

Pterostilbene Supplementation Inhibits Early Inflammatory Response and Oxidative Stress in UVB-Induced BALB/C Mice

(Suplementasi Pterostilbene Merencat Tindak Balas Keradangan Awal dan Tekanan Oksidatif pada Tikus BALB/C yang Diaruh-UVB)

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ABSTRACT

Extended dermal exposure of ultraviolet B (UVB) can induce erythema, hyperpigmentation, epidermal hyperplasia and cancer. Natural active compound such as pterostilbene (PS) is a potential UV-protecting agent as it has broad biological activities. This study aimed to evaluate the photoprotective effect of pterostilbene on ultraviolet-B-induced BALB/c mice. Twenty-four female mice were randomised into four groups (n=6/group): vehicle control (VC); UVB irradiated only (UVB only); UVB irradiation treated with pterostilbene 10 mg/kg (PS10+UVB); (iv) UVB irradiation treated with pterostilbene 20 mg/kg (PS20+UVB). The PS treatments were given for 14 days, and UVB was given at dose 250 mJ/cm² on days 9, 11, and 13 of the treatment periods. The results showed that PS lessened redness and scaling on the skin of UVB-irradiated mice. The skinfold thickness and epidermal thickness in the PS-treated group were significantly reduced ($p < 0.05$) in comparison with those in the UVB only group. The PS10 and PS20 groups (5.927 ± 0.354 and 5.660 ± 0.765 nmol/g, respectively) demonstrated significantly decreased MDA levels ($P < 0.05$) relative to the UVB only group (13.343 ± 1.350 nmol/g). The GSH level in both PS10 (0.555 ± 0.020 μ mol/mg) and PS20 (0.568 ± 0.055 μ mol/mg) groups increased significantly ($p < 0.05$) compared with that in the UVB only group (0.376 ± 0.025 μ mol/mg). SOD activity in the PS20 group (1.388 ± 0.172 U/min/mg) increased significantly ($p < 0.05$) compared with that in the UVB only group (0.561 ± 0.034 U/min/mg). Histological observation showed that PS reduced leukocyte infiltration and epidermal hyperplasia. Hence, oral PS may exert a photoprotective effect by acting as an anti-inflammatory and antioxidant agent on UVB-irradiated mice skin.

Keywords: Inflammation; oxidative stress; photoprotection; pterostilbene; UVB

ABSTRAK

Pendedahan kulit terhadap cahaya ultraungu B (UVB) secara berpanjangan boleh menyebabkan eritema, hiperpigmentasi, hiperplasia epidermis dan kanser. Sebatian aktif semula jadi seperti pterostilbene (PS) berpotensi untuk digunakan sebagai agen perlindungan UV kerana ia mempunyai aktiviti biologi yang luas. Kajian ini bertujuan untuk menilai kesan pemfotolindungan pterostilbene terhadap tikus yang diaruh sinaran UVB. Dua puluh empat ekor tikus betina dibahagikan kepada empat kumpulan secara rawak: kumpulan kawalan negatif (n=6), tanpa sinaran UVB dan rawatan; disinarkan UVB sahaja (n=6); disinarkan UVB dan dirawat dengan pterostilbene 10 mg/kg (PS10+UVB); disinarkan UVB dan dirawat dengan pterostilbene 20 mg/kg (PS20+UVB). Rawatan PS diberikan selama 14 hari, manakala pendedahan kepada UVB diberikan dengan dos 250 mJ/cm² pada hari ke-9, -11, dan -13 dalam tempoh rawatan. PS telah mengurangkan kulit bersisik dan eritema pada tikus aruhan UVB. PS telah mengurangkan ketebalan lipatan kulit tikus secara signifikan ($p < 0.05$) berbanding dengan kumpulan tikus yang disinarkan UVB sahaja. Ketebalan epidermis juga menurun dengan signifikan ($p < 0.05$) berbanding dengan kumpulan UVB sahaja. Kumpulan PS10 (5.927 ± 0.354 nmol/g) dan PS20 (5.660 ± 0.765 nmol/g) telah menunjukkan penurunan paras MDA yang signifikan ($p < 0.05$) berbanding kumpulan tikus yang disinarkan UVB sahaja (13.343 ± 1.350 nmol/g). Aras GSH bagi kumpulan PS10 (0.555 ± 0.02 μ mol/mol) dan PS20 (0.568 ± 0.055 μ mol/mol) telah meningkat secara signifikan ($p < 0.05$) berbanding dengan kumpulan tikus yang disinarkan UVB sahaja (0.376 ± 0.026 μ mol/mol). Kumpulan PS20 (1.388 ± 0.171 U/min/mg) telah menunjukkan peningkatan aktiviti SOD secara signifikan ($p < 0.05$) berbanding dengan kumpulan tikus yang disinarkan UVB sahaja (0.561 ± 0.034 U/min/mg). Pemerhatian histologi menunjukkan bahawa PS mengurangkan penyusupan leukosit dan hiperplasia epidermis. Oleh itu, PS oral boleh memberikan kesan pemfotolindungan dengan bertindak sebagai agen anti-radang dan antioksidan pada kulit tikus yang disinarkan UVB.

