International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:8, No:11, 2014

Ficus deltoidea Extract Protects HaCaT Keratinocytes from UVB Irradiation-Induced Inflammation

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Abstract*—Ficus deltoidea* from the Moraceae family is a popular medicinal herb in Malaysia. It possesses strong antioxidant and anti-inflammatory properties. In the present study, the anti-inflammatory effects of *F. deltoidea* extract on UVB-irradiated HaCaT Keratinocytes were investigated. HaCaT Keratinocytes were UVB-irradiated (12.5 mJ/cm^3) and were treated with 0.05, 0.08 or 0.1% of *F. deltoidea* extract. Cell viability following UVB irradiation was significantly higher in the groups treated with the *F. deltoidea* extract at doses of 0.05, 0.08 or 0.1% than in control group with UVB irradiation only. Tumor necrosis factor-α (TNF-α), interleukin-1α (IL-1α), interleukin-6 (IL-6) and cyclooxygenase (COX-2) play primary roles in the inflammation process upon UV irradiation and are known to be stimulated by UVB irradiation. Treatment with the *F. deltoidea* extract dramatically inhibited the UV-induced TNF-α, IL-1α, IL-6, and COX-2 expression. These results suggest that the *F. deltoidea* extract inhibits the production of pro-inflammatory cytokines and may be an effective protective agent for the treatment of skin diseases.

Keywords*—Ficus deltoidea*, anti-inflammatory activity, cytokines, COX-2.

I. Introduction

SKIN changes over time due to environmental factors, nutrition, and other factors. The main problem is UV irradiation from prolonged exposure of the skin to the sun. Chronic UV irradiation can induce DNA damage, leading to increased levels of reactive oxygen species (ROS) within the cell [1], [2]. The generation of ROS leads to a consequent signalling pathway in inflammation process and other biochemical reactions related to oxidative cell damages. Previous studies reported that UVB-irradiated skin keratinocytes activate the expression of various pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin-1α (IL-1α), IL-6, IL-10, and IL-8 [3], [4]. A prolonged or frequent increase in the levels of these cytokines could elicit harmful effects that could lead to chronic inflammation and epidermal hyperproliferation. Recent scientific studies strongly suggest the continuous up-regulation of these proinflammatory mediators during intrinsic ageing and the photoageing process due to an age-related redox imbalance that activates many pro-inflammatory signalling pathways, including the NF-κB signalling pathway [5]. Nevertheless, UVB exposure prominently enhances the level of cyclooxygenase-2 (COX-2) expression in cultured keratinocytes, leading to the UVB-induced synthesis of the inflammatory mediator prostaglandin E2 (PGE2) and photocarcinogenesis [3]-[7].

Recently, the use of botanical extracts in skin care and cosmetic products has gained consumer interest. *Ficus deltoidea* from the Moraceae family is a popular medicinal herb of Malaysia. Women have used this plant for decades as a postpartum treatment to aid in contracting the muscles of the uterus and vaginal canal. In addition, each plant part of *F. deltoidea* is known to possess medicinal properties, such as controlling LDL oxygenation, preventing blood clots, reducing blood sugar levels, decreasing blood pressure, and assisting the effectiveness of vitamin C in controlling nitric acid [8], [9].

Ficus species are rich in flavonoids, tannins, triterpenoids, proanthocyanins, and phenols, which are known to be responsible for the strong antioxidant properties that aid in the prevention and therapy of various oxidative stress-related diseases, such as neurodegenerative and hepatic diseases [10], [11]. Furthermore, some studies have shown various biological activities of *F. deltoidea*, including antioxidant, antinociceptive, photo-cytotoxic, and antidiabetic activities [9], [12], [13]. Therefore, the purpose of the present study was to investigate the protective effects of *F. deltoidea* against UVB-induce inflammation in HaCaT Keratinocytes.

