Journal of Natural Medicines (2021) 75:623–632
https://doi.org/10.1007/s11418-021-01509-x

ORIGINAL PAPER

Ellagitannins from *Rosa roxburghii* suppress poly(I:C)-induced IL-8 production in human keratinocytes

Satoru Takayama[1,2] · Miho Kawanishi[3] · Kosei Yamauchi[3] · Daiki Tokumitsu[4] · Hiroyuki Kojima[2] · Teruaki Masutani[2] · Arunasiri Iddamalgoda[2] · Tohru Mitsunaga[3] · Hiroyuki Tanaka[1,4]

Received: 5 November 2020 / Accepted: 27 March 2021 / Published online: 8 April 2021

Abstract
The anti-inflammatory effects of a 50% aqueous extract of *Rosa roxburghii* fruit (RRFE) and two ellagitannins (strictinin and casuarictin) isolated from the RRFE were evaluated in a cell model of skin inflammation induced by self-RNA released from epidermal cells damaged by UV ray (UVR) irradiation. The RRFE inhibited interleukin-8 (IL-8) mRNA expression in normal human epidermal keratinocytes (NHEKs) stimulated with polyinosinic:polycytidylic acid (poly(I:C)), a ligand of toll-like receptor-3 (TLR-3). The plant-derived anti-inflammatory agents, dipotassium glycyrrhizinate (GK2) and allantoin, had no influence on the IL-8 expression. The purified compounds, strictinin and casuarictin, inhibited the IL-8 mRNA expression and IL-8 release induced in NHEKs by poly(I:C). These ellagitannins were thus found to be responsible for the biological activity exhibited by the RRFE. This study demonstrates that RRFE and isolated RRFE compounds show promise as ingredients for products formulated to improve skin disorders induced by UVR irradiation.

Keywords *Rosa roxburghii* · Strictinin · Casuarictin · Anti-inflammatory effects · TLR-3 · IL-8

Introduction

Ultraviolet rays (UVRs) are the extrinsic factor responsible for the extensive skin damage caused by sunlight. Excessive UVR irradiation, especially UVB, damages the epidermal layer (epidermal cells) and induces inflammation accompanied by erythema (sunburn) and edema in the acute phase [1]. Chronic UVR irradiation over the course of years prolongs inflammation, causing rough skin, spots, dryness, reduced skin elasticity, wrinkles, and peripheral vasodilation [2]. The inflammatory response is an important physiological function in biological defense, but needs to be regulated when excessive or chronic.

When exposed to UVR irradiation, epidermal cells produce large amounts of inflammatory cytokines such as interleukin-1 (IL-1) [3, 4], interleukin-6 (IL-6) [5], tumor necrosis factor-α (TNF-α) [6, 7], and interleukin-8 (IL-8) [8]. These cytokines induce vasodilation in the dermis, which in turn reddens the skin [9] and promotes vascular permeability and the ensuing processes of edema and neutrophil infiltration [10, 11]. Bernard et al. [12] identified non-coding RNAs such as U1-RNA, a molecule with double-stranded domains released by UVR-damaged cells, as an important source of the inflammatory cytokines that lead to erythema. They also found that U1-RNA induced the production of inflammatory cytokines from epidermal cells, a cell type not severely damaged by UVR, via toll-like receptor 3 (TLR-3) [12]. Their experimental results showed that an intradermal injection of U1-RNA induced redness, swelling, and the production of TNF-α and IL-6 in the skin of wild-type mice, but not the skin of TLR-3−/− mice. The production of inflammatory cytokines can be disrupted by inhibiting the uptake of self-RNAs (which act

✉ Satoru Takayama
takayama-satoru@ichimaru.co.jp

✉ Hiroyuki Tanaka
hirotnk@gifu-pu.ac.jp

1 Medical Science Division, United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu City, Gifu 501-1194, Japan

2 Research and Development Department, Ichimaru Pharcos Co., Ltd, 318-1 Asagi, Motosu City, Gifu 501-0475, Japan

3 Department of Applied Life Science, Faculty of Applied Biological Science, Gifu University, 1-1 Yanagido, Gifu City, Gifu 501-1194, Japan

4 Laboratory of Pharmacology, Department of Bioactive Molecules, Gifu Pharmaceutical University, 1-25-4 Daigaku-nishi, Gifu City, Gifu 501-1196, Japan

as damage-associated molecular patterns (DAMPs)) via the endocytosis of epidermal cells, as most TLR-3 in human epidermal cells is expressed in the endosomes [13]. It can also be disrupted by inhibiting the binding between RNA and TLR-3, or by inhibiting downstream signaling after the RNA binds to TLR-3. Disrupting the production of inflammatory cytokines is thus thought to be effective in preventing skin disorders caused by UVR irradiation.

Botanicals are a rich source of biologically efficacious compounds, and botanical-derived products are widely used all over the world as herbal medicines, external preparations, and health foods. Among them, polyphenols containing ellagitannin have attracted great attention for their antioxidant [14], vasorelaxant [15], anti-inflammatory [15, 16], anti-hyperlipidemic [17], and anti-diabetic [18] actions. Ellagitannins are found in only a few fruits, such as strawberry, raspberry, pomegranate, and muscadine grape [19]. Fumagalli et al. [20] reported that two ellagitannins derived from strawberry, casuarictin and agrimoniin, suppressed IL-8 production in TNF-α-stimulated gastric adenocarcinoma cells (AGS) in an in vitro model of human gastritis induced by *Helicobacter pylori* infection.

Rosa roxburghii fruit is rich in tannins, which gives the fruit an astringent character and exerts hemostatic, analgesic, and antiseptic effects that have been applied in folk medicines and cosmetics [21]. Having found that *R. roxburghii* fruit contains ellagitannins such as alnusiin, casuarictin, and tellimagrandin II [22], we have been investigating whether a *R. roxburghii* fruit extract (RRFE) reduces skin inflammation induced by UVR irradiation.

In this study we (1) sought to evaluate whether RRFE reduces the inflammation caused by self-RNAs released from epidermal cells that have been significantly damaged by UVR. We did so by evaluating the effect of the RRFE in suppressing the gene expression of IL-8 using normal human epidermal keratinocytes (NHEKs) stimulated with polyinosinic:polycytidylic acid (poly(I:C)), a ligand of TLR-3 [23]. We also examined whether dipotassium glycyrrhizinate (GK2) [24], a known suppressor of the erythema induced by UVR irradiation, and allantoin [25], a suppressor of eczema, have similar effects. GK2 and allantoin are plant-derived anti-inflammatory agents that have been conventionally formulated in external preparations. (2) To identify the ellagitannins contained in the RRFE, and to show that they were active ingredients, we evaluated the inhibitory effects of the RRFE on IL-8 gene expression and IL-8 protein release.

