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CONCISE COMMUNICATION

WILEY Experimental Dermatology

Gromwell (*Lithospermum erythrorhizon*) root extract protects against glycation and related inflammatory and oxidative stress while offering UV absorption capability

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Abstract

Glycation and advanced glycation end products (AGE) damage skin which is compounded by AGE-induced oxidative stress and inflammation. Lip and facial skin could be susceptible to glycation damage as they are chronically stressed. As Gromwell (*Lithospermum erythrorhizon*) root (GR) has an extensive traditional medicine history that includes providing multiple skin benefits, our objective was to determine whether GR extract and its base naphthoquinone, shikonin, might protect skin by inhibiting glycation, increasing oxidative defenses, suppressing inflammatory responses and offering ultraviolet (UV) absorptive potential in lip and facial cosmetic matrices. We show GR extract and shikonin dose-dependently inhibited glycation and enhanced oxidative defenses through nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element activation. Inflammatory targets, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and tumor necrosis factor alpha, were suppressed by GR extract and shikonin. Glyoxalase 1 (GLO1) and glutathione synthesis genes were significantly upregulated by GR extract and shikonin. GR extract boosted higher wavelength UV absorption in select cosmetic matrices. Rationale for the use of GR extract and shikonin are supported by our research. By inhibiting glycation, modulating oxidative stress, suppressing inflammation and UV-absorptive properties, GR extract and shikonin potentially offer multiple skin benefits.

KEYWORDS
carbonyl stress, cell culture, colour cosmetics, skin care, UV absorption

1 | BACKGROUND

Gromwell (*Lithospermum erythrorhizon)* root's (GR) extensive traditional history, dating to China's Ming dynasty (1368-1644), includes skin benefits and utilization as a natural fabric dye.[1,2] Naphthoquinones, including shikonin, have been identified as the major bioactive phytochemicals and contribute to GR's unique colour and odour.[1,3] GR and shikonin possess health benefits including: repression of hepatic lipogenesis through AMP-activated protein kinase (AMPKα) regulation;[4] treatment for autoimmune diseases; neuro- and cardioprotective effects; chemotherapeutics; glucose metabolism; and antimicrobial activity.[3] In skin, GR extracts protect against UVB-induced inflammatory and apoptotic signals in human keratinocytes;[5] reduce inflammation and increase ceramide production in atopic dermatitis;[6] improve moisturization and barrier function;[7] and promote wound healing.[8]

Glycation is the non-enzymatic reaction between carbonyl compounds and proteins, lipids and nucleic acids. Once formed, advanced glycation end products (AGE) can generate reactive oxygen species (ROS) and inflammatory responses through the receptor for advanced glycation end products (RAGE) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling.[9,10] Glycation and AGE can also be enhanced by ROS and induce ROS generation,[11] while age, genetics and UV exposure accelerate skin glycation.[12] In skin models, AGE reduced dermal thickness, enhanced epithelial proliferation,[13] altered collagen fibre mechanical dynamics[14] and disrupted barrier homoeostasis.[15] AGE can cause skin discolouration,[16] loss of elasticity,[17] tissue damage and accelerate the ageing phenotype.[18]

Lip skin is unique from surrounding facial skin. The lip transitions from keratinized to mucosal epithelium, and dependent on the anatomical zone, lack hair follicles, sweat glands and often sebaceous glands.[19–21] The lip has distinct barrier properties indicated by elevated transepidermal water loss (TEWL), reduced hydration, elevated blood flow and increased surface temperature.[22,23] With ageing, lip shape transitions from a voluminous youthful appearance to a thinner elongated profile accompanied by fragmented collagen and elastin, and atrophied epidermal and dermal layers.[24] Lip and facial skin are constantly exposed to solar radiation, pollution, climate and intrinsic stressors.[25] Given the unique structure of the lip, and its susceptible barrier integrity, it is plausible AGE and their associated oxidative and inflammatory stresses will be more detrimental to lip tissue at an earlier age than to other facial features.

2 | QUESTIONS ADDRESSED

Our objective was to determine whether GR extract and shikonin would inhibit glycation and glycation-related inflammation pathways, and activate antioxidant defenses. Given the complex chemistry of GR and its unique ability to provide natural skin colour enhancement, research expanded to evaluate its potential to provide UV absorption in lip and facial cosmetic applications.