II. Materials and Methods

A. Preparation of Sample

Ground *F. deltoidea* leaves (5 kg) were extracted with boiling water (80 L) for 2 h. After removal of the solid parts by filtration, the extracted solution was spray-dried using a pilot spray dryer (Niro A/S, GEA Group, Soeborg Denmark. The resultant powder was used to determine the anti-photoageing effects. Working solutions of the sample were prepared as follows: the dried powder was dissolved in DMSO to a final concentration of 1% (w/v) and sterilized via filtration. The resulting filtrate was stored at -20°C.

B. Cell Culture

The immortalized human keratinocytes (HaCaT) were grown in Dulbecco's modified essential medium (DMEM) (Welgene, Daegu, South Korea), containing 10% FBS (Welgene, Daegu, South Korea) and 1% antibiotics (Welgene, Daegu, South Korea). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

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C. UV Irradiation and Treatment

The HaCaT cells were grown at a density of 1×10^5 cells/well in a 12-well plate or a 6-well plate and cultured in DMEM for 24 h. The medium was then removed and replaced with PBS. The HaCaT cells were then exposed to UVB irradiation at 312 nm and 12.5 mJ/cm^2 or 15 mJ/cm^2. Following the UVB irradiation, the cells were cultured in serum-free DMEM in the absence or presence of the *F. deltoidea* extract or dexamethasone (Sigma, USA) for 24 h.

D. Cell Viability Assay

Following the treatment with the *F. deltoidea* extract, the culture medium was removed and incubated with an MTT solution at 37°C for 90 min. The solution was replaced with a 0.04 N HCl/isopropyl alcohol solution, and the resulting solution was further incubated at room temperature for 30 min. The harvested solution was centrifuged at 13,000 rpm for 5 min. The absorbance of the supernatant was measured at 570 nm using a microplate reader (Perkin Elmer, USA).

E. Enzyme-Linked Immunosorbent Assay (ELISA)

Following UVB irradiation, the HaCaT cells were cultured in serum-free DMEM in the presence or absence of the *F. deltoidea* extract or dexamethasone for 24 h, and the culture supernatant was obtained. The levels of human TNF-α, IL-1α and IL-6 were measured using commercial ELISA kits (Invitrogen International, Camarillo, CA) according to the manufacturer's protocol.

F. Western Blot Analysis

After 24 h of culturing, the cells were washed with PBS and harvested with RIPA cell lysis buffer supplemented with protease inhibitor. The total protein concentration was first determined using the Bradford assay. Equal amounts of protein were analyzed using 8% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transferred to a PVDF membrane. The membrane was blocked with 5% skim milk in TTBS for 1 h and incubated with primary antibodies. COX-2 antibody was added at a dilution of 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight.

G. Statistical Analysis

An assessment of the statistical significance was performed using Student's t-test. All results are represented as the average ± SEM of the combined data from replicate experiments.

III. Results

A. Effects of F. Deltoidea on HaCaT Keratinocyte Cell Viability

F. deltoidea was added to HaCaT cells to analyze their effects on cell viability. Following 12.5 mJ/cm2 UVB irradiation, HaCaT cells were treated with *F. deltoidea* extract for 24 h. As shown in Fig. 1, the viability of the UVB-induced HaCaT cells was inhibited by 29%. Treatment with *F. deltoidea* extracts in a concentration range of 50-100 µg/ml provide slight protection to HaCaT cells from cell damage caused by UVB irradiation, but only with extracts at a concentration of 50 µg/ml was considered statistically significant ($\rho < 0.05$). Based on this observation, further experiments were performed within these concentration ranges.