Kata kunci: Keradangan; pemfotolindungan; pterostilbene; tekanan oksidatif; UVB

INTRODUCTION

Skin is an external and the most susceptible organ that is continuously exposed to harmful environments such as solar ultraviolet (UV) radiation (Afaq 2011). UV radiation can be divided into three regions, namely, UVA (315-400 nm), UVB (280-315 nm), and UVC (200-280 nm). Amongst these UV regions, UVB is 1,000 times stronger than UVA in causing sunburn (Wacker & Holick 2013). UVB is also highly mutagenic as it can directly damage DNA through the formation of cyclobutane pyrimidine dimers and absorption of photoproducts (Ramasamy et al. 2017). Its radiation can penetrate the basal layer of the epidermis and affect the epidermal cells. UVB can also alter the proliferation, differentiation and metabolism of these cells and cause epidermal hyperplasia (D'Orazio et al. 2013).

Over-exposure of skin to UVB radiation will damage the cells through formation of reactive oxygen species (ROS). Overproduction of ROS may lead to lipid peroxidation of cell membranes and DNA and protein damage to tissues (Afaq & Mukhtar 2006). ROS generation also decreases the levels of endogenous antioxidants, such as catalase, superoxide dismutase and reduced glutathione (GSH), causing an imbalance in the oxidant/antioxidant activities and reducing the ability of the skin to repair the damaged cells due to ROS (Afaq & Mukhtar 2006; Gonzales-Castañeda et al. 2011).

In recent years, natural active compounds have also gained considerable attention in dermatological and cosmeceutical fields due to their broad biological activities. Pterostilbene is a natural active compound that can be isolated from blueberries, leaves of *Vitis vinifera* and heartwood of *Pterocarpus marsupium* and *Pterocarpus santalinus* (Chakraborty et al. 2010; Ma et al. 2019). Pterostilbene is a dimethylated analog of resveratrol; it has two methoxy groups and one hydroxyl group that allow it to have greater lipophilicity and higher potential for cellular uptake in comparison with resveratrol (Kapetanovic et al. 2011). Pterostilbene exerts strong antioxidant, anti-inflammatory, anticancer, and chemo-preventive properties. Pterostilbene has also been reported to inhibit metastatic activity in B16 melanoma cells (Ferrer et al. 2005). However, no study has examined the photoprotective effect of oral supplementation of pterostilbene on an *in vivo* model. Therefore, this study aimed to determine the photoprotective effects of oral pterostilbene in an UVB-induced BALB/c mice model.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Twenty-four female BALB/c mice were purchased from the Faculty of Veterinary Medicine, Universiti Putra Malaysia. The animals were allowed to acclimatise for 1 week and maintained in standard conditions (12 h light/dark cycle and 20.3-23.1 °C) during the experimental cycle and fed with standard laboratory food and water

ad libitum. The animals were kept at the animal house of the Department of Biomedical Science, Centre of Health and Applied Sciences, Universiti Kebangsaan Malaysia. All experimental procedures were reviewed and approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC; UKMAEC NO: FSK/2017/AHMAD ROHI/22-NOV./887-NOV.-2017-JULY-2018) and the guidelines were strictly followed.