3 | EXPERIMENTAL DESIGN

The biological activity of GR extract and shikonin was assessed by: a fluorometric, biochemical glycation assay involving ribose and albumin incubated under accelerated conditions; cell-based luciferase reporter assays to measure the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway and NFκB activation; ELISA for the quantification of tumor necrosis factor alpha (TNFα) secretion following cell treatment with a TNFα inducer; and qRT-PCR for gene expression. All cell-based assays were developed and validated to maximize sensitivity and robustness. The UV predictive performance of cosmetic matrices containing GR extract was analysed using in vitro UV-A solar simulation and a UV transmittance analyser. Detailed material and method descriptions are in the Supporting Information (Appendix S1 and Table S1).

4 | RESULTS

GR extract and shikonin dose-dependently inhibited the formation of glycation intermediates between ribose and albumin (Figure 1A). Maximum glycation inhibition for GR extract, shikonin and aminoguanidine (AG) was 87%, 83% and 88%, respectively. Calculated IC_{50}

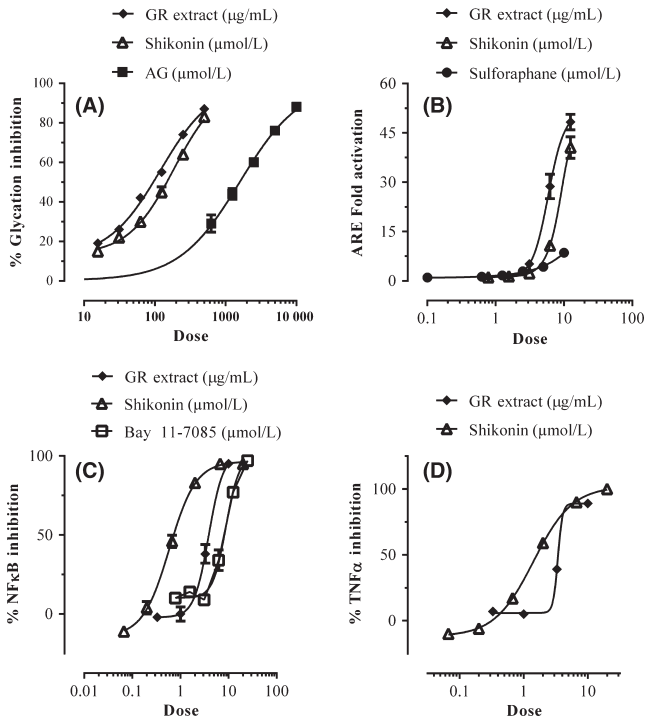

FIGURE 1 Dose-Response Curves of GR Extract and Shikonin. A, Glycation inhibition as measured when ribose and BSA, with and without treatments, were incubated at 65°C for 48 h, followed by fluorescence measurement (340 ex/410 em). GR extract was tested from 0 to 500 μg/mL, shikonin from 0 to 500 μmol/L and AG from 0 to 10 mmol/L. Data are expressed as mean inhibition ± SEM and are from two independent experiments performed in triplicate. B, Nrf2/ARE pathway activation by GR extract (0-25 μg/mL) and shikonin (0-25 μmol/L). HepG2 cells were stably transfected with multiple repeats of the ARE consensus sequence and a luciferase reporter construct and treated for 48 h. Following cell lysis, d-luciferin substrate was added and luminescence was measured. Results are expressed as mean fold change ± SEM compared with a vehicle-only (media and DSMO) treated control. Data were from two independent experiments performed in triplicate. C, Inhibition of NFκB activation. A549 cells were stably transfected with an NFκB response element luciferase construct and treated with GR extract (0.3-10 μg/mL) or shikonin (0.08-20 μmol/L) prior to NFκB activation with IL-1β (50 pg/mL). The cells were treated for 6 h and then lysed, and d-luciferin substrate was added. Data are expressed as mean per cent inhibition ± SEM, with background response (media only) subtracted from all treatments, compared with positive control cells treated with IL-1β only. D, Suppression of TNFα secretion. THP-1 cells were treated for 18 h with GR extract (0.3-10 μg/mL) or shikonin (0.08-20 μmol/L) and Pam3Csk4 (10 ng/mL). Cell culture supernatants were assayed for the presence of TNFα by ELISA. Results are from three separate experiments run in triplicate, and data are reported as mean per cent inhibition ± SEM

values for GR extract, shikonin and AG were 119 μg/mL, 186 μmol/L (54 μg/mL) and 1.6 mmol/L (197 μg/mL), respectively. GR extract was approximately two-fold more potent, and shikonin was >3-fold more potent than AG at inhibiting the glycation reaction.