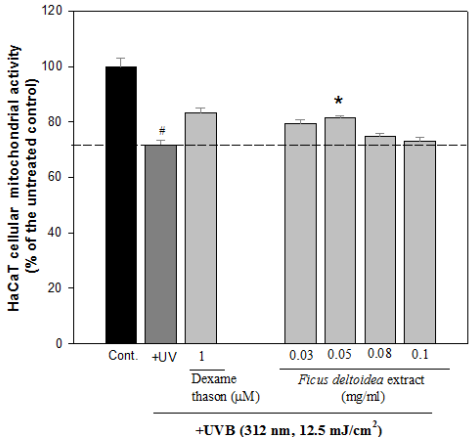

Fig. 1 The effects of *F. deltoidea* on cell viability reduced by UVB irradiation. HaCaT keratinocytes after exposure to UVB irradiation of 12.5 mJ/cm2 at 312 nm were treated with *F. deltoidea* extract at concentration of 0.03, 0.05, 0.08 and 0.1 mg/ml and dexamethasone at concentration of 1 µM for 24 h. The cell viability was analyzed with the MTT assay described in section 2.4. Values represent the means ± S.E.M. of three determinations. *P<0.05 compared to the untreated control (#)

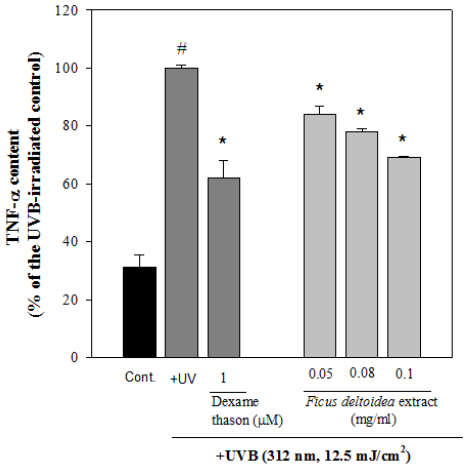

Fig. 2 Inhibition of UVB-induced TNF-α expression by F. deltoidea extract. HaCaT keratinocytes after exposure to UVB irradiation of 12.5 mJ/cm2 at 312 nm were treated with F. deltoidea at concentration of 0.05, 0.08 and 0.1 mg/ml and dexamethasone at concentration of 1 µM for 24 h. The secreted TNF-α content in culture media was measured by ELISA and represent as percent of UVB-irradiated control. Values represent the means ± S.E.M. of three determinations. *P<0.05 compared to the UVB-irradiated control (#)

A. F. Deltoidea Extract Reduced TNF-α, IL-1α and IL-6 Secretion Induced by UVB Irradiation

To investigate the UV-induced pro-inflammatory cytokine TNF-α, IL-1α, and IL-6 expressions in cultured HaCaT cells, the cells were exposed to 12.5 mJ/cm2 UVB irradiation. After UVB irradiation, the cells were treated with the *F. deltoidea* extract within the chosen concentration ranges of the cell viability study. Dexamethasone is a potent anti-inflammatory agent that was used as a positive control.

UVB irradiation increased the TNF-α content by more than two-fold compared with that of untreated cells (Fig. 2). Treatment of the UVB–irradiated HaCaT cells with the *F. deltoidea* extract for 24 h significantly reduced the elevated TNF-α secretions in a dose-dependent manner. The level of UVB-induced TNF-α secretion was reduced by 30% upon treatment with 100 µg/ml *F. deltoidea* extract.

F. deltoidea was also able to inhibit the IL-1α expression caused by UVB irradiation. After UVB irradiation, IL-1α production was increased three-fold compared with the control that was not exposed to UV irradiation (Fig. 3). This elevated level of IL-1α was significantly reduced to a level commensurate with that of the UVB-untreated control. Notably, the level of UVB-induced IL-1α secretion was inhibited by 70% upon treatment with 100 µg/ml *F. deltoidea* extract.

