation controls (red bars)

Fig. 5 Effect of CVE-added emulsion on the antioxidant capacity of the skin exposed to 4.14 J/cm² UVB radiation. For O₂⁻ level (A) and catalase activity (B), samples were collected at 2 h after the end of the UVB exposure. Bars represent means $\pm$ SEM of two experiments ($n=5$). One-way ANOVA analysis and Tukey's as posthoc test (Prism software) with * $p<0.05$ versus naive (greens bars); # $p<0.05$ versus UVB stimulation controls (red bars)

3.6 Histological evaluation: epidermal thickness, sunburn cells, and collagen degradation

Epidermal thickening and apoptotic keratinocytes (*sunburn cells*) are typical features of the UVB-stimulated epidermis. Microscopic assessment of H&E-stained skin samples shows that the UVB-stimulation group presented thicker epidermis and higher number of *sunburn cells* than the naive group (Fig. 6A, B). Treatment with 5% CVE significantly lowered the number of sunburn cells and maintained the epidermal thickness at basal levels (Fig. 6A, B).

Intense UVB exposure leads to acute overexpression of MMP-9 in the dermis with consequent collagen degradation. Results from Masson's Trichrome stained samples showed high MMP-9 activity (Fig. 7A, B) along with significant collagen degradation (Fig. 7C, D) in skin collected from the UVB-stimulation group. Samples collected from the group treated with 5% CVE present much lower MMP-9 activity (Fig. 7A, B), with less collagen degradation (Fig. 7C, D), resulting in a protective effect that maintained the CVE 5% group similar to the naive group.

Data from this study shows that the topical treatment with CVE-added emulsion was efficient in reducing the expression of COX-2 and the excessive production of cytokines (TNF- α , IL-1 β , and IL-6), consequently reducing the accumulation of inflammatory cells and edema. The treatment also controlled epidermal thickness, the appearance of epidermal apoptotic (sunburn) cells, and dermal collagen degradation. Topical treatment with CVE added emulsion could also recover the skin's antioxidant potential, inhibit UVB-induced depletion of reduced glutathione (GSH), recover UVB-induced decrease of catalase activity, and prevent the formation of superoxide anion.

4 Discussion

Cutaneous inflammatory response to acute UVB overexposure has been well documented and comprises erythema, heat, edema, pain, and pruritus, followed by epidermal thickening [37, 38]. UVB exposure also induces an overwhelming ROS production, which causes a depletion of innate antioxidant defenses leading to oxidative stress. Together, inflammation and oxidative stress are considered triggers for many skin diseases and may be regarded as crucial inducers of carcinogenesis in UVB-harmed skin [39].

Prior works studying the phytochemical composition of the leaves and stems of *Cordia verbenacea* have already demonstrated the presence of phenolic acids, flavonoids, monoterpenes, and sesquiterpenes [3, 4, 6, 40–49] (Table 3). Ethanolic extract of the leaves has shown flavonoids, mucilages, tannins, cordialins, and essential oils [4, 5]. Rosmarinic acid was the main phenolic acid identified in ethanolic extracts [17, 50], but other phenolic acids, such as caffeic, gallic, and chlorogenic acid, were also found in methanolic extracts [6]. Flavonoids, such as artemitin, hydroxy-artemitin, quercetin, and rutin, were also present in ethanolic and methanolic extracts of the plant's aerial parts [3, 4, 6, 45, 47].

Phenolic compounds, including flavonoids and phenolic acids, have demonstrated their therapeutic potential as anti-inflammatory, antioxidant, and anti-tumorigenic substances [51, 52]. The monoterpene α -pinene and the sesquiterpenes α -humulene and trans-caryophyllene were the terpenes found in higher concentrations in *C. verbenacea*'s essential oil [42]. These sesquiterpenes were tested and considered effective in inhibiting inflammatory mediators [49, 53], and α -humulene became an active anti-inflammatory marker for