Figure 1B illustrates the dose-dependent activation of the Nrf2/ARE pathway by GR extract and shikonin. GR extract and shikonin activated the Nrf2/ARE pathway comparable to sulforaphane. The concentrations that resulted in a 50% activation (EC50) of the Nrf2/ARE pathway were 5.9 μg/mL for GR extract, 8.5 μmol/L (2.5 μg/mL) for shikonin and approximately 10 μmol/L (1.8 μg/mL) for sulforaphane, which had an ambiguous GraphPad Prism curve fit.

GR extract and shikonin dose-dependently suppressed IL-1β—induced activation of NFκB in A549 cells (Figure 1C). GR extract and shikonin completely blocked IL-1β—induced NFκB activation. While GR extract had similar potency to the positive control Bay 11-7085, shikonin was found to have over a 12-fold lower IC50 value than the control. The IC50 values for GR extract, shikonin and Bay 11-7085 were 3.9 μg/mL, 0.6 μmol/L (0.17 μg/mL) and 8.8 μmol/L (2.2 μg/mL), respectively. Consistent with these results, GR extract and shikonin dose-dependently inhibited Pam3Csk4 stimulation of TNFα production in THP-1 cells (Figure 1D). GR extract had nearly a 90% effect at inhibiting TNFα secretion at a dose of 10 μg/mL, with an IC50 value of 3.4 μg/mL. Shikonin's maximal inhibition of TNFα secretion was 100% at 20 μmol/L (5.8 μg/mL), with an IC50 value of 1.4 μmol/L (0.40 μg/mL).

Glyoxalase I (GLO1), glutamate-cysteine ligase (GCLC) and glutamate-cysteine ligase modifier subunit (GCLM) gene expression levels were measured in NHEK cells following GR extract and shikonin treatment. Significant upregulation occurred for all three genes with both treatments (Figure 2). GR extract (2.5 μg/mL) increased GLO1, GCLC and GCLM expression 3-, 10- and 25-fold, respectively (Figure 2A-C). Shikonin treatment (1 μmol/L) increased GLO1, GCLC and GCLM expression 2.5-, 6- and 15-fold, respectively (Figure 2D-F). The 10 μmol/L dose of shikonin did result in a significant increase in GCLC expression (Figure 2E).

Table S2 displays the critical wavelength (CW) values measured for various lipophilic cosmetic preparations, with and without GR extract. All matrices had critical wavelengths >370 nm.

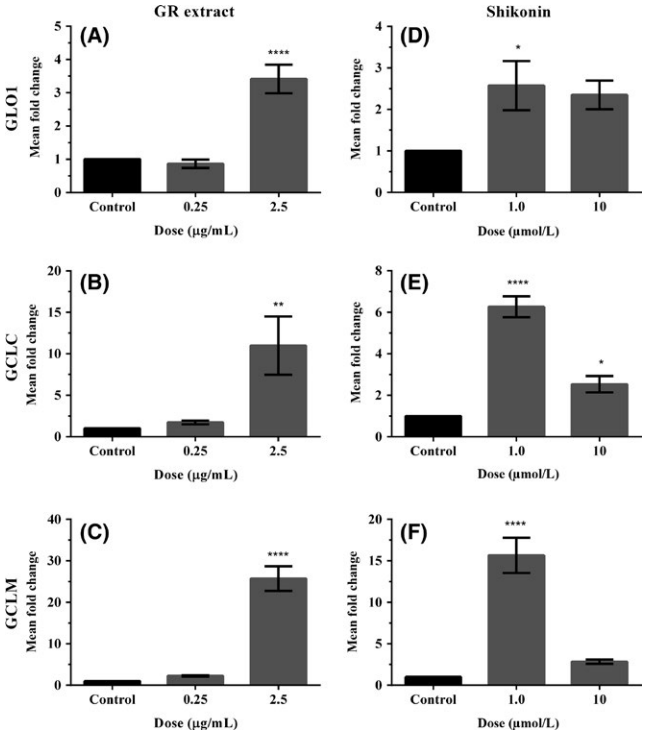

FIGURE 2 Gene Expression Changes of Glycation Detoxification Targets in Response to GR Extract (A-C) and Shikonin (D-F). NHEK cells were plated in six-well plates and treated for 18 h, followed by cell lysis, mRNA isolation and quantitation. One microgram of RNA was reverse transcribed followed by qPCR with primers for GLO1 (A & D), GCLC (B & E) and GCLM (C & F). Results represent mean fold change ± SEM. Comparisons to the control (media only) were performed with Dunnett's multiple comparison test. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{****}P \leq 0.0001$