The animals were randomly divided into four groups: vehicle control group (n=6), which was not exposed to UVB irradiation and not treated with pterostilbene; the exposure group (n=6), which was exposed to UVB irradiation only; and two treatment groups (n=6), which were exposed to UVB irradiation and treated with 10 and 20 mg/kg pterostilbene, respectively. Pterostilbene treatment was supplemented *via* oral force-feeding for 14 successive days (Park et al. 2012).

UVB IRRADIATION OF MICE

Mice were irradiated by using a UVB lamp at a wavelength of 312 nm and 15 watts (UVP, USA). In brief, mice from both groups (UVB only and pterostilbene treatment groups) were exposed to UVB irradiation for 3 min at a dose of 250 mJ/cm² on days 9, 11, and 13 of treatment after shaving the dorsal part of the skin by using an electric shaver (Phillips, Malaysia) (Park et al. 2012). UVB emission was monitored regularly before each UVB irradiation with an UVX UV radiometer equipped with a UVX-31 sensor (UVP, USA).

OBSERVATION OF SKIN MORPHOLOGY

Visible diversification of the dorsal skin in mice was examined and photographed at the end of the experiment. The dorsal part of the skin was observed for any morphological changes in skin photodamage.

MEASUREMENT OF SKIN OEDEMA

Skinfold thickness was measured to assess skin oedema (Kim 2016). The mid-dorsal skinfold thickness was measured in groups of six mice by lifting them up at the neck and pinching the skin. Skinfold thickness was measured by using a Harpenden skinfold caliper (Baty, United Kingdom).

EVALUATION OF OXIDATIVE STRESS AND ANTIOXIDANT STATUS

Oxidative stress marker malondialdehyde (MDA) was quantified based on the reaction of MDA with thiobarbituric acid at 100 °C to form thiobarbituric acid reactive substances, which were measured at 532 nm (Stocks & Dormandy 1971). Levels of antioxidants such as GSH were measured according to the Ellman method (Ellman 1959), and the level of superoxide dismutase (SOD) was measured based on its capacity to inhibit the reduction of ferricytochrome; the reaction was measured at 560 nm (Beyer & Fridovich 1987).

HISTOLOGICAL OBSERVATION AND DETERMINATION OF EPIDERMAL THICKNESS

The fixed skin specimens were dehydrated with increasing concentrations of ethanol, embedded in paraffin to form paraffin blocks sectioned at 5 μ m thickness and stained with haematoxylin and eosin (H&E) for histological evaluation. The epidermal thickness of stained sections was measured by ImageJ1.49 v software (NIH, Wayn Rasband, USA).

STATISTICAL ANALYSIS

Statistical analysis was conducted using Statistical Package for the Social Sciences (SPSS) version 23.0. The data were analysed by one-way ANOVA, followed by post-hoc Tukey's test to estimate the significance differences between groups. Differences were statistically significant at $p < 0.05$. Data were then expressed as the mean \pm standard error of mean (SEM).

RESULTS

PTEROSTILBENE ATTENUATES UVB-INDUCED EARLY INFLAMMATORY RESPONSE

The effects of pterostilbene supplementation on UVB-

irradiated mice were assessed by observing changes in skin morphology (Figure 1(A)-1(D)). We observed the presence of visible scaly skin in the UVB only group in comparison with the vehicle control group. The UVB only group also responded to erythema, hardening, and thickening of the skin in comparison with the vehicle control group (Figure 1(A) and 1(B)). Interestingly, both pterostilbene groups showed less erythema and no obvious skin scaling in comparison with the UVB only group (Figure 1(B)-1(D)). We also measured the skinfold thickness as an indicator of cutaneous edema. Figure 1(E) shows that the skinfold thickness of the UVB only group (1.725 ± 0.079 mm) increased significantly compared with that in the vehicle control group (1.000 ± 0.035 mm). However, both doses of pterostilbene (10 and 20 mg/kg) significantly inhibited UVB-induced cutaneous edema, showing skinfold thickness (1.200 ± 0.061 and 1.25 ± 0.045 mm, respectively) as compared with the UVB only group (1.725 ± 0.079 mm). In terms of the early inflammatory response, leukocyte infiltration was observed by H&E staining (Figure 1(F)-1(I)). Histological observations proved that the UVB only group showed a marked increase of leukocyte infiltration in the dermal layer of the skin in comparison with the vehicle control. Oral supplementation of pterostilbene decreased leukocyte infiltration in the dermis compared with the UVB only group.