Fig. 6 Effect of CVE-added emulsion on epidermal thickening and number of apoptotic keratinocytes (*sunburn cells*) in skin exposed to 4.14 J/cm² UVB radiation. Skin samples were collected 12 h after the end of the UVB exposure, and specimens were stained with H&E. Epidermal thickness images (A); apoptotic keratinocytes images (B) of all four test groups; compared epidermal thickness in micrometers per group (C); and compared number of apoptotic keratinocytes per group (D). Bars represent means $\pm$ SEM of two experiments ($n=5$). One-way ANOVA analysis and Tukey's as posthoc test (Prism software) with * $p<0.05$ versus naive (green bars); # $p<0.05$ versus UVB-stimulation controls (red bars)

a topical ointment containing *C. verbenacea* essential oil [2, 5]. The synergistic effect of the compounds present in *C. verbenacea* makes the ethanolic extract of its aerial parts a promising candidate for photochemoprotective agent to be investigated against UVB/ROS-induced damaged to the skin.

Edema and erythema function as primary signs of UVB-promoted damage. Earlier studies demonstrated that UVB promotes COX-2 expression in murine and human keratinocytes [54, 55]. Because COX-2 is a determinant enzyme for prostaglandins production, its expression represents a critical inflammation modulation factor. Vasodilation, venule permeability increase, and neutrophil chemotaxis are consequences of COX-2 derived prostaglandins' rise. Vasodilation in arterioles, capillaries, and venules in the dermis is responsible for erythema and for the increase in vase permeability with leakage of fluids to the surrounding tissues [56]. The increase in prostaglandins is also involved in the promotion and progression phases of UV-induced non-melanoma skin cancers (NMSCs) [54, 57]. The results obtained in this

work demonstrated CVE-added emulsion capacity to control UVB-induced upregulation of COX-2, which can be considered one mechanism through which UVB radiation incites the skin's inflammatory response. Accordingly, results demonstrated that the treatment with 5% CVE-added emulsion could control edema and neutrophil infiltration, although 1% CVE does not show the same effect, demonstrating the dose-dependent efficacy of CVE. A prior study had shown that the topical treatment with crude extract (in ethanol 70%) of *C. verbenacea* leaves could inhibit nystatin-induced paw edema in a dose-dependent fashion [4]. In another study, *Cordia verbenacea's* dichloromethane and ethanolic extracts, from where the flavonoid artemetin could be isolated, reduced the paw edema induced by carrageenan in mice [3]. In addition, a methanolic extract and rosmarinic acid isolated from it reduced the paw edema promoted by *Bothrops jararacussu* venom in mice [43]. UVB, carrageenan, and *Bothrops jararacussu* venom have different initiating mechanisms to promote edema [50], which suggests the importance of the

Fig. 7 Effect of CVE-added emulsion on MMP-9 activity and collagen damage in skin exposed to 4.14 J/cm² UVB. Skin samples were collected 12 h after the end of the UVB exposure. Image of acrylamide–SDS–gelatin zymography gel showing discolored zones that represent MMP-9 activity (A); compared MMP-9 activity per group (B); collagen fiber intensity evaluated as a percentage of staining, compared per group (C) and image of collagen fiber intensity

and bundles, shown in blue, of all four groups (D). Image (D) was analyzed and quantified by Image J Program to generate graph (C). Bars represent means $\pm$ SEM of two experiments ($n=5$). One-way ANOVA analysis and Tukey's *posthoc* test (Prism software) with $*p < 0.05$ versus naive (green bars); $\#p < 0.05$ versus UVB-stimulation controls (red bars)

