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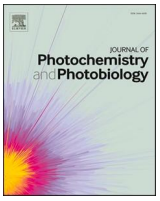

Modulation of UVB-induced oxidative stress and inflammation in skin keratinocytes (HaCaT) utilising unfermented rooibos and honeybush aqueous extracts

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ABSTRACT

Exposure to Ultraviolet B (UVB) radiation can trigger a diverse array of biological responses that have the potential to contribute to the onset of skin cancer. Natural compounds, such as tea polyphenols, have been shown to protect against UVB-induced damage by modulating oxidative stress, inflammatory response, and cell proliferation. The chemopreventive and anti-inflammatory properties of South African rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia spp.*) herbal teas have been shown to mainly target the early stages of cancer development through mechanisms that involve intracellular interleukin-1α (IL-1α) inhibition. Thus, the aim was to investigate the preventive effects of unfermented rooibos and honeybush aqueous extracts against UVB-induced oxidative stress and inflammation in HaCaTs. Honeybush was found to reduce the accumulation of UVB-induced IL-1α while maintaining cell viability and without affecting apoptosis. Furthermore, only honeybush extract was able to decrease the secretion of interleukin-6 (IL-6) caused by UVB exposure. Honeybush and rooibos extracts significantly decreased the secretion of UVB-induced interleukin-8 (IL-8). Except for rooibos extract at a concentration of 0.2 mg/mL, both extracts restored the expression of antioxidant genes to levels observed prior to UVB exposure. The anti-inflammatory effects of these herbal tea extracts are likely attributed to the antioxidant properties of their polyphenolic constituents, which modulate the oxidative stress-induced pathways governing inflammatory responses.

1. Introduction

Ultraviolet radiation is one of the exogenous stimuli that can alter innate and adaptive immune response in skin by causing irreversible damage that activates inflammatory and immunosuppressive signalling pathways leading to carcinogenesis [1]. The skin, being the body's largest and most exposed organ, is highly susceptible to the toxic effects of UVB radiation. Consequently, it serves as a primary site for the deleterious impact of UVB radiation. Additionally, the skin functions as an immune organ, playing a pivotal role in orchestrating the inflammatory response against UVB-induced damage. Early inflammatory events in skin are initiated in the epidermal layer by numerous signalling molecules including cytokines [2]. Keratinocytes, the predominant cell type within the epidermal layer, serve as a principal reservoir of cytokines such as tumor necrosis factor (TNF)-α, IL-6 and IL-8 with IL-1α being the most abundantly produced in these cells. IL-1α is constitutively produced as a biologically active precursor molecule and, due to its size of 31 kDa, remains cell associated and is only released into the extracellular environment in response to cellular injury or damage [3,4].

Following exposure to an inflammatory stimulus such as UVB radiation, IL-1α activates the production of pro–inflammatory mediators through autocrine and paracrine mechanisms involving nuclear and cell surface receptor (IL-1R) signalling, respectively [5]. The key cytokines IL-1α/β and TNF-α play a central role in instigating a signalling cascade that triggers the activation of genes and the generation of subsequent mediators. These mediators include cytokines, chemokines, growth factors, adhesion molecules, cyclooxygenase type 2 (COX-2), inducible nitric oxide synthase (iNOS), and various other pro-inflammatory factors. [6]. These pro-inflammatory factors facilitate the production of reactive oxygen species (ROS), recruiting immune cells which aid in the

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removal and repair of damaged cells and tissue [7]. However, with chronic UVB exposure, the inflammatory response can turn into a pathophysiological process that contributes to cancer development with ROS-producing immune cells exacerbating UVB-induced oxidative stress and promoting the malignant transformation of precancerous cells [8]. The involvement of UVB-induced ROS in inflammation has steered research toward exploring the anti-inflammatory properties of naturally occurring antioxidants, such as plant polyphenols [9–11].

One of the extensively investigated plants in terms of skin and biological activity is black and green tea (*Camellia sinensis*) and its major polyphenol, epigallocatechin gallate (EGCG) [12]. EGCG is known to protect against cancer development in skin by reducing UVB-induced ROS and inflammation in various *in vitro, in vivo* and clinical models [13]. South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia spp*), are also known to protect against cancer development in skin and one of the proposed mechanisms involve blocking the onset of inflammation in precancerous UVB damaged keratinocytes [14,15]. In an *in vitro* model of inflammation and chemoprevention, it was demonstrated that the anti-inflammatory effects of rooibos and honeybush extracts in UVB exposed keratinocytes are indirect and involves removal of damaged and inflamed cells, containing high levels of IL-1α, via apoptosis [15,16]. Since the indirect anti-inflammatory effect of these herbal extracts is associated with dose-dependent cytotoxicity, the ability of these extracts to directly inhibit pro-inflammatory cytokine production with minimal cytotoxic effects is of interest. The current study utilized a pre-exposure UVB/keratinocyte model to further assess the antioxidant and anti-inflammatory properties of rooibos and honeybush (*C. subternata*) aqueous extracts against the accumulation of pro-oxidant and inflammatory biomarkers, thereby reducing adverse cytotoxic interactive responses.

2. Materials and methods

2.1. Chemicals

ABTS (2,2′-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid), thiobarbituric acid (TBA), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2,4,5-tri(2-pyridyl)-S-triazine (TPTZ), gallic acid, and (+)-catechin (>96 %) were procured from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteau reagent, p-dimethylaminocinnamaldehyde (DAC), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), and all other analytical reagents were purchased from Merck Chemicals (Darmstadt, Germany). Heat-inactivated fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, California, USA). RPMI-1640 culture medium, l-glutamine, trypsin-versene, and Hank's buffered salt solution (HBSS) were sourced from Lonza (Basel, Switzerland). Dulbecco's phosphate-buffered saline (DPBS), dimethyl sulfoxide (DMSO), albumin bovine serum (BSA), and 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant IL-1α, IL-1ra, IL-6, IL-8 ELISA kits were acquired from R&D Systems (Minneapolis, Minnesota, USA). The CellTiter-Glo Luminescent cell viability assay and the Caspase-Glo® 3/7 Assay kits were obtained from Promega (Madison, Wisconsin, United States). Lipofectamine 3000 was purchased from Sigma-Aldrich (St. Louis, MO, USA). pTK-renilla, pGL2 Basic, and plasmid (p(IL6Kβ)350hu.IL6P-luc) were gifted by the Biochemistry Department of Stellenbosch University. The RNase-free DNase kit and RNeasy mini kit were procured from Qiagen (Hilden, Germany). The iScript First Strand cDNA synthesis kit and SsoAdvanced Universal SYBR Green were purchased from Bio-Rad (California, USA).

2.2. Plant material and preparation of the extracts

Unfermented rooibos was sourced from Rooibos Ltd in Clanwilliam, South Africa, while unfermented honeybush (*C. subternata*) was supplied by the Agricultural Research Council, Infruitec-Nietvoorbij in Stellenbosch, South Africa. Aqueous extracts from these plant materials were prepared by steeping 100 g of the plant material in 1 L of freshly boiled deionized water for 30 min. The resulting extracts were initially filtered through a single layer of cheesecloth to remove the majority of the plant material and subsequently subjected to stepwise filtration using Whatman No. 4 and No. 1 filters. The filtrates were then freeze-dried, and the lyophilized material was stored in desiccated form in amber vials at room temperature.

2.3. Chemical characterisation of extracts

2.3.1. HPLC analysis

The analysis of monomeric polyphenolic content was conducted at the Agricultural Research Council, Infruitec-Nietvoorbij in Stellenbosch, South Africa, following the methods outlined by Beelders et al. [17] for rooibos and Joubert and De Beer et al. [18] for the honeybush extracts. Polyphenol concentrations were quantified and expressed as μg/mg extract.

2.3.2. Antioxidant assays

Apart from the TBARS assay, all antioxidant assays were conducted using a Biotek Synergy HT microplate (Winooski, Vermont, USA). A 1 mg/mL solution of each extract was prepared in deionized water, and aliquots were preserved at −20 °C until required.

DPPH• radical scavenging assay: determining the ability of the antioxidant to scavenge free radicals, were done according to the method of Brand-Williams, Cuvelier and Berset [19].