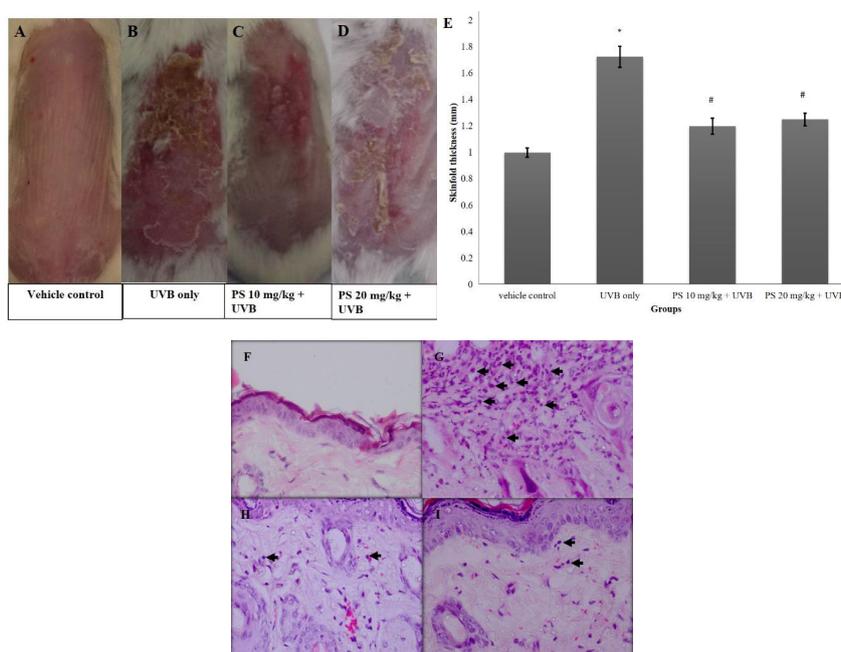


FIGURE 1. Effects of pterostilbene on early inflammatory response in UVB-irradiated mice. (A-D) Skin morphology was observed on day 15. (E). Evaluation of cutaneous oedema by measuring the skinfold thickness. Bar chart shows results of skinfold thickness in different groups represented by the mean \pm standard error mean (S.E.M) (n = 6). * Statistically significant difference in comparison with the vehicle control group ($P < 0.05$). # Statistically significant difference in comparison with the UVB exposure group ($P < 0.05$). (F-I). Histological observation to show leukocyte infiltration (arrow) in the dermis layer by using haematoxylin and eosin staining. (F) Vehicle control group; (G) UVB only group; (H) PS 10 mg/kg + UVB; (I) PS 20 mg/kg + UVB. Pictures are shown at a magnification of 40 \times . Scale bar: 100 μ m

PTEROSTILBENE AMELIORATES UVB-INDUCED
EPIDERMAL HYPERPLASIA

The epidermal thickness was measured and evaluated using ImageJ 1.49v software. The epidermal thickness of the UVB only group ($186.025 \pm 5.742 \mu\text{m}$) increased significantly compared with that of the vehicle control group ($31.466 \pm 2.079 \mu\text{m}$). Interestingly, the epidermal thickness of the 10 ($65.939 \pm 3.616 \mu\text{m}$) and 20 mg/kg ($54.372 \pm 2.354 \mu\text{m}$) pterostilbene groups decreased significantly compared with that of the UVB only group

($186.025 \pm 5.742 \mu\text{m}$). These findings could be supported with the histological analysis of the dorsal skin *via* H&E staining to evaluate epidermal hyperplasia. The vehicle control group showed the normal histology of the skin. However, the UVB only group showed obvious thickening of the epidermis of the skin in comparison with the vehicle control group. In addition, the UVB only group showed hyperkeratosis in the epidermis. However, the pterostilbene supplementation group showed a reduction in epidermal thickening as compared with the UVB only group (Figure 2).