Table 3 *Cordia verbenacea* phytochemical composition

Classification	Substance	Extraction method	References
Phenolic acids	Rosmarinic acid	Methanolic fraction of the chloroform macerate	[43]
		Crude extract obtained by maceration with ethanol 70% followed by percolation	[17]
	Caffeic acid, gallic acid, chlorogenic acid	Methanolic fraction of extract obtained by maceration with methanol	[6]
Flavonoids	Artemetin, hydroxy-artemetin, quercetin	Crude extract obtained by maceration with ethanol 70%	[3, 4, 45, 47]
	Artemetin	Soxhlet extraction using ethanol	[44]
	Rutin, quercetin	Methanolic fraction of extract obtained by maceration with methanol	[6]
Terpenes	Cordialin A	Extraction with acetone	[41]
	α -Pinene; trans-caryophyllene; allo-aromadendrene; α -humulene; beta-gurjurnene	Essential oil obtained by hydrodistillation	[42]
	α -Pinene, β -pinene, trans-caryophyllene, bicyclogermacrene	Essential oil obtained by hydrodistillation	[40]
	α -Humulene, allo-aromadendrene, trans-caryophyllene	Essential oil obtained by hydrodistillation	[46, 49]
	α -Humulene, allo-aromadendrene, trans-caryophyllene	Supercritical fluid extraction (SFE) with CO ₂ and ethanol	[44]

synergism among the *Cordia verbenacea*'s phytochemical compounds to its anti-inflammatory efficacy. Following this trend, a study conducted with *C. verbenacea* essential oil [46, 58] and other with supercritical fluid extract in human breast cell lines MCF-7 [59], both of which have very complex phytochemical composition, demonstrated the capacity of the plant to control UVB-induced overexpression of COX-2.

Keratinocytes constitute a major UVB target and play an essential role in UVB-induced inflammation, mainly by producing cytokines, including TNF- α , IL-1 β , and IL-6. TNF- α is a multi-functional cytokine that induces IL-1 β , IL-6, chemokines, and adhesion molecules. IL-6 is implicated in leukocyte infiltration and sunburn reaction [60, 61]. Results showed that UVB exposure significantly increased the expression of TNF- α , IL-1 β , and IL-6 in consonance with a previous study that demonstrated a rise of cytokines in human keratinocytes (HaCaT cells) after 400 mJ/cm² UVB irradiation [62]. UVB-induced cytokines released by keratinocytes recruit, activate, and promote the infiltration of leukocytes, such as neutrophils and monocytes into the skin. These leukocytes also release cytokines what causes the engagement of even more inflammatory cells. Furthermore, neutrophils recruited and activated by inflammatory cytokines produce ROS, further aggravating the inflammation and generating oxidative stress [63]. Our results show the inhibition of UVB-induced TNF- α , IL-1 β , and IL-6 in samples from mice treated with CVE-added emulsion. It's been postulated that plant polyphenols, such as the ones present in *C. verbenacea*, can modulate TNF- α and other inflammatory molecules [64, 65]. Furthermore, another assay presented that oral treatment with the sesquiterpenes α -humulene and trans-caryophyllene, isolated from *C. verbenacea*, was effective in controlling TNF- α , IL-1 β release generated by carrageenan paw injection in mice [49].

Because MPO occurs naturally inside neutrophils azurophilic granules, the enzyme's activity is considered a marker for epidermal neutrophil infiltration and a measure of the inflammation's severity. MPO is also responsible for reducing H₂O₂ in the presence of Cl to ClO⁻, a potent cytotoxic agent [66, 67]. In this sense, neutrophils and MPO represent additional ROS sources [38]. Our results show that treatment with CVE-added emulsion kept MPO activity at basal levels, indicating that the treatment can control neutrophil infiltration. The demonstrated ability to control COX-2 and cytokines suggests that CVE-added emulsion presents a wide range of protection by preventing further aggravation of the inflammatory response and oxidative stress, which together create the conditions for increased UVB damage and potential risk of carcinogenesis.

Mast cells are commonly found, in small numbers, in the normal dermis. These cells are known to release several inflammation mediators—such as histamine, interleukins,