Materials and methods

Materials

Poly(I:C) was purchased from Sigma-Aldrich (St Louis, MO). Dipotassium glycyrrhizinate (GK2) and allantoin were purchased from Fujifilm Wako Pure Chemical Co., Ltd (Tokyo, Japan). *Rosa roxburghii* fruits harvested in Hubei Province, China in 2017 (Lot No. 79Q4) were purchased from Matsuura Yakugyo Co., Ltd. (Nagoya, Japan).

Preparation of *Rosa roxburghii* fruit extract and fractionation, isolation, and identification of pure compounds from the extract

The *R. roxburghii* fruit extract (RRFE) used for the in vitro testing was prepared by the following method. The fruits were soaked in 50% ethanol (30 times their weight) at room temperature, filtered, and concentrated in an evaporator. The RRFE was prepared as a solution by adjusting the solid content to 11.6 mg/ml with 50% ethanol.

The active compounds were isolated and identified by the following steps. *Rosa roxburghii* fruits were soaked in 100% methanol (MeOH) (10 times their weight). The methanol extract (16.9 g) was separated by Sephadex LH-20 gel column (80 mm $\varphi \times 450$ mm) chromatography and eluted with solvent (MeOH: water = 9: 1 v/v) to obtain 8 fractions (Fr. 1–8). Fr. 8 (675.7 mg) was separated by Sephadex LH-20 gel column (35 mm $\varphi \times 450$ mm) chromatography and eluted with solvent (MeOH: water = 9: 1 → 8: 2 → 7: 3 → 6: 4 v/v) to obtain 10 fractions (Fr. 8–1 to Fr. 8–10) (Fig. 1).

Strictinin (CID 73330) (5.1 mg) and casuarictin (CID 73644) (27.6 mg) were isolated from Fr. 8–5 and Fr. 8–10, respectively, using a preparative HPLC (p-HPLC) method [ODS-3 (4.6 mm $\varphi \times 250$ mm) (MeOH: 0.05% TFA aq. = 5%: 95% → 50%: 50% (40 min)].

The isolated compounds were identified by ^{1}H–NMR, ^{13}C–NMR, ^{1}H–^{1}H COSY, HMQC, HMBC (JEOL ECA 600 NMR spectrometer), and MALDI-TOF–MS (Shimadzu Biotech Axima Resonance 2.9.1.20100121).

Strictinin (1–*O*–galloyl–4,6–*O*–hexahydroxy diphenoyl–*β*–D–glucopyranose): λ_{max} (H_2O: MeOH = 71: 29) nm (log ε): 218 (2.94), 269 (2.61). The molecular weight was determined by MALDI-TOF–MS as 657.0975 *m/z* $[M + Na]^+$ (Calcd for $C_{27}H_{22}NaO_{18}$: 657.0698) (Shimadzu Biotech Axima Resonance: Mode positive, Low 100 +, power:120). ^{1}H-NMR (Methanol-d_4, JEOL ECA-600, 600 MHz) δ: Glc, 3.59 (1H, t, *J* = 8.28, H-2), 3.70 (1H, t, *J* = 9.66, H-3), 3.80 (1H, d, *J* = 12.4, H-3), 4.02 (1H, dd, *J* = 5.52, 8.94, H-5), 4.77 (1H, t, *J* = 15.5, H-4), 5.21 (1H, dd, *J* = 6.9, 13.7, H-6α), 5.65 (1H, d, *J* = 8.22, H-1); 4,6-HHDP, 6.53 (1H, s, H-3‴), 6.67 (1H, s, H-3″); galloyl, 7.12 (2H, s, H-2′,6′). ^{13}C NMR ((Methanol-d_4, 150 MHz): δ Glc, 62.9 (C-6α), 71.9 (C-4), 72.3 (C-5), 73.4 (C-2), 74.7 (C-3), 94.9 (C-1); galloyl, 109.2 (C-2′, 6′), 119.2 (C-1′), 139.1 (C-4′), 145.2 (C-3, 5), 165.5 (C-7′); 4,6-HHDP, 106.9 (C-3‴), 107.3 (C-3″), 115.3(C-1‴), 115.5 (C-1″), 125.0, 125.2 (C-2″ or 2‴), 136.0 (C-5‴), 136.3 (C-5″), 143.4, 143.5 (C-4″ or

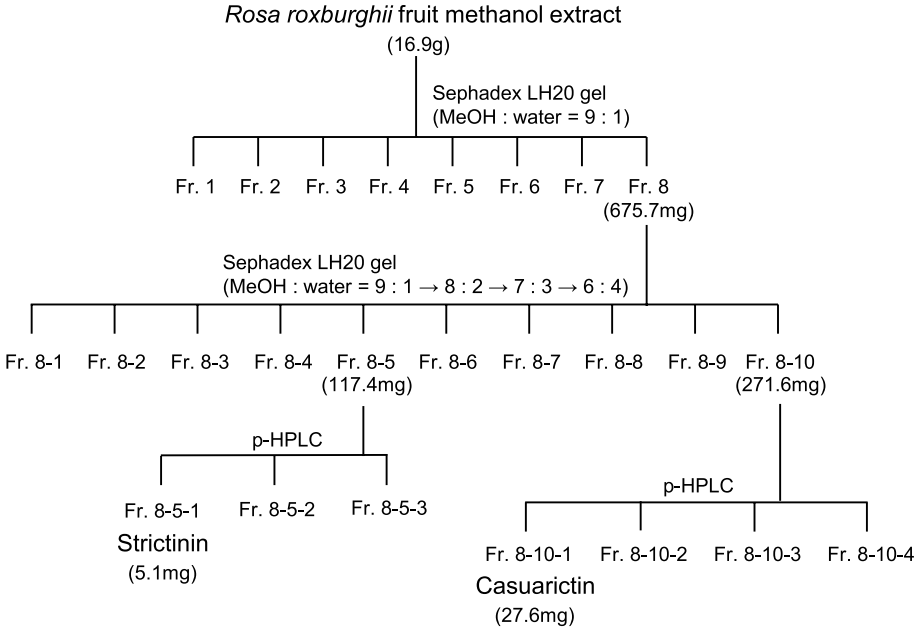

Fig. 1 Scheme for the fractionation and identification of active ingredients in the *Rosa roxburghii* fruit methanol extract

4‴), 144.5, 144.6 (C-6″ or 6‴), 168.3 (C-7″), 168.5 (C-7‴) (Fig. 2a).