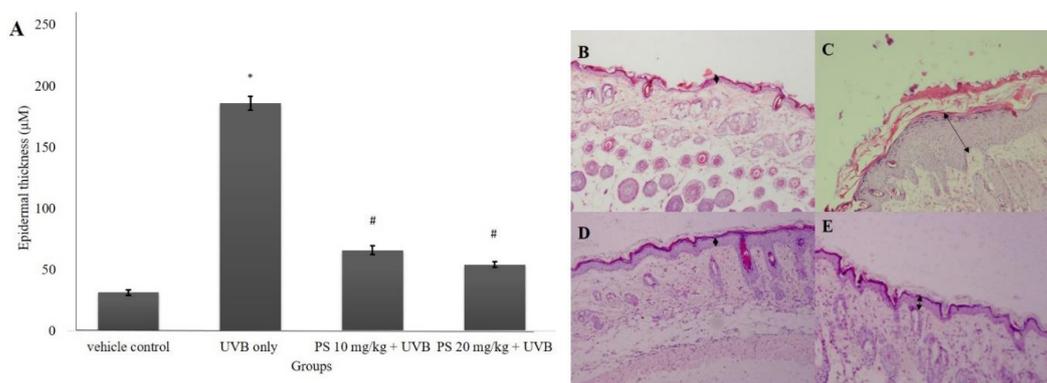


FIGURE 2. Effects of pterostilbene on epidermal hyperplasia in UVB-irradiated BALB/c mice. (A) Measurement of epidermal thickness by ImageJ1.49 v software. Bar chart shows the results of epidermal thickness in different groups represented by the mean \pm standard error mean (S.E.M) ($n = 6$). * Statistically significant difference in comparison with the vehicle control group ($P < 0.05$). # Statistically significant difference in comparison with the UVB exposure group ($P < 0.05$). (B–E) Histological observation to show epidermal hyperplasia (double sided arrow) by using haematoxylin and eosin staining. (B) vehicle control group; (C) UVB only group; (D) PS 10 mg/kg + UVB; (E) PS 20 mg/kg + UVB. Haematoxylin-eosin staining; Pictures are shown at a magnification of $10\times$. Scale bar: $100 \mu\text{m}$

PTEROSTILBENE DECREASES UVB-INDUCED OXIDATIVE
STRESS

UVB-induced oxidative stress was determined by evaluating the levels of MDA and antioxidants such as GSH and SOD. Figure 3(A) shows that the MDA level in the UVB only group ($13.343 \pm 1.350 \text{ nmol/g}$) increased significantly ($p < 0.05$) compared with that in the vehicle control group ($2.670 \pm 0.613 \text{ nmol/g}$). However, both the 10 and 20 mg/kg pterostilbene groups (5.927 ± 0.354

and $5.660 \pm 0.765 \text{ nmol/g}$, respectively) demonstrated significantly decreased MDA levels compared with the UVB only group ($13.343 \pm 1.350 \text{ nmol/g}$). Figure 3(B) shows that the GSH level in the UVB only group ($0.376 \pm 0.025 \mu\text{mol/mg}$) decreased significantly ($p < 0.05$) compared with that in the vehicle control group ($0.554 \pm 0.045 \mu\text{mol/mg}$). By contrast, both the 10 ($0.555 \pm 0.020 \mu\text{mol/mg}$) and 20 mg/kg ($0.568 \pm 0.055 \mu\text{mol/mg}$) pterostilbene groups showed significantly increased GSH levels ($p < 0.05$) as

compared with the UVB only group ($0.376 \pm 0.025 \mu\text{mol}/\text{mg}$). Figure 3(C) shows that the UVB only group ($0.561 \pm 0.034 \text{ U}/\text{min}/\text{mg}$) exhibited significantly decreased SOD activity ($p < 0.05$) compared with the vehicle control

group ($1.142 \pm 0.156 \text{ U}/\text{min}/\text{mg}$). However, the 20 mg/kg pterostilbene group ($1.388 \pm 0.172 \text{ U}/\text{min}/\text{mg}$) exhibited a significant increase in SOD activity ($p < 0.05$) compared with the UVB only group ($0.561 \pm 0.034 \text{ U}/\text{min}/\text{mg}$).