and leukotrienes, which have a well-recognized role in UVB-generated edema and erythema [68]. Our results demonstrated that UVB exposure increased the number of dermal mast cells, amplifying the inflammatory and oxidative response [69–71]. Histamine, released from these cells upon degranulation, induces PGE₂, increasing prostanoids release by keratinocytes. Histamine is also known as one of the mediators involved in UVB-induced immunosuppression. It may directly influence the migration of immune cells to UVB radiated sites and regulate their proliferation and cytokine profile [72]. CVE-added emulsion treatment maintained the number of mast cells at a basal level in UVB-stimulated mice, once again demonstrating the extract's broad and effective anti-inflammatory activity. In another study, *C. verbenacea* extract showed its histamine inhibitory activity by reducing histamine secretion from rat and guinea pig mast cells induced by ionophore A23187, concanavalin A, and the mast cell degranulating compound 48/80. The same study showed that the oral treatment inhibited the histamine secretion caused by ionophore A23187 in rats [73]. *C. verbenacea* essential oil and its main compounds already demonstrated anti-histamine effects in different in vivo models of allergy and inflammation [46, 49, 58]. Quercetin and rutin, two flavonoids found in *C. verbenacea*, inhibited IgE or phorbol-12-myristate 13-acetate and calcium ionophore A23187 (PMACI)-mediated histamine release in RBL-2H3 cells and decreased gene expression and production of all the proinflammatory cytokines after PMACI stimulation [74].

Therefore, in what concerns UVB-induced inflammation, our results show that CVE topical treatment restrained inflammatory response by controlling the overexpression of COX-2 from keratinocytes, the activation of neutrophils and mast cells, and the release of cytokines (TNF- α , IL-1 β , and IL-6) from keratinocytes and inflammatory cells. Because a chronic inflammatory microenvironment can lead to immunosuppression, the control of these inflammatory mechanisms suggests that CVE-added emulsion can also prevent immunosuppression, interrupting pathways that could lead to irreparable damage to DNA and carcinogenesis [71, 75].

Reactive oxygen species (ROS) are physiologically produced by immune cells as response activators to aggressions [76]. UVB radiation intensifies the development of these chemical species [77, 78]. Excessive ROS production leads to depletion of the tissue's natural antioxidants, limiting the cells' self-protecting capacity [79–82]. Under normal conditions, cells present a robust system for dealing with oxidative damage. The cell redox balance is maintained both by enzymatic and non-enzymatic natural antioxidant molecules. Examples of natural antioxidant enzymes are catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRx), and superoxide dismutase (SOD). Ascorbic acid, uric acid, reduced glutathione (GSH), glutathione, ubiquinol-10, and α -tocopherol are some non-enzymatic antioxidants [19].

CVE already demonstrated high antioxidant capacity *in vitro* and *in vivo* using per oral administration [50]. A comprehensive set of assays evaluated the antioxidant potential of CVE-added emulsion against UVB oxidative damage. FRAP and ABTS tests were used to determine the effects of CVE-added formulation on the skin's antioxidant capacity after UVB exposure. Both assays demonstrate that the treatment with an emulsion containing 5% CVE could maintain the antioxidant capacity close to basal levels. Since FRAP and ABTS correlate to the activity of scavenging species [27, 83], and *C. verbenacea* is loaded with ROS scavenging molecules, the results could suggest that CVE protective effect was due to the ROS scavenging activity of phenolic acids and flavonoids present in the extract. The scavenging capacity of these molecules is already well-recognized due to their multiple hydrogen donating groups and structural ability to incorporate electrons [84, 85]. Rosmarinic acid shows a particularly robust scavenging activity, even stronger than many flavonoids, due to its double catechol structure and hydrogen donating ability [86].

Among the non-enzymatic antioxidants found in the epidermis, GSH is one of the most relevant [87], not only because of its scavenging properties, but also because its redox state functions as an early and sensitive indicator of the cutaneous redox condition [24, 87, 88]. Studies show that UVB causes depletion of GSH in the skin [22, 24]. The enzyme catalase plays an essential part in the epidermal antioxidant system. It catalyzes the conversion of H_2O_2 to oxygen and water, preventing the propagation of the hydroxyl free radical ($\cdot\text{OH}$), a potent oxidant generated when there is an excess of H_2O_2 [89]. Several other studies show that skin exposure to acute UVB doses reduces catalase activity [90–92], a situation that leads to a higher risk for the generation of damage to DNA and macromolecules by hydroxyl radical [93]. The treatment with CVE demonstrated its protective effect by providing exogenous antioxidants, thus reducing the number of free radicals and minimizing the depletion of the skin's natural antioxidants, including GSH and catalase. Phenolics, including the ones present in CVE, can stimulate the induction of enzymes such as catalase and the regeneration of GSH through the induction of the enzyme glutathione reductase [94].