Casuarictin (1–*O*–galloyl–2,3–4,6–bis–*O*–hexahydroxydiphenoyl–*β*–D–Glucopyranose): λmax (H_2O: MeOH = 66: 34) nm (log *ε*): 218 (3.11), 265 (2.86); The molecular weight was determined by MALDI-TOF–MS as 959.3599 *m/z* $[M+Na]^+$ (Calcd for $C_{41}H_{28}NaO_{26}$: 959.0761). ^{1}H NMR (Acetone-d_6, 600 MHz) δ Glc, 3.85 (1H, d, *J* = 13.1, H-6), 4.48 (1H, dd, *J* = 6.2, 9.7, H-5), 5.15 (m, H-4), 5.16 (1H, m, H-2), 5.35 (1H, dd, *J* = 6.9, 13.1, H-6), 5.39 (1H, t, *J* = 9.7, H-3), 6.19 (1H, d, *J* = 8.9, H-1); galloyl, 7.15 (2H, s, H-2, 6); 2, 3-HHDP, 6.34 (1H, s, H-3′), 6.43 (1H, s, H-3); 4,6-HHDP, 6.52 (1H, s, H-3), 6.65 (1H, s, H-3′); ^{13}C NMR (Acetone-d6, JEOL ECA-600, 150 MHz): δ ppm Glc, 62.2 (C-6), 68.4 (C-4), 72.7 (C-5), 75.1 (C-2),76.4 (C-3), 91.4 (C-1); galloyl, 109.5 (C-2,6), 119.1 (C-1), 139.1 (C-4), 145.5 (C-3, 5), 164.2 (C-7); 2, 3-HHDP, 106.5 (C-3″, 3‴), 167.7 (C-7″), 168.4 (C-7‴); 4, 6-HHDP, 106.8 (C-3⁗), 107.5 (C-3⁗′), 167.0 (C-7⁗), 167.2 (C-7⁗′); HHDP, 113.3–115.2 (C-1″, 1‴, 1⁗, 1⁗′), 125.1–125.7 (C-2″, 2‴, 2⁗, 2⁗′), 135.4–135.8 (C-5″, 5‴, 5⁗, 5⁗′), 143.7–143.8 (C-6″, 6‴, 6⁗, 6⁗′), 144.3–144.5 (C-4″, 4‴, 4⁗, 4⁗′) (Fig. 2b).

Table 1 shows the amounts of strictinin and casuarictin contained in the RRFE and Fr. 8.

Cell culture

NHEKs purchased from Kurabo (Osaka, Japan) were grown in serum-free keratinocyte growth medium KBM-2 (Lonza, Walkerville, MD) at a low calcium concentration (0.06 mM) together with bovine pituitary extract (BPE), human recombinant epidermal growth factor (hEGF), insulin, transferrin, hydrocortisone, epinephrine, and gentamicin/amphotericin-B, at 37 ℃ in a humidified atmosphere of 5% CO_2. The medium was changed daily. When near confluence (70–90%), the cells were subcultured with

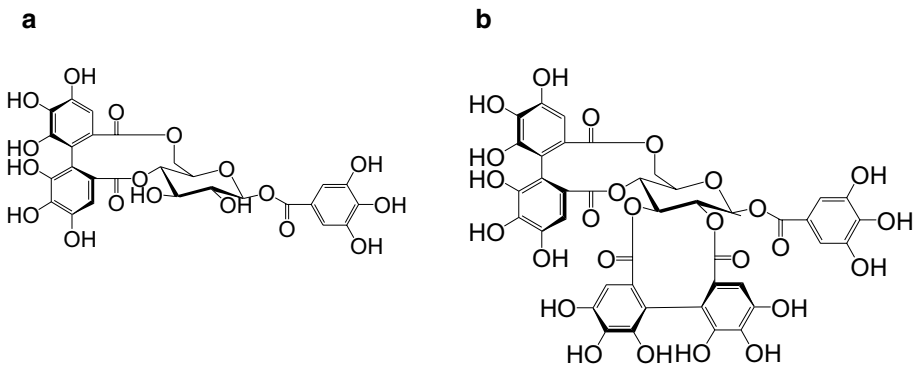

Fig. 2 Structures of the monomeric ellagitannins, **a** strictinin and **b** casuarictin

Table 1 The amounts of strictinin and casuarictin contained in 1 mg of solid component of the RRFE and Fr. 8

	Strictinin (μg)	Casuarictin (μg)
RRFE	4.92	4.26
Fr. 8	7.55	40.85

trypsin/ethylenediaminetetraacetic acid (EDTA). Cells from passages 3–6 were used for the experiments.

Treatment of the cells

When the NHEKs reached 80% confluence, the medium was replaced with KBM medium without hydrocortisone and epinephrine. After further culture for 24 h, the cells were treated with the RRFE at the indicated concentrations for 1 h and then exposed to poly(I:C) (1 μg/ml) for 4 h for gene expression analysis or for 24 h for protein expression analysis.

RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR)

After the cultured NHEKs were rinsed, total RNA was extracted with an RNeasy kit (QIAGEN) and quantified using NanoDrop software (Thermo Fisher Scientific, USA). cDNA was prepared from 50 ng of total RNA using a Prime Script RT Reagent Kit (Takara Bio, Japan) for RT-PCR. The RT-PCR procedure consisted of a reverse transcription reaction at 37 °C for 15 min followed by enzyme inactivation at 85 °C for 5 s.

Real-time quantitative PCR

Real-time quantitative PCR was performed using each specific primer and SYBR Premix EX Taq II (Takara Bio, Japan). IL-8 (forward primer sequence of 5′–CCACACTGC GCCAACA–3; reverse primer sequence of 5′–GCATCT TCACTGATTCTTGGAT–3) and ribosomal protein S18 (RPS18) for internal control (forward primer sequences of 5′–TTTGCGAGTACTCAACACCAACATC–3′; reverse primer sequence of 5′–GAGCATATCTTCGGCCCA CAC–3′) were purchased from Hokkaido System Science, Japan. The stability of RPS18 in the cells showed no variation under any of conditions used in this study. Real-time fluorescence detection was performed using a Thermal Cycler Dice Real Time System (Single PCR) (Takara Bio, Japan). The PCR cycling conditions were as follows: 95 °C for 30 s followed by 40 cycles at 95 °C for 5 s and 54–60 °C for 30 s.

Enzyme-linked immunosorbent assay (ELISA)

After the treatment, the cell supernatant was collected and stored at − 80 °C until measurement. Secreted IL-8 levels were estimated by ELISA assays using ELISA kits (R&D Systems, USA) according to the manufacturer's instructions.

Statistical analysis

The results were analyzed using SPSS 22.0 (IBM, USA). The data were collected from at least three independent experiments and expressed as means ± SD. For all results, the data were analyzed by analysis of variance (ANOVA). To perform multiple comparisons, Dunnett's test was used post hoc after ANOVA. A p value of < 0.05 was considered statistically significant. The levels of statistical significance were indicated as: *, $p < 0.05$; **, $p < 0.01$.

Results

RRFE suppresses IL-8 gene expression in poly(I:C)-treated NHEKs

We investigated whether the RRFE affected IL-8 gene expression in poly(I:C)-treated NHEKs. Poly(I:C) induced the expression of IL-8 in NHEKs significantly, and the RRFE suppressed IL-8 gene expression in a concentration-dependent manner (Fig. 3a). GK2 and allantoin, two anti-inflammatory components widely used in topically applied skin agents, showed no inhibitory effects on IL-8 gene expression (Fig. 3b and c).