FRAP: assesses the capacity of the samples to convert iron (III) into iron (II), following the modified microplate format method outlined by Benzie and Strain [20]. The results were quantified and reported as μmol Trolox equivalents/mg extract using the standard curve generated.

ORAC: assays were performed according to the method described by Huang et al. [21]. The radical scavenging activity of the extracts were determined using the cation ABTS decolourisation assay [22] and data were expressed as μmol TE/mg extract utilising a Trolox standard curve.

TBARS: Microsomes for assessing lipid peroxidation were derived from male Fischer 344 rats, and a microsomal fraction was isolated through a Sepharose 2B column, as previously detailed by Gelderblom et al. [23]. Inhibition of lipid peroxidation in rat liver microsomes by the extracts was evaluated using the procedure outlined by Snijman et al. [24]. The concentrations of each extract needed to attain a 50 % inhibition rate (IC_{50}) were calculated utilizing GraphPad Software Prism Version 5.00 for Windows (La Jolla, California, USA).

2.4. Keratinocyte cell culture

Spontaneously immortalized keratinocytes (HaCaT) were procured from CLS Cell Lines Service GmbH (Eppelheim, Germany). The cells were cultured in a humidified environment with 5 % CO_2 and 95 % air at 37 °C, using RPMI-1640 medium supplemented with 10 % FBS (v/v) and 2 mM l-glutamine.

2.4.1. UVB/Keratinocyte pre-exposure model

HaCaT cells were initially plated in RPMI-1640 medium with 10 % FBS and incubated at 37 °C for 6 hrs to allow cell attachment. An equal volume (100 μ in 96-well plates; 900 μL in 24 well plates) of fresh 0.5 % FBS: 0.5 % DMSO (v/v) RPMI medium containing different dilutions of *A. linearis* and *C. subternata* extracts were added to the different plates. The concentrations utilized for *A. linearis* (0.1 and 0.2 mg/mL) and *C. subternata* (0.2 and 0.4 mg/mL) were determined based on previous studies [16,25] The final DMSO concentration in the wells was 0.5 %. The cells were further incubated with the tea extracts for an additional 18 hrs. Following the removal of the cultured medium, the cells were exposed to UVB light at doses of 30 and 80 mJ/cm^2 [26] in DPBS (600 μL). Following irradiation, DPBS was replaced with fresh RPMI-1640 medium (100 μL in 96 well plates; 600 μL in 24 well plates)

containing 0.5 % FBS and 0.5 % DMSO (v/v), cells were then incubated for an additional 24 hrs. It is important to note that cells used to monitor ATP content and DCFDA were prepared differently, details will be provided under the section of cell growth parameters and oxidative stress, respectively.

2.4.2. Cell viability and intracellular antioxidant activity

Cells were seeded (3×10^4 cells in 100 μL medium per well) in white plates for ATP determination (Porvair Sciences, Shepperton, UK).

2.4.3. Cell viability

Cell viability was determined based on the quantification of adenosine triphosphate (ATP) (representing metabolically active cells) using the CellTitre Glo® luminescent cell viability assay (Promega, Wisconsin, United States) according to the manufacturer's instructions [27]. The luminescence signal (expressed as relative light units) was determined on a Veritas microplate Luminometer (Turner Biosystems, Madison, USA) and ATP production was expressed as a percentage (%) of the control cells.

2.4.4. Determination of oxidative status

Intracellular levels of ROS were quantified using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA), a lipophilic, initially non-fluorescent compound. H2DCFDA is converted into the hydrophilic alcohol H(2)DCF (dihydrodichlorofluorescein) and further oxidized into a fluorescent DCF (2′,7′-dichlorofluorescein) molecule when exposed to ROS [28]. After a 24-hour incubation of cells in black plates with rooibos and honeybush extracts, the RPMI media was substituted with 20 μM of DCFDA in PBS (100 μL), and the cells were allowed to incubate at 37 °C for 30 min. Following this incubation period, the cells were washed once and covered with PBS (100 μL), and the relative fluorescent units (RFU) were measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a microplate reader. The RFU values were standardized with respect to cell viability, and intracellular ROS levels were expressed as a fold increase relative to the control treatment. The quantification of total glutathione was performed using the GSH/GSSG Glo assay (Promega, Wisconsin, United States), as per manufacturer's instructions and the data was presented as a percentage in comparison to untreated and non-irradiated samples.

2.4.5. Cytokine production and apoptosis

Cells were seeded in 24-well plates (18×10^4 cells in 600 μL per well). After cells were exposed to the extracts, irradiated, and incubated, supernatants were transferred into fresh plates. Cells were then washed once with DPBS and lysed by adding 0.5 % Triton-X-100 (v/v) in DPBS, pH 7.4, and exposed to one freeze-thaw cycle. Supernatants and cell lysates, stored at −80 °C, were utilised for cytokine production (IL-1α, IL-1ra, IL-6, IL-8).

2.4.6. Cytokine production

IL-1α content in cell lysates and IL-1ra, IL-6 and IL-8 content in the supernatant, prepared as described above, was determined with IL-1α, IL-ra, IL-6 and IL-8 ELISA kits according to the manufacturer's instructions. Data was analysed using the standard curve generated from Gen5 Data Analysis Software (version 2 for Windows). All cytokine levels were expressed as pg/mL.

2.4.7. Caspase-3 activity

Apoptosis was determined with the Caspase-Glo® 3/7 Assay kit. Analyses were conducted using a Veritas microplate luminometer and relative light units used to calculate the fold increase compared to the control treatment regimens.

2.4.8. Pre-UVB model for RNA extraction and gene expression

HaCaT cells were plated in 35 mm petri dishes at a density of 45×10^4 cells per plate in 1 mL RPMI-1640 medium containing 10 % FBS and incubated at 37 °C for 6 hrs to allow the cells to attach. An equal volume (1 mL per petri dish for RNA extractions) of fresh RPMI-1640 medium containing 0.5 % FBS, 0.5 % DMSO (v/v) and respective concentrations of rooibos and honeybush extracts was added to the plates. Cells were incubated for an additional 18 hrs [24 hr (-UVB)]. After removing the cultured medium, cells were exposed to UVB light (30 or 80 mJ/cm^2) using the UVLink UV crosslinker (UVitec limited, UK) in DPBS (600 μL). However, it was noticed that 80 mJ/cm^2 was more toxic to the cells exposed in a petri than in a 96 well or 24 well plate, thus moving forward, experiments conducted in petri dishes, were irradiated with 30 mJ/cm^2. Following irradiation, DPBS was replaced with fresh RPMI-1640 medium (1 mL per petri dish) containing 0.5 % FBS and 0.5 % DMSO (v/v), cells were then incubated for an additional 24 hrs [48 hr (-UVB) & 48 hr (+UVB)] (Fig. 1). Cells were then washed once with DPBS and trypsinated for 15 min. The cells were then centrifuged, and the cell pellet was then collected in a microtube for RNA extraction.

2.4.9. qPCR analysis of selected inflammatory and antioxidant genes

Gene expression analysis of IL-1α, IL-6, IL-8, GPX1, Nrf2, and CAT was carried out using quantitative PCR (qPCR). RNA was extracted from cell pellets using the RNeasy mini kit, following the manufacturer's instructions, which included on-column DNase digestion (RNase-free DNase kit; Qiagen). The quantity and quality of the total RNA were assessed using a NanoDrop spectrophotometer (Thermo Scientific) and an Agilent 2100 BioAnalyzer (Agilent Technologies). Samples with RIN values above 7 were considered suitable for real-time PCR analysis and stored at −80 °C. A two-step qPCR procedure was employed to investigate gene expression profiles. First-strand cDNA was synthesized using the iScript First Strand cDNA synthesis kit (Bio-Rad, California, USA). Each reaction mixture contained total RNA (750 ng), 5x iScript reaction mix (4 μL), and iScript Reverse Transcriptase (1 μL) in a final volume of 20 μL, using RNase-free water. The cDNA synthesis was carried out in a T100 thermal cycler (Bio-Rad) following the manufacturer's instructions, and the resulting samples were stored at −20 °C. Samples incubated in the absence of reverse transcriptase served as experimental controls. Transcript abundances were determined using a reaction mix comprising 1 μL of each cDNA, 2x SsoAdvanced Universal SYBR Green Supermix (4 μL) (Bio-Rad), 10 μM of each primer, in a final volume of 10 μL, with RNase-free water. All assays were conducted using the CFX96 touch qPCR system (Bio-Rad) under the following conditions: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and then at 60 °C for 1 min. A melting curve analysis ranging from 72 °C to 95 °C was performed to differentiate between primer dimers, nonspecific amplification, and target gene amplification. All samples were analysed in triplicate. The relative expression of the genes was determined using a reference gene (GAPDH) selected to normalize all data, and the expression ratio was calculated using the 2ˆ-ΔCt method [29].