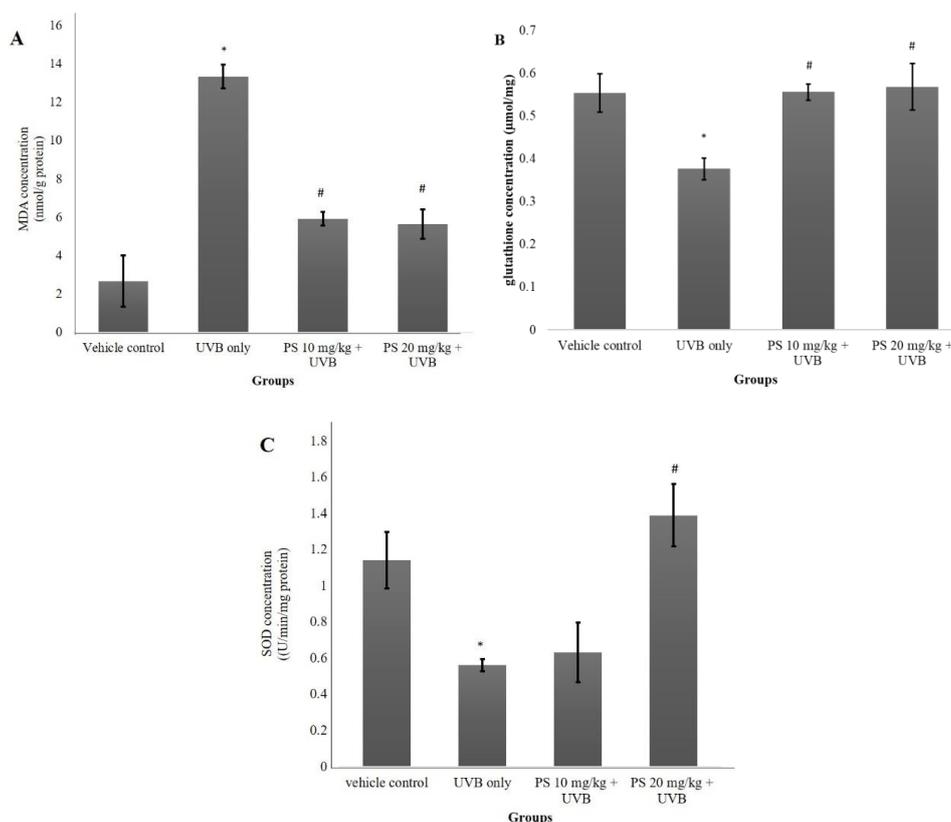


FIGURE 3. Effects of pterostilbene supplementation on oxidative stress in UV-irradiated BALB/c mice. (A) Bar chart shows results of the malondialdehyde (MDA) concentration in different groups represented by the mean \pm standard error mean (S.E.M) ($n = 6$). (B) Bar chart shows results of the glutathione concentration in different groups represented by the mean \pm standard error mean (S.E.M) ($n = 6$). (C) Bar chart shows the results of the glutathione concentration in different groups represented by the mean \pm standard error mean (S.E.M) ($n = 6$). * Statistically significant difference in comparison with the vehicle control group ($P < 0.05$). # Statistically significant difference in comparison with the UVB exposure group ($P < 0.05$)

DISCUSSION

UV radiation present in sunlight is a class I carcinogen (Mogensen & Jemec 2010). Extended exposure to UVB radiation may lead to significant biological complications to human skin, such as erythema, hyperpigmentation,

peeling or dryness of skin and skin cancer (Nagapan et al. 2019). Therefore, there is current interest in identifying natural active compounds from plants that might prove to be of superior efficacy in protecting skin from photodamage (Nagapan et al. 2018a). Pterostilbene, a natural bioactive

compound from blueberries, heartwood of *P. marsupium* and heartwood of *P. santalinus*, exhibits high oral bioavailability due to the presence of two methoxy groups, which leads to improved lipophilicity and cellular transit. In addition, pterostilbene increases the metabolic stability of molecules (Chen et al. 2017; Ghazali et al. 2012; Lin et al. 2009). Pterostilbene exhibits preventive and therapeutic properties against numerous human diseases due to its antioxidant capacity to reduce and scavenge ROS production (McCormack & McFadden 2013). Hence, this study aimed to determine the photoprotective effects of pterostilbene supplementation on UVB-irradiated BALB/c mice.