Isolation and identification of the active compounds in RRFE that inhibit IL-8 gene expression

Upon observing the inhibitory activity of the RRFE on IL-8 gene expression (Fig. 3a), we decided to fractionate the extract to isolate the active compounds. Sephadex LH-20 gel column chromatography of the *R. roxburghii* fruit methanol extract yielded 8 fractions (Fig. 1). IL-8 gene expression was inhibited in the NHEKs treated with Fr. 5–8 (Fig. 4). Fr. 8, a fraction with high activity and low impurities, was further fractionated, and two hydrolyzed tannins, namely, strictinin and casuarictin, were isolated.

Strictinin and casuarictin suppress IL-8 gene expression and IL-8 release in poly(I:C)-treated NHEKs

We investigated the effects of strictinin and casuarictin on IL-8 gene expression and IL-8 release in NHEKs treated with poly(I:C). Each tannin suppressed IL-8 gene

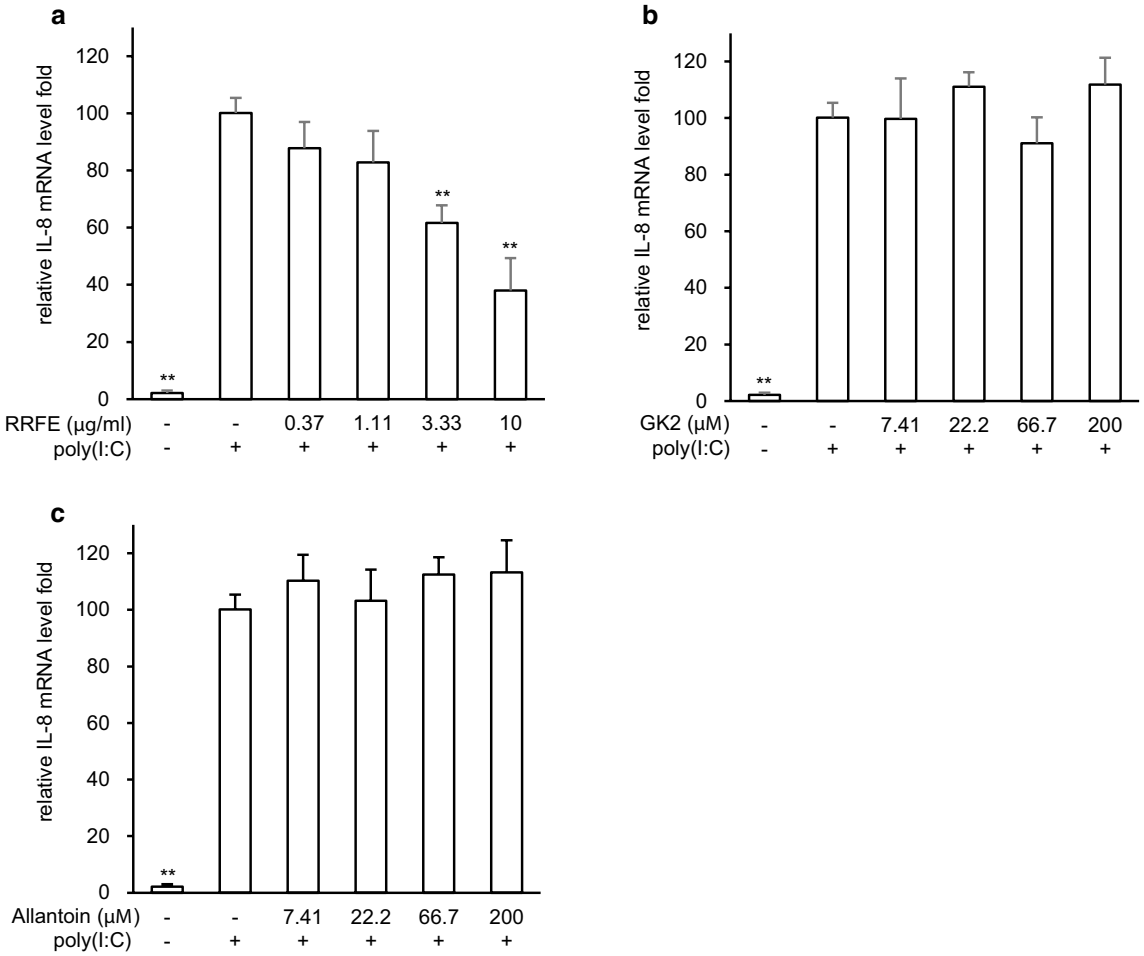

Fig. 3 Effects of RRFE, GK2, and allantoin on IL-8 gene expression in poly(I:C)-treated NHEKs. NHEKs were pretreated with **a** RRFE (0.37–10 μg/ml), **b** GK2 (7.41–200 μM), or **c** allantoin (7.41–200 μM) for 1 h and stimulated with poly(I:C) (1 μg/ml) for 4 h. The IL-8 mRNA expression was analyzed by quantitative real-time PCR. The relative expression level of IL-8 in the poly(I:C)-treated NHEKs was normalized by the housekeeping gene, RPS18. Data are expressed as means ± standard deviation ($n = 4$). $^{**}p < 0.01$ versus poly(I:C) alone, Dunnett's test

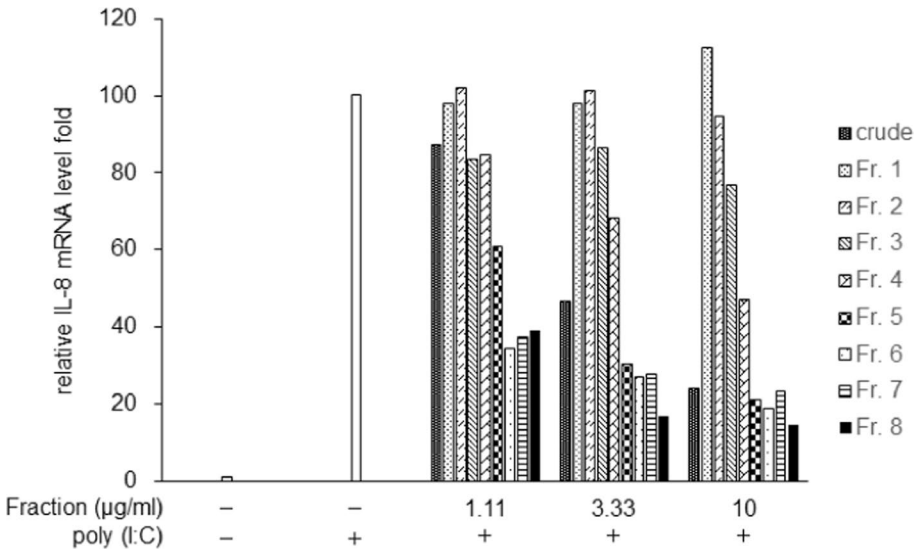

Fig. 4 IL-8 inhibitory activity of RRFE fractions obtained by Sephadex LH-20 gel column chromatography. NHEKs were pretreated with a methanol extract (crude) or fraction (1.11–10 μg/ml) for 1 h and then stimulated with poly(I:C) (1 μg/ml) for 4 h. The IL-8 mRNA expression level was analyzed by quantitative real-time PCR. One value is shown for each fraction concentration ($n = 1$)