2.4.10. NF-kB reporter plasmid assay

The NF-kB assay was performed according to Louw-du Toit et al. [30]. HaCaT cells were seeded in 24-well plates at a density of 100 000 cells per well in 500 μL RPMI-1640 supplemented with 2 mM l-glutamine and 10 % FBS. The cells were then incubated for 24 hrs, where after a transfection mixture was added dropwise to the wells. The transfection mixture consisted of Lipofectamine 3000, serum free RPMI-1640, pTK-renilla and either an empty vector (pGL2 Basic) or the reporter plasmid (p(IL6Kβ)350hu.IL6P-luc). After a 30-hr incubation with the transfection mixture, the cells were treated with the previously used concentrations of rooibos and honeybush extracts and incubated for a further 18 hrs. The extracts were removed and replaced with 500 μL DPBS. One plate was then exposed to UVB (30 mJ/cm^2) while the other plate was unexposed. The DPBS was removed and replaced with fresh media. After an incubation of 24 hrs, the cells were harvested by removing the media, adding 80 μL passive lysis buffer per well and shaking the plate for 15 min. The Promega Dual Luciferase Reporter Assay kit was used according to the manufacturer's instructions. The

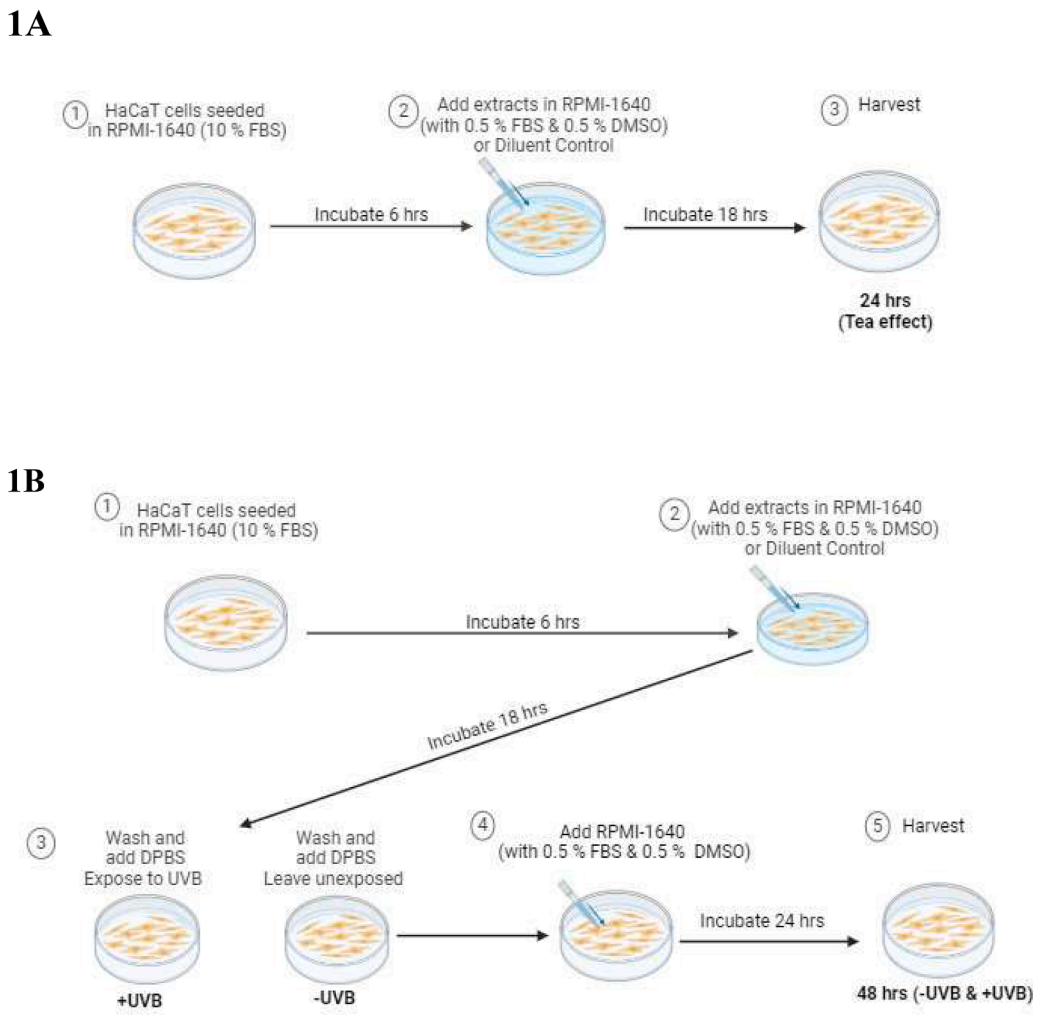

Fig. 1. Experimental setup for UVB/Keratinocyte pre-exposure model. (**1A**) HaCaT cells were seeded and allowed to bind for 6 hrs (1). Rooibos extracts, honeybush extracts or diluent control (0.5 % DMSO) were added in RPMI-1640 (with 0.5 % FBS) media for 18 hrs (2), before harvesting of cells for RNA extraction [24 hr (-UVB)] to investigate the effect of tea compared to diluent control on the cells (3). (**2B**) HaCaT cells were seeded and allowed to bind for 6 hrs (1). Rooibos extracts, honeybush extracts or diluent control (0.5 % DMSO) were added in RPMI-1640 (with 0.5 % FBS) media for 18 hrs (2), DPBS was added, and cells were exposed or left unexposed to UVB (4). Recuperation from the UVB exposure was allowed by adding RPMI-1640 (0.5 % FBS, 0.5 % DMSO) and incubating for a further 24 hrs (5) and cells harvested at 48 hrs to investigate the effect of UVB on cells. Image was generated using BioRender (BioRender.com).

lysate (20 μL) was transferred to a white 96-well plate and 50 μL LARII reagent was added. The luminescence was read using a Veritas microplate luminometer. Stop and Glo reagent (50 μL) was added, and the luminescence read again. The lysate (10 μL) was also used to determine the total soluble proteins. Data was analysed by plotting Luciferase Activity (relative luminescence units)/Total soluble protein.

2.5. Statistical analysis

Analysis of variance (ANOVA) was utilized to determine the statistically significant impacts of treatment on antioxidant and chemical characterization data. In the case of unbalanced data, automatic Tukey-Cramer adjustments were applied. Statistical significance was established at a significance level of $p < 0.05$. Descriptive statistics conducted on the data revealed that all sample groups exhibited normal distribution, as confirmed by the Kolmogorov–Smirnov Test for biological data. Furthermore, for multiple pairwise comparisons between all groups, the post-hoc Tukey's Studentized Range Test was employed. Due to the unbalanced nature of the data, the Tukey–Cramer adjustment was automatically implemented. When only two groups were involved, Group differences were evaluated using Student's T-test, with statistical significance being denoted at $p < 0.05$.