CVE-added emulsion treated group presented superoxide anion ($\text{O}_2^{\cdot-}$) levels significantly lower than the UVB-stimulation group. Metabolic processes and exogenous sources, especially UV radiation, can produce $\text{O}_2^{\cdot-}$, which results from the donation of one electron to oxygen [95]. This radical can promote the formation of several other reactive species generating a chain of reactions that promote oxidative stress. For instance, $\text{O}_2^{\cdot-}$ can directly or indirectly produce hydroxyl radicals implicated in lipid peroxidation [96]. Furthermore, $\text{O}_2^{\cdot-}$ induces cytokines, such as IL-1 β and TNF- α , triggering inflammation and amplifying tissue damage.

Cytokines, in turn, activate phagocytes that will produce more $\text{O}_2^{\cdot-}$. This mechanism contributes to maintaining a feedback cycle of oxidation inductors and inflammation mediators, increasing UVB damage even further [25, 97]. Therefore, CVE's ability to sustain the skin's innate antioxidant defenses, enhance skin's $\text{O}_2^{\cdot-}$ scavenging capacity, control neutrophil infiltration, and regulate cytokine release may explain the lower levels of $\text{O}_2^{\cdot-}$ in samples collected from CVE-added emulsion treated group.

UV-induced ROS are associated with the initiation, promotion, and progression of skin cancer. ROS promotes DNA damage and modifications of the proto-oncogene ras and tumor suppressor gene p53 [98]. Furthermore, the inflammatory and immunological responses generated by ROS have crucial roles in carcinogenesis. For instance, the ROS-related over-expression of COX-2 and consequent rise in PGE₂ are linked to the genesis of many cancer types. COX-2 inhibition has a strong potential for cancer intervention and chemoprevention; in fact, COX-2 selective inhibitors were initially developed as anti-cancer drugs [99]. Because of its connection to the increase of ROS production, especially superoxide anion, COX-2 activity is also implicated in amplifying oxidative stress [100]. An earlier study demonstrated a promising inhibitory activity of COX-2 and PGE₂ production by flavonoids with a catechol group located in B ring [101], which is the case of quercetin, rutin, and artemetin, some of the flavonoids found in *C. verbenacea*. Gallic and caffeic acids, also identified in *C. verbenacea*, were among the compounds that demonstrated over 50% inhibition of COX-2 using Cayman's COX fluorescent inhibitor screening assay [102]. Rosmarinic acid, the main phenolic acid found in the extract tested by this work, demonstrated effectiveness by regulating the cytokine/chemokine gene expression in HCat cells after UVB irradiation [65]. This effect is associated with its capacity for inhibiting lipoxygenases and cyclooxygenases and controlling the complement cascade [103].

Keratinocytes operate under a tightly regulated proliferation program that can be altered by UVB radiation [104]. Epidermal thickening, an epidermal self-protection mechanism against this powerful radiation, is frequently observed in experiments with hairless mice exposed to acute doses of UVB [25, 92, 105], serving as a parameter to estimate the intensity of UVB exposure [31]. CVE's ability to control epidermal hyperplasia reflects its overall UVB protective effect due to anti-inflammatory and antioxidants activities. Apoptotic keratinocytes or *sunburn cells* are another common characteristics of excessive epidermal UVB exposure. They appear in H&E-stained skin sections as shrunken cells with a condensed nucleus and eosinophilic cytoplasm, located mainly near the basal layer [106, 107]. The presence of *sunburn cells* is related to UVB-generated irreparable damage introduced by

overwhelming mutations to DNA. These mutations can be promoted either directly by UVB or indirectly by UVB-induced ROS [20, 104]. Apoptosis occurs to limit the survival rate of irreparably mutated or damaged cells that can develop into carcinogenic cells [108, 109]. Polyphenols present in CVE have shown cytoprotective capacity against UVB damage by modulating the cell's antioxidant systems [110]. Phenolic acids, such as caffeic, gallic, and rosmarinic, are linked to the regulation of apoptosis and control of the cell cycle [52]. Besides their ROS scavenging efficacy, flavonoids present anti-tumoral activity, because they can initiate DNA repair mechanisms, induce apoptosis, and inhibit metastasis by targeting various signaling pathways [94, 111–113].