5 | CONCLUSIONS

Given elevated glycation negatively affects skin health,[26,27] inhibiting glycation and preventing AGE-generated ROS and inflammation are paramount for preserving skin integrity.[25] Due to the chronic external stressors lip and facial skin are exposed to,[25] and lip skin's susceptible barrier and thinner stratum corneum,[23] cosmetics containing GR extract potentially offer protective skin benefits. We show for the first time that GR extract and shikonin inhibit the glycation reaction. In addition, our results showing GR extract and shikonin boosting oxidative defenses through activation of the Nrf2/ARE pathway, suppression of NFκB and TNFα inflammatory targets, and upregulation of detoxification genes support the rationale for GR's traditional uses.

Activation of the Nrf2 pathway and glutathione synthesis prior to glycation promotes detoxification of glycation-related carbonyls.[28] Antioxidant benefits of shikonin have been described,[3] including activation of the Nrf2/ARE pathway.[29] We have shown GR extract possesses similar activation of the Nrf2/ARE pathway, comparable to the known Nrf2/ARE inducer, sulforaphane.[30] GLO1, an ARE-containing glycation detoxification gene,[31] was significantly upregulated by GR extract and shikonin in human keratinocytes. GCLC and GCLM, subunits of glutamate-cysteine ligase (GCL), the rate limiting enzyme for glutathione production,[32] were significantly upregulated in human keratinocytes following GR extract and shikonin treatment. Shikonin's maximal response at 1 μmol/L was followed by noticeable reduction in expression of all three genes at 10 μmol/L. Huang et al[33] showed increased expression of GCLM with 1 μmol/L shikonin treatment in human endothelial cells, while cellular viability was not impacted. Shikonin above 1 μmol/L might activate alternate pathways leading to the reduced expression of GCLC and GCLM, as glutathione synthesis and GCLC and GCLM regulation are highly complex and influenced by several pathways.[32]

The anti-inflammatory properties of GR extract and shikonin are well documented,[1,3,5] and our results showing NFκB suppression

and reduced TNFα generation in cellular models support these findings. Low-dose treatment with GR extract and shikonin completely inhibited NFκB activation and TNFα production, suggesting these are potent inhibitors of inflammatory signalling. RAGE activation by AGE induces an inflammatory cascade involving NFκB, and prolonged inflammatory signalling is implicated in accelerating the ageing phenotype.[34]

UV exposure enhances glycation-related skin ageing[12] and is propagated by the accompanying oxidative and inflammatory stresses.[25] Topical technologies to protect against UV damage are paramount for healthy skin. The Food and Drug Administration's monographed in vitro method requires an integrated absorptive profile for calculation of a topical product's critical wavelength. A calculated critical wavelength of ≥370 nm is necessary to claim broad-spectrum UVA and UVB protection for any new product registration.[35] GR extract, in cosmetic matrices designed for lip and skin applications, or in combination with avobenzone, surpasses this threshold, offering potential broad-spectrum protection. In vivo sun protection factor testing is necessary to assess GR's full sunscreen protective potential.

Through in vitro experimentation, we have shown GR extract and shikonin inhibit glycation. GR extract and shikonin's ability to activate oxidative defense mechanisms and suppress inflammatory targets are critical to protect against glycation, making these naturally derived technologies attractive for cosmetic applications. Coupled with its broad-spectrum UV absorption and natural colourant capabilities, GR extract provides desirable lip and facial skin health benefits.

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CONFLICT OF INTERESTS

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AUTHOR CONTRIBUTION

KG, PA, DF and JK performed experiments, analysed data and assisted in writing the manuscript. JR developed assays. KG and RV wrote the manuscript. JR and RV and assisted in research design and critically revised the manuscript.

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SUPPORTING INFORMATION

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

APPENDIX S1 Materials and methods

TABLE S1 Naphthoquinone composition of the supercritical CO_2 GR extract from Flavex®

TABLE S2 UV predictive performance of cosmetic applications for the skin and lips

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