3. Results

3.1. Antioxidant and phenolic contents of extracts

The HPLC analysis of polyphenolic compounds in the rooibos extract revealed that the predominant polyphenols were the dihydrochalcones, aspalathin and nothofagin, the flavonol, bioquercetin, and the flavones, isoorientin and orientin (Table 1). The rooibos extract also contained quantifiable levels of rutin, isoquercitrin, and hyperoside, as well as vitexin, isovitexin, luteoloside and PPAG. The honeybush extract exhibited a different polyphenolic composition, with the major compounds being the xanthones, mangiferin and isomangiferin. Statistical analysis indicated a significant difference ($p < 0.05$) between the two extracts in terms of their free radical scavenging activity against ABTS and DPPH (Table 1), with the rooibos extract demonstrating a significantly stronger response in both assays compared to the honeybush extract. Furthermore, the antioxidant capacity (ORAC) and ferric iron reducing potential (FRAP) of the rooibos extracts were also found to be significantly higher ($p < 0.05$) than those of the honeybush extract. Additionally, the rooibos extract exhibited greater inhibition of iron-induced peroxidation (LPO), as evidenced by a significantly lower IC_{50} value compared to the honeybush extract.

Table 1
Phenolic composition and antioxidant activity of extracts in various assays.

Compound[a]/Parameter	Rooibos (Rg)	Honeybush (Hg)[b]
Aspalathin	9.153	–
Nothofagin	0.756	–
Orientin	1.051	–
Isoorientin	0.680	–
Vitexin	0.123	–
Isovitexin	0.128	–
Luteoloside	0.049	–
PPAG	0.607	–
Bioquercetin	0.806	–
Rutin	0.374	–
Hyperoside	0.211	–
Isoquercitrin	0.248	–
Mangiferin	–	2.211
Isomangiferin	–	0.786
IDG	–	1.047
IMG	–	0.877
MMG	–	0.204
Eriocitrin	–	0.372
Hesperidin	–	0.629
HPDG	–	0.487
PDG	–	1.487
Vicenin-2	–	0.227
Scolymoside	–	0.640
Total quantified compounds	14.186	8.966
ABTS (μmol TE/mg extract)	3.32 ± 0.12a[c]	2.65 ± 0.16b
DPPH (μmol TE/mg extract)	1.92 ± 0.06a	1.47 ± 0.02b
ORAC (μmol TE/mg extract)	4.17 ± 0.25a	3.49 ± 0.25b
FRAP (μmol TE/mg extract)	1.51 ± 0.03a	1.08 ± 0.02b
TBARS (IC_{50} mg/mL)	0.15 ± 0.04a	0.32 ± 0.03b

Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity; FRAP, ferric reducing antioxidant power; HPDG, 3′,5′-di-β-d-glucopyranosyl-3-hydroxyphloretin; IC_{50}, concentration needed to obtain 50 % inhibition; IDG, 3-β-d-glucopyranosyl-4-*O*-β-d-glucopyranosyliriflophenone; IMG, 3-β-d-glucopyranosyliriflophenone; MMG, 3-β-d-glucopyranosylmaclurin; ORAC, oxygen radical absorbance capacity; PDG, 3′,5′-di-β-d-glucopyranosylphloretin; PPAG, *Z*-2-(β-d-glucopyranosyloxy)−3-phenylpropenoic acid; TBARS, thiobarbituric acid reactive substances; TE, Trolox equivalents.

[a] g/100 g extract.

[b] *Cyclopia subternata*.

[c] Antioxidant values in a row with different letter were significantly different ($P < 0.05$).

3.2. Modulation of cell viability and apoptosis

Following an 18-hour pre-exposure period [24 hr (-UVB)], neither the rooibos (Rg) nor the honeybush (Hg) extracts demonstrated any significant impact on cell viability (Fig. 2A). Additionally, no significant changes in ATP production (cell viability) were observed after 24 hrs extract exposure and an additional 24 hrs in medium [48 hr (-UVB)] to rooibos at a concentration of 0.1 mg/mL and to both concentrations of the honeybush extract. However, when rooibos extract was used at a concentration of 0.2 mg/mL, a significant ($p< 0.05$) decrease in cell viability was noted at 48 hr (-UVB). Nevertheless, the percentage of viable cells remained above 80 % (Fig. 2A). Upon UVB exposure [48 hr (+UVB)], the UVB control group displayed a significantly ($p< 0.05$) lower cell viability compared to the control group without UVB exposure [48 hr (-UVB)]. However, when cells were pre-treated with the rooibos and honeybush extracts, no significant difference in cell viability was observed compared to the control group exposed to UVB.

The impact of UVB and tea treatment on apoptosis was assessed by analysing caspase 3/7 activity. The results indicated that at 24 hr (-UVB) honeybush extract significantly ($p< 0.05$) increased apoptosis by 2-fold at both concentrations tested (Fig. 2B). In contrast, the rooibos extracts had no significant effect on caspase activity. After a 48-hour tea exposure period [48 hr (-UVB)], no significant difference in caspase activity was observed between the control group and the tea treatments. Upon UVB exposure [48 hr (+UVB)], the UVB control group exhibited a significant ($p< 0.05$) 2-fold increase in caspase 3/7 activity. The rooibos extract showed a slight increase in caspase activity relative to control when the cells were pre-treated with 0.1 mg/mL extract, although not statistically significant. In contrast, honeybush extract at both concentrations demonstrated protective effects against apoptosis induced by UVB exposure compared to the control cells. Rooibos extract at a concentration of 0.2 mg/mL exhibited similar protective effects. The protective effect of HaCaT cells by the suppression of apoptosis treated with an herbal extract before UVB radiation has been observed previously [31,32].

3.3. In vitro antioxidant properties of rooibos and honeybush extracts in UVB-irradiated keratinocytes

The assessment of oxidative stress modulation in UVB-exposed HaCaT cells involved the quantification of ROS levels measured in terms of DCF fluorescent units, as well as the determination of total glutathione (GSH) levels (Fig. 3). Before subjecting the keratinocytes to UVB exposure, pre-treatment with the extracts [24 hr (-UVB)] did not induce any significant alterations in ROS levels (Fig. 3A). However, after exposure to UVB [48 hr (+UVB)], ROS levels increased significantly ($p< 0.05$) in the control cells exposed to UVB only compared to the unexposed cells. Pre-treatment with rooibos extract at a concentration of 0.2 mg/mL resulted in a significant ($p< 0.05$) reduction in UVB-induced elevation of ROS levels. Conversely, pre-treatment with honeybush extracts did not have any significant impact on UVB-induced elevation ROS levels.

Regarding GSH (Fig. 3B), UVB exposure significantly ($p< 0.05$) reduced total GSH levels by 20 % in untreated cells. This decrease was significantly ($p< 0.05$) counteracted by pre-treatment with honeybush (0.2 and 0.4 mg/mL) and rooibos (0.1 mg/mL). However, at a higher

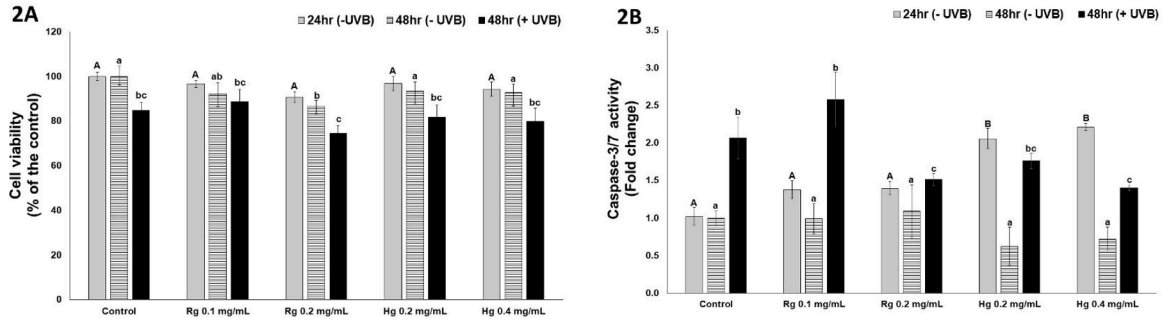

Fig. 2. Effect of rooibos (Rg) and honeybush (Hg) extracts on cell viability (2A) and apoptosis (2B) in HaCaT cells. Values represent mean ± standard deviation of five determinations of at least two experiments. Statistical analyses of the different extract concentrations and control were compared independently for each timepoint. Statistically significant differences ($p< 0.05$) between the means are indicated by upper-case letters for 24 hr (-UVB) and different lower-case letters for 48 hr (-UVB) and 48 hr (+UVB). Cell viability: % ATP production; Apoptosis: caspase 3/7 activity fold change.