Neutrophils, keratinocytes, mast cells, and endothelial cells produce metalloproteinases (MMPs) [81, 114]. ROS can also activate MMP gene expression by stimulating the protein kinase cascade [63]. UVB radiation stimulates the production of inflammatory mediators and ROS, thus increasing the expression of MMPs, specifically MMP-1, MMP-3, and MMP-9 [81]. These MMPs are associated with skin aging, intensification of inflammation, and cancer development [115, 116]. MMP-9 is mainly linked to the degradation of collagen IV, which is an essential component of the basement membrane, and elastin fibers [117]. According to the observed results, CVE can influence the UVB-induced inflammatory and oxidative network of events that culminate with overexpression of MMP-9 and damage to collagen, suggesting a possible role of CVE topical treatment protecting the skin against these hallmarks of photoaging.

Because *C. verbenacea* extract contains polyphenolic compounds that can absorb UVB radiation, when applied topically, the formulation enriched with CVE can have sunscreen activity, preventing the interaction between the radiation and the skin, hence reducing inflammation and oxidative stress [51]. Flavonoids and phenolic acids, such as those present in *C. verbenacea*, are theoretically capable of absorbing UV radiation due to their phenolic structure. Furthermore, quercetin, rutin, rosmarinic, and caffeic have experimentally demonstrated UV absorption in a range of varied UV wavelengths [118, 119]. Given this, it's essential to consider that we observed a low in vitro sun protection factor (SPF) of the present topical formulation containing CVE using a previously described method [120] (data not shown). In addition, the phytochemicals present in *C. verbenacea* have demonstrated anti-inflammatory and antioxidant activities in various evaluation models and different types of treatments, as priorly exposed in this text [4, 46, 47, 73, 121, 122]. In addition, finally, commercial sunscreens without antioxidants do not fully mimic the antioxidant activity observed with the current topical formulation [123] thus, suggesting that *C. verbenacea* photochemoprotective activity is tightly related to its biological antioxidant properties.

5 Conclusion

The synergistic effect of the compounds present in *C. verbenacea* promote its photochemoprotective activity, which includes: the antioxidant activity by scavenging of UVB-induced ROS and protecting the natural antioxidant system of the skin; the control of the inflammatory responses to UVB by immune cells; and the modulation the skin's proliferative and metabolic stress reactions to radiation. Because of the intricate network linking inflammation, oxidative stress, and skin cancer, the present results point to CVE as a promising photochemoprotection strategy and indicates the importance of further pre-clinical and clinical studies elucidating a possible role of *C. verbenacea* in the prevention of UVB-induced skin cancer and evaluating the benefits of the of sunscreen formulations containing CVE against acute UVB effects.

Glossary

ABTS+	2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
CAT	Catalase
COX	Cicloxigenase
CVE	<i>Cordia verbenacea</i> ethanolic leaves extract
DTNB	5,5'-Dithiobis (2-nitrobenzoic acid)
ELISA	Enzyme-Linked Immunosorbent Assay
FRAP	Ferric Reducing Antioxidant Power
GSH	Reduced glutathione
HaCaT	Aneuploid immortal keratinocyte cell
HTAB	Hexadecyltrimethylammonium bromide
IL	Interleukin
LT	Leukotriene
MCF-7	Breast cancer cell line
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
NBT	Nitroblue tetrazolium
NMSC	Non-melanoma skin cancer
$\cdot\text{OH}$	Hydroxyl free radical
$\text{O}_2^{\cdot-}$	Superoxide anion
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error mean
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
TNF- α	Tumor necrosis factor- α
UV	Ultraviolet

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Availability of data and materials The data that support the findings of this study are available from the corresponding author, [RC], upon reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interests.

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