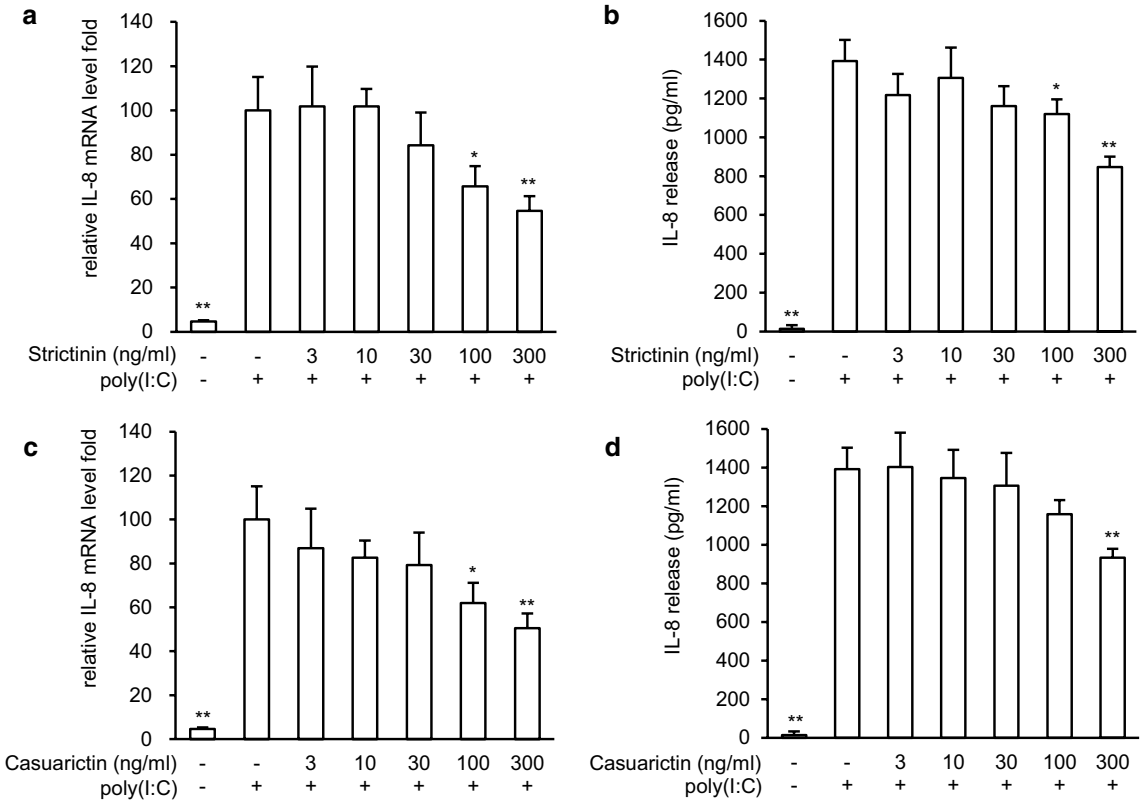

Fig. 5 Effects of strictinin and casuarictin on IL-8 gene expression and IL-8 release in poly(I:C)-treated NHEKs. NHEKs were treated with strictinin or casuarictin for 1 h and incubated **a**, **c** for 4 h or **b**, **d** for 24 h in the presence of 1 μg/ml poly(I:C). The IL-8 mRNA expression was analyzed by quantitative real-time PCR. The IL-8 protein in the supernatant was quantified by the IL-8 ELISA method. Data are expressed as means ± standard deviation ($n = 4$). *$p < 0.05$, **$p < 0.01$ versus poly(I:C) alone, Dunnett's test

Table 2 The amounts of strictinin and casuarictin contained in the RRFE

RRFE (μg/ml)	0.37	1.11	3.33	10
Strictinin (ng/ml)	1.83	5.47	16.4	49.2
Casuarictin (ng/ml)	1.58	4.73	14.2	42.6

Table 3 The amounts of strictinin and casuarictin contained in Fr. 8

Fr. 8 (μg/ml)	1.11	3.33	10
Strictinin (ng/ml)	8.4	25.2	75.5
Casuarictin (ng/ml)	45.4	136.2	408.5

expression in the poly(I:C)-treated NHEKs when applied at concentration above 100 ng/mL (Fig. 5a, c). A similar tendency was observed in the suppression of IL-8 release (Fig. 5b, d).

On the other hand, the amounts of strictinin and casuarictin contained in the RRFE and Fr. 8 that significantly suppressed IL-8 gene expression in the poly(I:C)-treated NHEKs were lower than the amounts of strictinin and casuarictin that achieved comparable effects when administered alone (Tables 2, 3). We speculated that the coexistence of strictinin and casuarictin in the RRFE or Fr. 8 may have strengthened the suppression of IL-8 gene expression through a synergistic effect. To investigate, we conducted an experiment to confirm whether the combined use of strictinin and casuarictin affected the IL-8 gene expression and IL-8 production in poly(I:C)-treated NHEKs.

When the strictinin-to-casuarictin ratio was set to 1:1 in the RRFE, IL-8 gene expression and IL-8 release were both significantly inhibited by the compounds applied in combination at 100 ng/ml concentrations (Fig. 6a, b). While we also expected to see significant effects from the compounds in combination at the lower concentrations of 30 ng/ml, IL-8 gene expression was not inhibited under that condition (Fig. 6a, b). When the strictinin-to-casuarictin ratio was set to 1:5 in Fr. 8, the combined compounds applied at respective concentrations of 10 ng/ml and 50 ng/ml showed additive and significant inhibitory effects on IL-8 gene expression and IL-8 release (Fig. 6c, d).