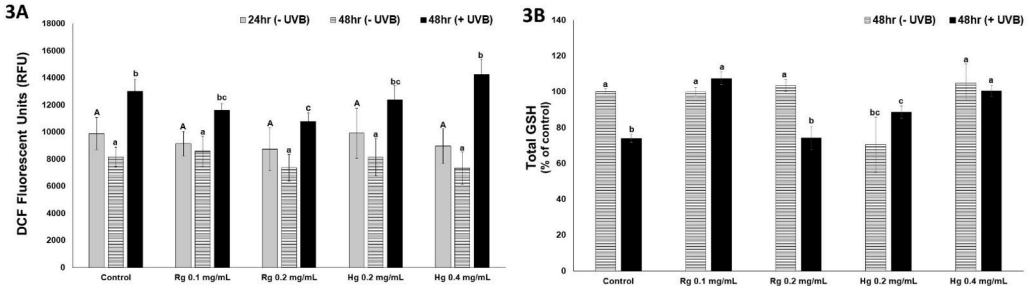

Fig. 3. Effect of rooibos (Rg) and honeybush (Hg) extracts on oxidative stress measuring ROS levels using DCF (3A) and Total GSH (3B) in HaCaT cells. Values represent mean ± standard deviation of five determinations of at least two experiments. Statistical analyses of the different extract concentrations and control were compared independently for each timepoint. Statistically significant differences ($p< 0.05$) between the means are indicated by upper-case letters for 24 hr (-UVB) and different lower-case letters for 48 hr (-UVB) and 48 hr (+UVB).

concentration of 0.2 mg/mL, rooibos extract did not mitigate the effect of UVB on GSH levels.

The expression of genes encoding for certain role players in the defence against oxidative stress, such as *GPX1* and *CAT* were assessed in the pre-exposure model using qPCR. In the absence of UVB exposure [48 hr (-UVB)], rooibos increased the expression of *GPX1* compared to the control, whereas honeybush at 0.4 mg/mL significantly ($p< 0.05$) decreased the *GPX1* expression (Fig. 4A) but at 0.2 mg/mL showed no effect. UVB exposure led to a significant ($p< 0.05$) reduction in *GPX1* gene expression in the control group. However, pre-treatment of HaCaT cells with herbal tea extracts resulted in a significant increase in *GPX1* expression. Specifically, honeybush extracts at concentrations of 0.4 and 0.2 mg/mL ($p< 0.05$), as well as rooibos extract at a concentration of 0.1 mg/mL ($p< 0.05$), demonstrated the ability to protect the UVB-induced decrease in *GPX1* expression. In contrast, rooibos extract at a concentration of 0.2 mg/mL did not exhibit any significant effect on modulating the UVB response.

In the absence of UVB exposure [48 hr (-UVB)], all the extracts elicited a significant ($p< 0.05$) decrease in the expression of catalase (*CAT*), except for the rooibos extract at a concentration of 0.2 mg/mL, which did not exert any notable effect (Fig. 4B). Following the exposure of the keratinocytes to UVB, *CAT* gene expression decreased significantly ($p< 0.05$) in the +UVB control cells. Pre-treatment with both extracts resulted in a significant increase relative to control cells in the expression of the catalase (*CAT*) gene. Specifically, rooibos extract at a concentration of 0.2 mg/mL and honeybush extract at a concentration of 0.2 mg/mL restored the expression levels to a similar magnitude as those observed without UVB exposure in control cells.

3.4. Effect of the extracts on UVB-induced NRF2 and NF-κB activation

NF-κB activation (Fig. 5A) was not significantly affected by rooibos, while honeybush significantly ($p< 0.05$) decreased activation at 0.4 mg/mL and increased activation at 0.2 mg/mL [48 hr (-UVB)]. Following UVB exposure [48 hr (+UVB)], activation of NF-κB increased significantly ($p< 0.05$) by approximately 5-fold in the UVB exposed cells compared to the control cells. Both rooibos and honeybush extracts exhibited significant ($p< 0.05$) inhibition of this activation, with rooibos demonstrating a dose-dependent response. Notably, honeybush extract displayed a higher response in inhibiting UVB-induced NF-κB activation.

In the absence of UVB, neither rooibos nor honeybush had any significant effect on the expression of Nrf2 (Fig. 5B). Exposure to UVB increased ($p< 0.05$) Nrf2 gene expression more than 3-fold in the UVB-exposed control cells compared to the unexposed cells, while pre-treatment with 0.2 and 0.4 mg/mL honeybush and 0.1 mg/mL rooibos extracts significantly decreased Nrf2 expression ($p< 0.05$).

3.5. Anti-inflammatory properties of rooibos and honeybush extracts in UVB-irradiated keratinocytes

Intracellular IL-1α protein concentration at 24 hr (-UVB) showed no significant difference between the control cells and the cells after treatment with the extracts (Fig. 6A). IL-1α concentration decreased after 48 hrs [48 hr (-UVB)] with no significant differences between control and the HaCaT cells treated with tea extracts. After UVB exposure [48 hr (+UVB)], IL-1α protein concentration in the UVB-exposed control increased significantly (**$p< 0.05$**) compared to the non-exposed control cells at 48 hr [48 hr (-UVB)]. Honeybush (both concentrations) and rooibos (0.2 mg/mL) extracts were able to significantly (**$p< 0.05$**) decrease the production of IL-1α relative to the cells only exposed to UVB. Both rooibos and honeybush extracts demonstrated a significant (**$p< 0.05$**) inhibition of IL-1α gene expression relative to control cells prior to UVB exposure (Fig. 6B). Notably, the honeybush extracts exhibited a significantly (**$p< 0.05$**) stronger effect compared to the rooibos extracts. Exposure to UVB increased (**$p< 0.05$**) the expression of IL-1α approximately 2-fold in untreated keratinocytes. Treatment with honeybush and rooibos extracts resulted in a significant (**$p< 0.05$**) decrease in IL-1α gene expression following UVB exposure compared to

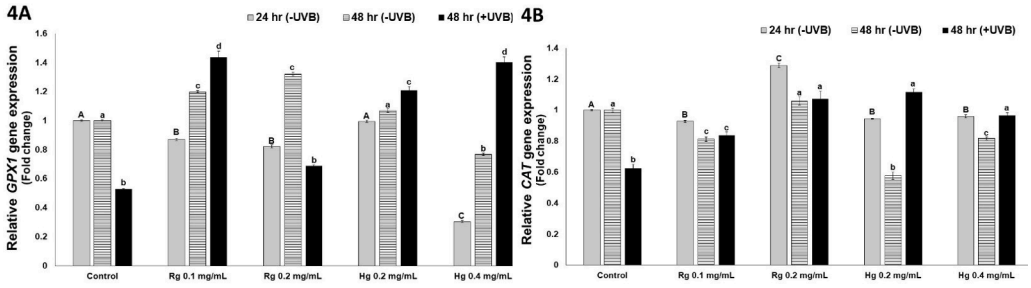

Fig. 4. Effect rooibos (Rg) and honeybush (Hg) extracts on oxidative stress gene expression of *GPX1* (4A) and *CAT* (4B) in HaCaT cells. Values represent mean ± standard deviation of three determinations of at least two experiments. Statistical analyses of the different extract concentrations and control were compared independently for each timepoint. Statistically significant differences ($p< 0.05$) between the means are indicated by upper-case letters for 24 hr (-UVB) and different lower-case letters for 48 hr (-UVB) and 48 hr (+UVB).