A previous study reported that UVB exposure may lead to alterations in skin morphology such as erythema, peeling, and scaling of the skin (Petrova et al. 2011). These clinical manifestations are early signs of the inflammatory response. In this study, we also found similar clinical manifestations such as skin scaling and erythema in the UVB only group. Interestingly, the oral supplementation pterostilbene groups showed less erythema and no obvious skin scaling compared with the other groups. The formation of cutaneous oedema is one of the signs of acute UVB photodamage. Edema occurs when extracellular fluid accumulates due to excess leakage from the hyperpermeability of blood vessels (Sawane et al. 2011). Edema can be evaluated by measuring skinfold thickness (Kim 2016). In the present study, we discovered that pterostilbene reduced UVB-induced cutaneous oedema significantly compared with the UVB only group. On the basis of a previous study, pterostilbene was found to reduce ear skin oedema on hexavalent chromium-induced mice (Wang et al. 2018). Furthermore, cutaneous oedema was reported to be accompanied with significant leukocyte infiltration in the dermis layer of the skin. Our histological study showed that the UVB only group showed an increase in leukocyte infiltration in the dermis layer. However, the pterostilbene group exhibited markedly reduced infiltration of leukocytes in the dermal layer. Therefore, our findings suggested that pterostilbene supplementation could attenuate the early inflammatory response induced by UVB irradiation.

Epidermal hyperplasia is a histological hallmark for acute photodamage. Thickening of the epidermis occurs when UVB causes cell cycle arrest in keratinocytes. This mechanism is a protective effect to repair damaged cells (Marrot & Meunier 2008; Ramasamy et al. 2017). However, prolonged exposure to UVB rapidly activates epidermal growth factor receptor (EGFR) and disrupts the cell cycle arrest that would progressively thicken the

epidermal layer of the skin (El-abaseri et al. 2006). In the present paper, we reported that the UVB only group showed an increase in epidermal thickness as compared with the vehicle control group. Moreover, the UVB only group showed obvious epidermal hyperplasia in comparison with the vehicle control group. Interestingly, both pterostilbene groups showed a reduction in epidermal thickness and epidermal hyperplasia in comparison with the UVB only group. The postulated mechanism is that pterostilbene supplementation could control the activation of EGFR and induce cell cycle arrest in keratinocytes, thereby ameliorating UVB-induced epidermal hyperplasia. Previous studies reported that pterostilbene reduces benzo[a]pyrene-induced epidermal hyperplasia (Singh et al. 2017). Topical application of pterostilbene also leads to a reduction in epidermal hyperplasia in UVB-irradiated hairless mice (Sirerol et al. 2015). Other stilbenes such as resveratrol also ameliorate epidermal hyperplasia in UVB-induced BALB/c mice (Nagapan et al. 2018b).

Acute exposure of UVB to skin can lead to oxidative stress by free radicals and ROS. This mechanism occurs when endogenous antioxidants such as SOD and GSH are depleted and lipid peroxidation activity increases. In addition, the main target of ROS on skin is lipids of the stratum corneum, which produces squalene peroxides; these peroxides cause the peroxidation of the cellular membrane of lipids (Auffray 2007). In this study, we demonstrated that UVB irradiation increased lipid peroxidation activity and decreased the contents of antioxidants such as SOD and GSH. However, these results showed that pterostilbene could protect cells by increasing the levels of antioxidants such as SOD and GSH while decreasing lipid peroxidation activity *via* the antioxidant defence mechanism. Pterostilbene is a potent activator of Nrf2 in which it controls the expression of genes whose protein products are involved in the detoxication and elimination of reactive oxidants and electrophilic agents *via* the Nrf2 signalling pathway. Previous studies showed that pterostilbene has been proven to have strong antioxidant capacity and free radical scavenging ability (Acharya & Ghaskadbi 2013; Hasiah et al. 2011). The topical application of pterostilbene has also been reported to increase the SOD and GSH levels in UVB-irradiated hairless mice (Sirerol et al. 2015). These findings demonstrated that 20 mg/kg pterostilbene showed a maximum response (efficacy) as compared with 10 mg/kg pterostilbene. Based on the other findings in an *in vivo* model, 20 mg/kg pterostilbene showed better hepatoprotective effects in dimethylnitrosamine-induced liver fibrosis in rats than 10 mg/kg pterostilbene (Lee et al. 2012).

CONCLUSION

In summary, pterostilbene supplementation could reduce the early inflammatory response and oxidative stress in UVB-irradiated BALB/c mice. Therefore, pterostilbene has the potential to be developed as a natural alternative for photoprotection.

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