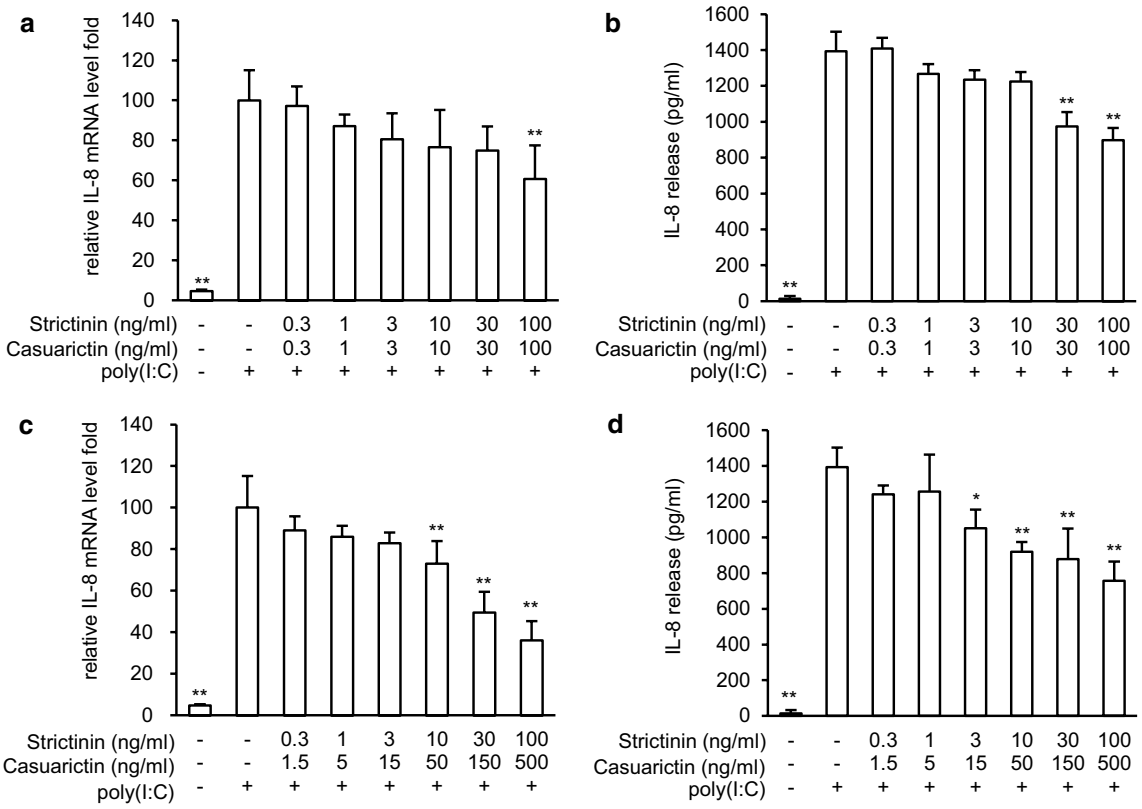

Fig. 6 Effects of the combined treatment of strictinin and casuarictin on IL-8 gene expression and IL-8 release in poly(I:C)-treated NHEKs. The NHEKs were treated with strictinin and casuarictin in combination for 1 h at concentration ratios of **a**, **b** 1:1 and **c**, **d** 1:5 and incubated **a**, **c** for 4 h and **b**, **d** for 24 h in the presence of 1 μg/ml poly(I:C). The IL-8 mRNA expression was analyzed by quantitative real-time PCR. The IL-8 protein in the supernatant was quantified by the IL-8 ELISA method. Data are expressed as means ± standard deviation ($n = 4$). $*p < 0.05$, $**p < 0.01$ versus poly(I:C) alone, Dunnett's test

Discussion

The RRFE significantly suppressed IL-8 gene expression in poly(I:C)-stimulated NHEKs in our experiments (Fig. 3). Chemokines are generally thought to be important mediators of UV-induced inflammatory responses [26]. The production of IL-8 in UVB-irradiated epidermal cells has been reported both in vitro [8, 27] and in vivo [28]. IL-8 effectively activates neutrophils, migrates to the site of inflammation, and exacerbates the inflammation by generating a cytokine storm. Our results indicate that the topical application of RRFE to skin before sun exposure can block the cytokine cascade by suppressing IL-8 production in the UV-damaged epidermis, and thereby reduce skin inflammation.

Casuarictin has been reported to suppress IL-8 production by inhibiting the NF-κB pathway in AGS stimulated with TNF-α [20]. Both the casuarictin and strictinin contained in the RRFE in our present experiments suppressed IL-8 gene expression in NHEKs stimulated by poly(I:C), a TLR-3 ligand (Fig. 5). While it was reported that strictinin inhibited the NO production and iNOS expression in the macrophage-like cell line, RAW 264.7, and that orally administered strictinin contained in *Pimenta racemosa* leave extract exhibited anti-inflammatory activity using rat footpad edema induced by carrageenan, our present study is the first report to demonstrate the anti-dermatitis effect at least in poly(I:C) treated NHEKs [29, 30].

Casuarictin has been found to suppress IL-8 expression by strongly inhibiting the NF-κB pathway in TNF-α stimulated AGS, while leaving the activator protein 1 (AP-1) pathway unchanged [20]. In NHEKs stimulated by poly(I:C), in contrast, the AP-1 pathway appears to contribute more strongly to the poly(I:C)-induced IL-8 gene expression than the NF-κB pathway, as the IL-8 expression peaks within 4 h after the poly(I:C) exposure [8]. The early control of AP-1 is thought to be crucial in preventing the exacerbation of inflammation after UVR irradiation, as AP-1 is produced within 30 min after UVR exposure [31], and IL-8 gene expression is first detected within 1 h [8]. A future question to explore is whether strictinin and casuarictin can regulate the expression of AP-1.

Strictinin and casuarictin, the two ellagitannins isolated and identified from the RRFE, inhibited IL-8 gene expression and IL-8 protein release in poly(I:C)-stimulated NHEKs

when administered alone at high concentrations (Fig. 5) or in combination at low concentrations (Fig. 6c, d). Both ellagitannins can thus be considered active ingredients of RRFE. No sufficient effects were observed, meanwhile, in the combination test using the amounts of strictinin and casuarictin contained in RRFE naturally—amounts that were expected to show significant IL-8 gene expression inhibitory activity (Table 2), (Fig. 6a, b). This dose discrepancy is thought to be partly explained by factors in RRFE that enhance the efficacy of strictinin and casuarictin. The search for such factors will be a subject for future study.

GK2 has been reported to directly bind to high-mobility group box-1 protein (HMGB1), a non-histone DNA binding protein and a type of DAMP released by cells during inflammation [32]. Blocking the binding to HMGB1 receptors (TLR-2, 4) [33] and receptors for advanced glycation end products (RAGE) [34] inhibits the NF-κB signal and suppresses the inflammatory response. Allantoin has been reported to suppress carrageenan-induced footpad edema [35], and thus may inhibit the inflammatory response induced by carrageenan via TLR-4 [36, 37]. The absence of any suppressive activity of GK2 or allantoin against the TLR-3-mediated IL-8 expression using poly(I:C) (Fig. 3) is thought to be explained by the binding selectivity of GK2 and allantoin with respect to the pattern recognition receptors.

Dexamethasone, a steroid used for the treatment of sunburn, suppresses IL-8 expression in several ways. Strong IL-8 suppression can take place via the TLR-3-toll/IL-1 receptor domain containing the adapter-inducing interferon-β (TRIF) signaling pathway when epidermal cells are stimulated with poly(I:C) [38], or via other IL-8 production pathways such as the suppression of secondary IL-8 production by IL-1β released from epidermal cells by TLR-3-mediated Caspase-4 activation [39, 40]. While strictinin and casuarictin suppress IL-8 expression via the TLR-3-TRIF signaling pathway, they entirely lack the ability to suppress IL-8 secondarily produced via other pathways. As such, we believe that the IL-8 mRNA expression at 4 h after poly(I:C) stimulation (Figs. 5a, c and 6a, c) was suppressed more strongly than the IL-8 release at 24 h after stimulation (Figs. 5b, d and 6b, d).