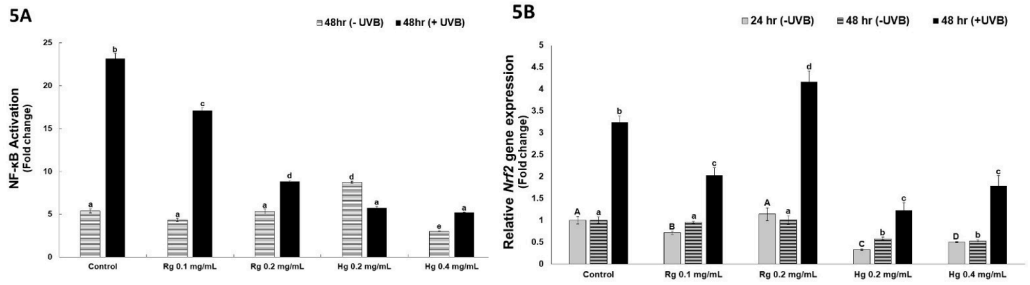

Fig. 5. Effect of rooibos (Rg) and honeybush (Hg) extracts on NF-kB activation (5A) and Nrf2 gene expression (5B) in HaCaT cells. Values represent mean ± standard deviation of three determinations of at least two experiments. Statistical analyses of the different extract concentrations and control were compared independently for each timepoint. Statistically significant differences ($p < 0.05$) between the means are indicated by upper-case letters for 24 hr (-UVB) and different lower-case letters for 48 hr (-UVB) and 48 hr (+UVB).

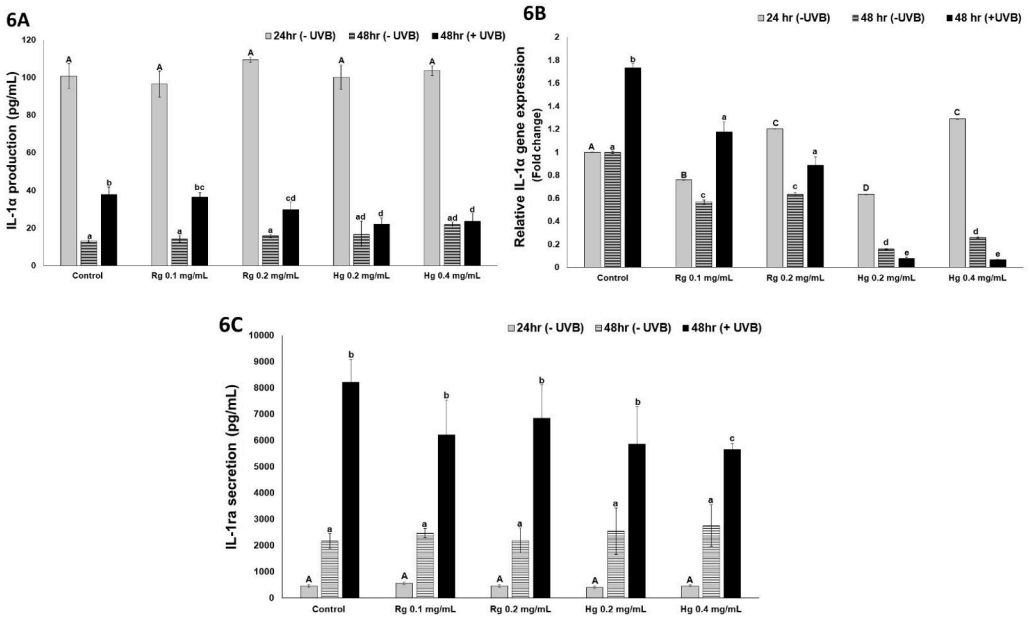

Fig. 6. Effect of rooibos (Rg) and honeybush (Hg) extracts on IL-1α protein production (**6A**), IL-1α gene expression (**6B**) and IL-1ra protein secretion (**6C**) in HaCaT cells. Values represent mean ± standard deviation of three determinations of at least two experiments. Statistical analyses of the different extract concentrations and control were compared independently for each timepoint. Statistically significant differences ($p < 0.05$) between the means are indicated by upper-case letters for 24 hr (-UVB) and different lower-case letters for 48 hr (-UVB) and 48 hr (+UVB).

control cells. The magnitude of the decrease was dose-dependent for rooibos extract, whereas honeybush extract exhibited a greater ($p < 0.05$) reduction in IL-1α gene expression compared to the cells pre-treated with the rooibos extracts.

The secreted IL-1ra protein concentration was not significantly affected after 24 hrs (-UVB) by either extract (Fig. 6C). Although, the protein level was increased 4-fold after 48 hrs (-UVB), there was no significant difference amongst the different tea treatments or when compared to control cells. After UVB exposure [48 hr (+UVB)], the protein concentration significantly ($p < 0.05$) increased in the UVB

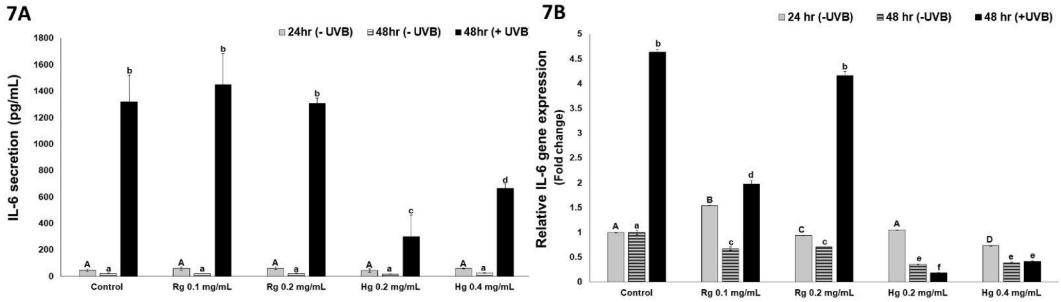

Fig. 7. Effect of rooibos (Rg) and honeybush (Hg) extracts on IL-6 protein secretion (**7A**) and IL-6 gene expression (**7B**) in HaCaT cells. Values represent mean ± standard deviation of five determinations of at least two experiments. Statistical analyses of the different extract concentrations and control were compared independently for each timepoint. Statistically significant differences ($p < 0.05$) between the means are indicated by upper-case letters for 24 hr (-UVB) and different lower-case letters for 48 hr (-UVB) and 48 hr (+UVB).

control cells. Only 0.4 mg/mL honeybush extract significantly ($p < 0.05$) decreased IL-1ra secretion relative to the control cells after UVB exposure.

Minimal levels of extracellular IL-6 protein were detected at both 24 hrs and 48 hrs in the absence of UVB exposure (Fig. 7A) with no significant changes observed between the treatments and control. After UVB exposure [48 hr (+UVB)], IL-6 protein secretion was significantly ($p < 0.05$) increased. Only the honeybush extracts significantly decreased the secretion of IL-6 relative to control, with 0.2 mg/mL ($p < 0.05$) exhibiting significantly higher inhibition than 0.4 mg/mL ($p < 0.05$). The was no significant difference in the gene expression of IL-6 in cells not exposed to UVB (Fig. 7B). However, following UVB exposure, IL-6 gene expression was significantly ($p < 0.05$) increased 4.5-fold in the UVB control cells with rooibos extract (0.1 mg/mL) and to a larger extent honeybush extracts (0.2 and 0.4 mg/mL) significantly ($p < 0.05$) reducing this increase in IL-6 gene expression.

The secretion of IL-8 protein was significantly ($p < 0.05$) decreased by both rooibos and honeybush extracts at both concentrations at 24 hr (-UVB) (Fig. 8A). However, after 48 hrs [48 hr (-UVB)], honeybush at 0.4 mg/mL was the only treatment to increase ($p < 0.05$) the concentration of IL-8 protein relative to control. After UVB exposure, IL-8 secretion increased significantly in the UVB exposed control cells ($p < 0.05$), however tea pre-treatment lowered IL-8 secretion, but only significantly with 0.4 mg/mL honeybush compared to the respective control. Regarding IL-8 gene expression, without UVB the extracts showed no significant effect on IL-8 gene expression (Fig. 8B). UVB resulted in a 10-fold increase ($p < 0.05$) in IL-8 gene expression in the control, which rooibos at 0.2 mg/mL further significantly ($p < 0.05$) increased. However, honeybush (0.2 and 0.4 mg/mL) and rooibos (0.1 mg/mL) significantly ($p < 0.05$) inhibited the increase in IL-8 gene expression seen in control cells.