While it remains unclear whether strictinin and casuarictin inhibit poly(I:C), suppress endocytosis, or inhibit the signal transduction system of TLR-3, both compounds suppress inflammation originating from self-RNAs released from UVR-damaged cells that resist control by the plant-derived anti-inflammatory agents used previously. It was reported that topical treatment on human reconstituted skin with pomegranate extract, which contains ellagitannnins, punicalin, pedunculagin and punicalagin as its major constituent, resulted in inhibition of UVB-induced formation of cyclobutane pyrimidine dimers (CPD) and 8–dihydro–2′–deoxyguanosine (8–OHdG) [41]. In our clinical trials, it has been shown that pre-application of lotion-containing RRFE-suppressed erythema caused by UV irradiation (data not shown). From these, it is expected that Strictinin, Casuarictin and RRFE have some degree of cutaneous absorption. However, we do not know the exact percutaneous absorption of these compounds, and their measurement is for further study.

We found that the RRFE and its strictinin and casuarictin components suppressed IL-8 expression, a biomarker of the cytotoxicity of UV irradiation induced by TLR-3 ligand. RRFE and its strictinin and casuarictin components are potentially beneficial to the skin, and thus provide a safe and natural strategy for the development of new topical anti-inflammatory agents.

Acknowledgements We are grateful to Prof. Hiroshi Ueda at Gifu University for his critical discussion and advice on the preparation of this manuscript. We would like to thank Mr. Simon Johnson for his skillful assistance in the preparation of this manuscript.

Declarations

Conflict of interests The authors declare no potential conflicts of interest.

References

1. McGregor JM, Hawk JLM (1999) Acute effects of ultraviolet radiation on the skin. In: Freedberg IM, Eisen AZ, Wolff K, Austen KF, Goldsmith LA, Katz SI, Fitzpatrick TB (eds) Dermatology in general medicine, vol 5. McGraw-Hill, New York, pp 1562–1568
2. Gilchrest BA (1989) Skin aging and photoaging: an overview. J Am Acad Dermatol 21:610–613. https://doi.org/10.1016/s0190-9622(89)70227-9
3. Ansel JC, Luger TA, Green I (1983) The effect of in vitro and in vivo UV irradiation on the production of ETAF activity by human and murine keratinocytes. J Invest Dermatol 81:519–523. https://doi.org/10.1111/1523-1747.ep12522862
4. Luger TA, Sztein MB, Schmidt JA, Murphy P, Grabner G, Oppenheim JJ (1983) Properties of murine and human epidermal cell-derived thymocyte-activating factor. Fed Proc 42:2772–2776
5. Urbanski A, Schwarz T, Neuner P, Krutmann J, Kirnbauer R, Kock A, Luger TA (1990) Ultraviolet light induces increased circulating interleukin-6 in humans. J Invest Dermatol 94:808–811. https://doi.org/10.1111/1523-1747.ep12874666
6. Oxholm A, Oxholm P, Staberg B, Bendtzen K (1988) Immunohistological detection of interleukin I-like molecules and tumor necrosis factor in human epidermis before and after UVB-irradiation in vivo. Br J Dermatol 118:369–376. https://doi.org/10.1111/j.1365-2133.1988.tb02430.x
7. Kock A, Schwarz T, Kirnbauer R, Urbanski A, Perry P, Ansel JC, Luger TA (1990) Human keratinocytes are a source for tumor necrosis factor alpha: evidence for synthesis and release upon stimulation with endotoxin or ultraviolet light. J Exp Med 172:1609–1614. https://doi.org/10.1084/jem.172.6.1609
8. Kondo S, Kono T, Sauder DN, McKenzie RC (1993) IL-8 gene expression and production in human keratinocytes and their