4. Discussion

UVB radiation, known to act as an initiator and promoter, is regarded as one of the major causative agents of skin cancer [33]. Photocarcinogenesis is characterised by three distinct stages: initiation, promotion and progression. Due to its reversible nature, the promotion stage has been identified as a suitable target for chemoprevention studies that use sub-cytotoxic concentrations of natural compounds, such as polyphenols, to impede the process of skin carcinogenesis [34]. Chemoprevention studies have indicated that rooibos and honeybush herbal extracts may effectively target the early stages of carcinogenesis in skin [15,35,36].

Rooibos and honeybush herbal tea extracts, with their unique polyphenol components, are known to exhibit a plethora of biological properties that are associated with cancer prevention, including antioxidant, anti-inflammatory, immunomodulatory, anti-proliferative and anti-cancer activity [35,36]. The anti-tumour activity of rooibos and honeybush extracts against UVB-induced skin carcinogenesis has been demonstrated *in vivo* and associated with distinctive polyphenolic interactions that target pathophysiological processes in cancer promotion [14,37]. Chemoprevention studies have described various mechanisms of action for the herbal teas in skin, with the removal of IL-1α containing cells via apoptosis being identified as one of the strategies that may effectively block the onset of inflammation; thereby targeting the early stages of skin carcinogenesis [15]. However, dose-dependent cytotoxic effects (apoptosis) associated with this indirect anti-inflammatory effect against IL-1α has been of concern. Consequently, the ability of the herbal tea extracts to directly modulate inflammatory activity in UVB-exposed cells with minimal adverse cytotoxic effects is of interest. The objective of the present investigation was to assess the anti-inflammatory effects on cytokine (IL-1α, IL-1ra, IL-6 and IL-8) protein and gene expression levels as well as NF-κB activity, a key transcription factor in inflammation. Furthermore, we also evaluated the impact on cell viability, apoptosis and oxidative status (DCFDA, GSH, CAT, GPX1 and Nrf2). For the purpose of this study, precise sub-cytotoxic concentrations ($<IC_{50}$) of rooibos and honeybush extracts [15] were carefully chosen to ensure the absence of cytotoxic effects on keratinocytes prior to UVB exposure [15,25].

Since the biological properties of herbal tea extracts are often attributed to the distinctive combinations of polyphenols present within the extracts [38–41] and seasonal variations are known to affect the level of compounds in plants, it is therefore crucial to perform a chemical analysis of the extracts being utilized to characterise the specific polyphenols present and evaluate their antioxidant activity. Thus, current extracts were screened for polyphenolic content and validated for antioxidant activity before their application in non-irradiated and UVB irradiated keratinocytes. In the rooibos extract, HPLC analysis corroborated the presence of dihydrochalcones, aspalathin and nothofagin, flavones, isoorientin and orientin, as well as flavonols, rutin, isoquercitrin, and hyperoside (Table 1). Aspalathin constituted the major rooibos flavonoid (9.15 %), a level of which could reach up to 12 % depending on the source of unfermented plant material [17]. The major compounds often associated with the honeybush extract included xanthones, mangiferin and isomangiferin, and the flavanone, hesperidin. In the current extract, mangiferin was, as expected, the major compound present while, PDG IMG and IDG were more abundant than hesperidin. The phenolic profile of the current honeybush extract was consistent with previous findings [15,42].

The antioxidant characterization revealed the distinct effectiveness of the unique polyphenol content in the two herbal extracts in terms of their activity. Honeybush displayed an overall lower antioxidant activity in comparison to rooibos, as indicated in Table 1. This observation aligns with the general understanding that the primary polyphenols found in honeybush exhibit lower activity, as reported previously [35]. Previous research has shown that the major flavonoid in rooibos, aspalathin, is an exceptionally active radical scavenger in the ABTS assay, outperforming

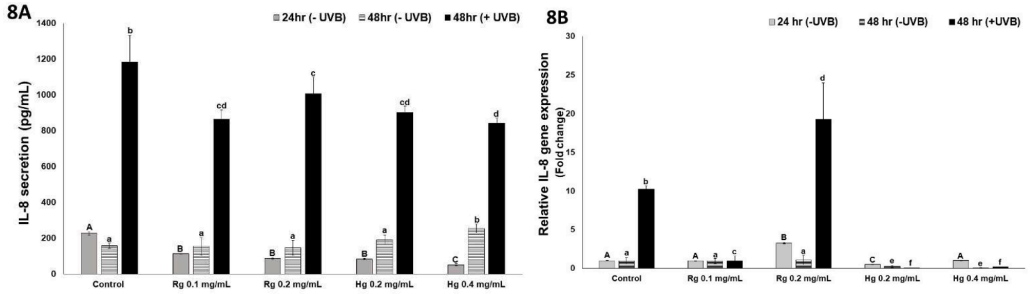

Fig. 8. Effect of rooibos (Rg) and honeybush (Hg) extracts on IL-8 protein secretion (**8A**) and IL-8 gene expression (**8B**) in HaCaT cells. Values represent mean ± standard deviation of five determinations of at least two experiments. Statistical analyses of the different extract concentrations and control were compared independently for each timepoint. Statistically significant differences ($p < 0.05$) between the means are indicated by upper-case letters for 24 hr (-UVB) and different lower-case letters for 48 hr (-UVB) and 48 hr (+UVB).

numerous other rooibos polyphenols [24]. It is plausible that, given its significantly higher concentration, aspalathin plays a pivotal role in the antioxidant activity of rooibos, particularly due to its high electron-donating capacity in the ABTS and DPPH radical scavenging assays. In contrast, the honeybush extract, as examined in our study, exhibited significantly lower radical-donating ability in comparison to rooibos (Table 1). Interestingly, it has been reported to have similar characteristics to aqueous extracts of green tea and rooibos [26]. The antioxidant properties of these extracts were further explored in their capacity to scavenge peroxyl radicals, as assessed by the ORAC assay. Rooibos demonstrated superior peroxyl scavenging capabilities compared to honeybush. The hydrogen-donating capacity of rooibos can be attributed to the structural characteristics of its flavones, such as orientin and iso-orientin, as well as flavonols like rutin and iso-quercitrin. The presence of the unsaturated 2,3 bond and the 4-oxo function in the C-ring contributes to radical stabilization [43]. Consequently, rooibos exhibits the potential for higher antioxidant capacity and may have a more significant impact on mitigating oxidative stress.

As shown in Fig. 9, oxidative stress is a significant consequence of chronic UVB exposure and is thus believed to be a potential target for anti-inflammatory agents, such as herbal tea extracts from rooibos and honeybush. UVB induces excess production of ROS, thus causing an imbalance in cellular oxygen; as state known as oxidative stress [44,45]. In this regard, glutathione (GSH), glutathione peroxidase (GPX), catalase (CAT) and Nrf2 are prominent markers to evaluate the combating or controlling of oxidative stress [46].

In the current study, total ROS production was increased by UVB in the keratinocytes by more than 50 % and only significantly reduced by rooibos at 0.2 mg/mL, similar to the antioxidant properties of *Antidesma thwaitesianum* fruit extracts in a keratinocyte/UVB model [50]. The UVB-induced reductions in the antioxidant marker GSH, as well as *GPX1* and *CAT* gene expression, were effectively counteracted and restored to pre-UVB levels by both extracts. Notably, honeybush extract demonstrated greater efficacy as both concentrations exhibited a restorative response on all three biomarkers. In contrast, the lower concentration of rooibos extract (0.1 mg/mL) was effective on all three biomarkers, while the higher concentration did not exert the same protective effect. This phenomenon may be attributed to the possibility that the heightened concentration of rooibos extract is conducive to a pro-oxidative role. The recovery of GSH levels correlates to a previous study wherein chalcone derivatives from rooibos returned GSH to levels prior to UVB exposure in keratinocytes as well as in melanocytes [51]. Furthermore, UVB induced reduction of antioxidant enzyme (CAT and SOD) activity were shown to be restored by honeybush treatment in HaCaT cells in a previous study [52]. The overall *in vitro* antioxidant activity of honeybush and rooibos derived herbal extracts have been show previously by demonstrating their modulation of GSH levels [51] and *GPX1* [53] and *CAT* [52] expression in HaCaT cells and melanocytes.

Nrf2 is another important transcription factor in the regulation of ROS production. Oxidative stress triggers the binding of Nrf2 to genes encoding for antioxidant enzymes such as glutathione synthase and glutamate-cysteine ligase catalytic subunit [54]. Therefore, the upregulation of Nrf2 is important in regulating oxidative stress by aiding in the removal of excess ROS [47]. The expression of Nrf2 was increased more than 3-fold by UVB exposure in the current study. This was also shown in a study investigating the amelioration of UVB-induced inflammation by elderberry [45]. Rooibos at 0.2 mg/mL further increased the expression of Nrf2, indicating an increased potential to promote the antioxidant response mechanisms. The interplay between Nrf2 and NF-kB, regarding inflammation, is of interest in relation to the effect of the two herbal extracts as well as dose-dependent effects. For example, rooibos at 0.2 mg/mL decreased the activation of NF-kB, while also increasing the expression of Nrf2. This is in contrast to the lower dose of rooibos (0.1 mg/mL) which elicited an opposite response. Therefore, this dose interaction within the same herbal extract is of interest which could play a role in either promoting or inhibiting the inflammatory response.

The antioxidant properties of the extracts have therefore been implicated to play a determining role in the modulation of inflammation [55,56] Several mechanisms have been proposed whereby polyphenols inhibit UVB-induced cytokine induction in keratinocytes thereby reducing inflammation. These are mainly related to the induction of oxidative stress and the activation of the Toll-like receptors and NF-κB through signal transduction [57]. It is known that polyphenols protect against the depletion of antioxidants in membranes due to their low redox potential [58], thus assisting in the removal of excess ROS and leading to a decrease in the activation of NF-κB (Fig. 9). The inhibition of UVB-induced NF-κB activation by both extracts, with honeybush showing stronger activity than rooibos, was demonstrated in this study, thus showing the anti-inflammatory ability of both extracts in keratinocytes. Of the rooibos flavonoids, luteolin, quercetin and its rutinoside, rutin, have been reported to reduce the UVB-induced expression of

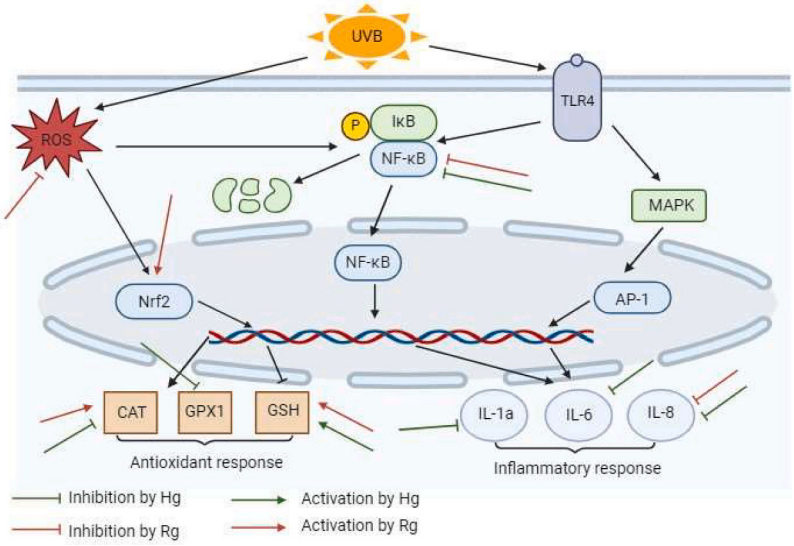

Fig. 9. A simplified schematic of the pathway critical to the activation of the antioxidant and inflammatory responses. Exposure to UVB increases the production of ROS, which activates the antioxidant response via the activation of NF-kB and an increase in Nrf2. Toll-like receptors 4 is also activated by UVB exposure and via the MAPK pathway and the activation of NF-kB, which enhances the inflammatory response [47-49].

NF-κB in keratinocytes without affecting ATP production and cell viability [59–61]. Mangiferin, the major xanthone present in honeybush, has also shown inhibitory effects against the activation of NF-κB, associated with chronic inflammation in mice [62,63].

When evaluating the anti-inflammatory properties of the extracts in the pre-UVB model, both rooibos and honeybush extracts validated their anti-inflammatory activity against UVB-induced intracellular IL-1α protein expression and provided evidence that the herbal tea extracts directly inhibited UVB-induced gene expression of this pro-inflammatory cytokine. The anti-inflammatory effect of the herbal tea extracts against IL-1α was associated with minimal cytotoxic effects against cell viability and apoptosis. These results provide novel insight into the anti-inflammatory mechanisms of rooibos and honeybush extracts in UVB exposed cells by showing that the extracts directly inhibit the synthesis of IL-1α without affecting cell viability as opposed to decreasing IL-1α levels through the removal of damaged cells via apoptosis [15]. Of interest, the anti-inflammatory activity of both herbal extracts did not elicit a strong response with regards to the antagonist, IL-1ra. In addition, rooibos also did not alter the release of IL-6. Aspalathin and nothofagin, from rooibos, are known anti-inflammatory compounds that supress the production of IL-6 and TNF-α *in vitro* and *in vivo* [64]. In the current study rooibos only had significant effects on the gene expression of IL-1α (both doses) and IL-6 (0.1 mg/mL). A differential effect was observed on IL-8 gene expression, with the 0.1 mg/mL dose decreasing expression and the 0.2 mg/mL dose increasing expression. This is a response specific to rooibos, and one could postulate that at a higher dose rooibos could be affecting other signalling pathways involved in inflammation thereby exacerbating the pro-inflammatory signalling from UVB exposure. This shows the importance of concentration selection with regards to rooibos and its anti-inflammatory activity.

The honeybush extracts demonstrated greater efficacy in mitigating the UVB-induced elevation of all cytokine levels and corresponding gene expression. Generally, both concentrations of honeybush extract exhibited similar reductions in most cytokines, with the exception of the 0.2 mg/mL concentration that had a significantly greater impact on IL-6 levels and its gene expression. The major polyphenolic constituent in honeybush, mangiferin, has been shown to have anti-inflammatory activity via its inhibitory effect on NF-κB and its subsequent suppression of IL-6 expression in mice [62,65].

It has also been shown that rooibos polyphenols, aspalathin and nothofagin, inhibit the lipopolysaccharide (LPS)-induced expression of toll-like receptors, which in turn reduces the activation of NF-κB [66]. The inhibition of LPS-induced toll-like receptor expression by mangiferin, from honeybush, has also been demonstrated in mice [65]. Thus, the effects of whole extracts of rooibos and honeybush on UVB-induced toll-like receptor expression could shed further light on the mechanism of action of these extracts in their ability to reduce the inflammatory response.

Several limitations should be considered in the interpretation of our findings. Firstly, the use of HaCaT cells as an experimental model may not fully replicate the intricate physiological responses of primary keratinocytes *in vivo*, potentially compromising the translational relevance of the results. Secondly, the selected UVB radiation dose may not accurately mirror real-world exposure scenarios, introducing discrepancies in the observed effects. Additionally, the interpretation of anti-inflammatory effects observed *in vitro* may not capture the complexity of inflammatory processes *in vivo*, where multiple cell types and signaling pathways are involved. These limitations underscore the need for cautious interpretation and further validation of our findings.

In conclusion, the current study highlighted the antioxidant and anti-inflammatory properties of honeybush and rooibos aqueous extracts, their total polyphenol content as well as differences in polyphenol components. The rooibos extract had a greater antioxidant response, including activation Nrf2, whereas honeybush seemed more effective in attenuating the UVB-induced inflammatory cytokine response and corresponding gene expression. However, it also appeared that the effects observed are concentration or dose-related, which is more evident with rooibos than with the honeybush extract. The differences also show the importance of the careful consideration needed when selecting herbal tea extract concentrations in the investigation of their effects *in vitro*.

CRediT authorship contribution statement

Lana Keet: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Tandeka Magcwebeba:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Stefan Abel:** Writing – review & editing, Visualization, Supervision, Formal analysis, Conceptualization. **Ann Louw:** Writing – review & editing, Writing – original draft, Supervision. **Wentzel Gelderblom:** Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Mariska Lilly:** Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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