modulation by UVB. J Invest Dermatol 101:690–694. https://doi.org/10.1111/1523-1747.ep12371677
9. Clydesdale GJ, Dandie GW, Muller HK (2001) Ultraviolet light induced injury: immunological and inflammatory effects. Immunol Cell Biol 79:547–568. https://doi.org/10.1046/j.1440-1711.2001.01047.x
10. Cooper KD, Duraiswamy N, Hammerberg C, Allen E, Kimbrough-Green C, Dillon W, Thomas D (1993) Neutrophils, differentiated macrophages, and monocyte/macrophage antigen presenting cells infiltrate murine epidermis after UV injury. J Invest Dermatol 101:155–163. https://doi.org/10.1111/1523-1747.ep12363639
11. Ouhtit A, Muller HK, Davis DW, Ullrich SE, McConkey D, Ananthaswamy HN (2000) Temporal events in skin injury and the early adaptive responses in ultraviolet-irradiated mouse skin. Am J Pathol 156:201–207. https://doi.org/10.1016/s0002-9440(10)64720-7
12. Bernard JJ, Cowing-Zitron C, Nakatsuji T, Muehleisen B, Muto J, Borkowski AW, Martinez L, Greidinger EL, Yu BD, Gallo RL (2012) Ultraviolet radiation damages self noncoding RNA and is detected by TLR3. Nat Med 18:1286–1290. https://doi.org/10.1038/nm.2861
13. Vu AT, Chen X, Xie Y, Kamijo S, Ushio H, Kawasaki J, Hara M, Ikeda S, Okumura K, Ogawa H, Takai T (2011) Extracellular double-stranded RNA induces TSLP via an endosomal acidification- and NF-kappaB-dependent pathway in human keratinocytes. J Invest Dermatol 131:2205–2212. https://doi.org/10.1038/jid.2011.185
14. Alvarez-Suarez JM, Dekanski D, Ristic S, Radonjic NV, Petronijevic ND, Giampieri F, Astolfi P, Gonzalez-Paramas AM, Santos-Buelga C, Tulipani S, Quiles JL, Mezzetti B, Battino M (2011) Strawberry polyphenols attenuate ethanol-induced gastric lesions in rats by activation of antioxidant enzymes and attenuation of MDA increase. PLoS ONE 6:e25878. https://doi.org/10.1371/journal.pone.0025878
15. Edirisinghe I, Banaszewski K, Cappozzo J, Sandhya K, Ellis CL, Tadapaneni R, Kappagoda CT, Burton-Freeman BM (2011) Strawberry anthocyanin and its association with postprandial inflammation and insulin. Br J Nutr 106:913–922. https://doi.org/10.1017/S0007114511001176
16. Joseph SV, Edirisinghe I, Burton-Freeman BM (2014) Berries: anti-inflammatory effects in humans. J Agric Food Chem 62:3886–3903. https://doi.org/10.1021/jf4044056
17. Zunino SJ, Parelman MA, Freytag TL, Stephensen CB, Kelley DS, Mackey BE, Woodhouse LR, Bonnel EL (2012) Effects of dietary strawberry powder on blood lipids and inflammatory markers in obese human subjects. Br J Nutr 108:900–909. https://doi.org/10.1017/S0007114511006027
18. Parelman MA, Storms DH, Kirschke CP, Huang L, Zunino SJ (2012) Dietary strawberry powder reduces blood glucose concentrations in obese and lean C57BL/6 mice, and selectively lowers plasma C-reactive protein in lean mice. Br J Nutr 108:1789–1799. https://doi.org/10.1017/S0007114512000037
19. Koponen JM, Happonen AM, Mattila PH, Torronen AR (2007) Contents of anthocyanins and ellagitannins in selected foods consumed in Finland. J Agric Food Chem 55:1612–1619. https://doi.org/10.1021/jf062897a
20. Fumagalli M, Sangiovanni E, Vrhovsek U, Piazza S, Colombo E, Gasperotti M, Mattivi F, De Fabiani E, Dell'Agli M (2016) Strawberry tannins inhibit IL-8 secretion in a cell model of gastric inflammation. Pharmacol Res 111:703–712. https://doi.org/10.1016/j.phrs.2016.07.028
21. Duke JA, Ayensu ES (1985) Medicinal plants of China. Reference Publications Inc, Algonac, Michigan
22. Yoshida T, Chen XM, Hatano T, Fukushima M, Okuda T (1987) Tannins and related polyphenols of Rosaceous medicinal plants. IV. Roxbins A and B from *Rosa roxburghii* fruits. Chem Pharm Bull 35:1817–1822
23. Borkowski AW, Park K, Uchida Y, Gallo RL (2013) Activation of TLR3 in keratinocytes increases expression of genes involved in formation of the epidermis, lipid accumulation, and epidermal organelles. J Invest Dermatol 133:2031–2040. https://doi.org/10.1038/jid.2013.39
24. Puglia C, Rizza L, Offerta A, Gasparri F, Giannini V, Bonina F (2013) Formulation strategies to modulate the topical delivery of anti-inflammatory compounds. J Cosmet Sci 64:341–353
25. Draelos ZD (2016) A pilot study investigating the efficacy of botanical anti-inflammatory agents in an OTC eczema therapy. J Cosmet Dermatol 15:117–119. https://doi.org/10.1111/jocd.12199
26. Borkowski AW, Gallo RL (2014) UVB radiation illuminates the role of TLR3 in the epidermis. J Invest Dermatol 134:2315–2320. https://doi.org/10.1038/jid.2014.167
27. Norris DA, Lyons MB, Middleton MH, Yohn JJ, Kashihara-Sawami M (1990) Ultraviolet radiation can either suppress or induce expression of intercellular adhesion molecule 1 (ICAM-1) on the surface of cultured human keratinocytes. J Invest Dermatol 95:132–138. https://doi.org/10.1111/1523-1747.ep12477877
28. Strickland I, Rhodes LE, Flanagan BF, Friedmann PS (1997) TNF-alpha and IL-8 are upregulated in the epidermis of normal human skin after UVB exposure: correlation with neutrophil accumulation and E-selectin expression. J Invest Dermatol 108:763–768. https://doi.org/10.1111/1523-1747.ep12292156
29. Lee CJ, Chen LG, Liang WL, Wang CC (2010) Anti-inflammatory effects of *Punica granatum* Linne in vitro and in vivo. Food Chem 118:315–322. https://doi.org/10.1016/j.foodchem.2009.04.123
30. Moharram FA, Al-Gendy AA, El-Shenawy SM et al (2018) Phenolic profile, anti-inflammatory, antinociceptive, anti-ulcerogenic and hepatoprotective activities of *Pimenta racemosa* leaves. BMC Complement Altern Med 18:208. https://doi.org/10.1186/s12906-018-2260-3
31. Shah G, Ghosh R, Amstad PA, Cerutti PA (1993) Mechanism of induction of c-fos by ultraviolet B (290–320 nm) in mouse JB6 epidermal cells. Cancer Res 53:38–45
32. Mollica L, De Marchis F, Spitaleri A, Dallacosta C, Pennacchini D, Zamai M, Agresti A, Trisciuoglio L, Musco G, Bianchi ME (2007) Glycyrrhizin binds to high-mobility group box 1 protein and inhibits its cytokine activities. Chem Biol 14:431–441. https://doi.org/10.1016/j.chembiol.2007.03.007
33. Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, Abraham E (2004) Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J Biol Chem 279:7370–7377. https://doi.org/10.1074/jbc.M306793200
34. Liliensiek B, Weigand MA, Bierhaus A, Nicklas W, Kasper M, Hofer S, Plachky J, Grone HJ, Kurschus FC, Schmidt AM, Yan SD, Martin E, Schleicher E, Stern DM, Hammerling GG, Nawroth PP, Arnold B (2004) Receptor for advanced glycation end products (RAGE) regulates sepsis but not the adaptive immune response. J Clin Invest 113:1641–1650. https://doi.org/10.1172/JCI18704
35. Florentino IF, Silva DPB, Galdino PM, Lino RC, Martins JLR, Silva DM, de Paula JR, Tresvenzol LMF, Costa EA (2016) Antinociceptive and anti-inflammatory effects of *Memora nodosa* and allantoin in mice. J Ethnopharmacol 186:298–304. https://doi.org/10.1016/j.jep.2016.04.010
36. Bhattacharyya S, Gill R, Chen ML, Zhang F, Linhardt RJ, Dudeja PK, Tobacman JK (2008) Toll-like receptor 4 mediates induction of the Bcl10-NFkappaB-interleukin-8 inflammatory pathway by carrageenan in human intestinal epithelial cells. J Biol Chem 283:10550–10558. https://doi.org/10.1074/jbc.M708833200
37. Tsuji RF, Hoshino K, Noro Y, Tsuji NM, Kurokawa T, Masuda T, Akira S, Nowak B (2003) Suppression of allergic reaction by lambda-carrageenan: toll-like receptor 4/MyD88-dependent and

-independent modulation of immunity. Clin Exp Allergy 33:249–258. https://doi.org/10.1046/j.1365-2222.2003.01575.x
38. Aries MF, Hernandez-Pigeon H, Vaissiere C, Delga H, Caruana A, Leveque M, Bourrain M, Ravard Helffer K, Chol B, Nguyen T, Bessou-Touya S, Castex-Rizzi N (2016) Anti-inflammatory and immunomodulatory effects of *Aquaphilus dolomiae* extract on in vitro models. Clin Cosmet Investig Dermatol 9:421–434. https://doi.org/10.2147/CCID.S113180
39. Grimstad O, Husebye H, Espevik T (2013) TLR3 mediates release of IL-1beta and cell death in keratinocytes in a caspase-4 dependent manner. J Dermatol Sci 72:45–53. https://doi.org/10.1016/j.jdermsci.2013.05.006
40. Bian ZM, Elner SG, Elner VM (2009) Dual involvement of caspase-4 in inflammatory and ER stress-induced apoptotic responses in human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 50:6006–6014. https://doi.org/10.1167/iovs.09-3628
41. Afaq F, Zaid MA, Khan N, Dreher M, Mukhtar H (2009) Protective effect of pomegranate-derived products on UVB-mediated damage in human reconstituted skin. Exp Dermatol 18:553–561. https://doi.org/10.1111/j.1600-0625.2008.00829.x