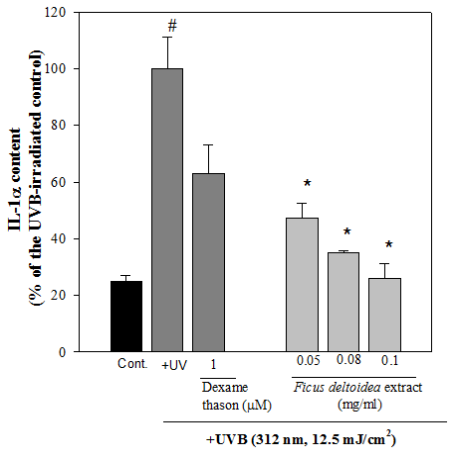

Fig. 3 Inhibition of UVB-induced IL-1α expression by *F. deltoidea* extract. HaCaT keratinocytes after exposure to UVB irradiation of 12.5 mJ/cm2 at 312 nm were treated with *F. deltoidea* at concentration of 0.05, 0.08 and 0.1 mg/ml and dexamethasone at concentration of 1 µM for 24 h. The secreted IL-1α content in culture media was measured by ELISA and represent as percent of UVB-irradiated control. Values represent the means ± S.E.M. of three determinations. *P<0.05 compared to the UVB-irradiated control (#)

The protective effects of the *F. deltoidea* extract against UVB-induced inflammation were also investigated in terms of IL-6 expression. As for IL-1α, the *F. deltoidea* extract reduced the production of IL-6 in a dose-dependent manner compared with the control (Fig. 4). The level of UVB-induced IL-6 secretion was significantly inhibited by 60% upon treatment with 100 µg/ml *F. deltoidea* extract.

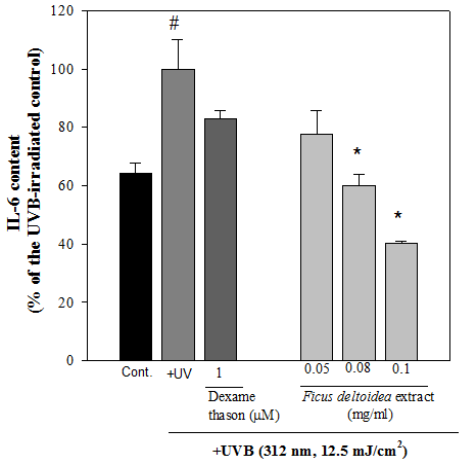

Fig. 4 Inhibition of UVB-induced IL-6 expression by *F. deltoidea* extract. HaCaT keratinocytes after exposure to UVB irradiation of 12.5 mJ/cm2 at 312 nm were treated with *F. deltoidea* at concentration of 0.05, 0.08 and 0.1 mg/ml and dexamethasone at concentration of 1 µM for 24 h. The secreted IL-6 content in culture media was measured by ELISA and represent as percent of UVB-irradiated control. Values represent the means ± S.E.M. of three determinations. *P<0.05 compared to the UVB-irradiated control (#)

Treatment with dexamethasone (1 µM) reduced the TNF-α and IL-1α secretion by approximately 40% and 45%, respectively, whereas the IL-6 secretion was reduced by approximately 20%. However, the *F. deltoidea* extract exhibited better inhibition of IL-1α and IL-6 compared with the commercial inflammatory drug dexamethasone. These data clearly demonstrate that the *F. deltoidea* extract could be used as an anti-inflammatory agent for alleviating skin inflammatory conditions.

B. F. Deltoidea Extract Decreased UVB-Induced COX-2 Expression

To analyze whether *F. deltoidea* extract could down regulate expression of COX-2, western blot analysis was performed. Elevated levels of COX-2 protein are also a biomarker for inflammation because this enzyme catalyses the biosynthesis of the inflammatory mediator PGE_2 [11], [13]. Cultured HaCaT cells were treated with the *F. deltoidea* extract at concentrations of 50, 80, and 100 µg/ml after exposure to 15 mJ/cm^2 UVB light.

The cell lysates were harvested 24 h after the *F. deltoidea* extract treatment to evaluate the COX-2 expression (Fig. 5). The data clearly demonstrated that UVB irradiation markedly enhanced COX-2 expression in HaCaT cells. This enhancement was reduced following treatment with the *F. deltoidea* extract. When treated with the *F. deltoidea* extract at a concentration of 100 µg/ml, the COX-2 expression was nearly abolished. This result further confirmed that the *F. deltoidea* extract could be an effective regulator of anti-inflammation processes.

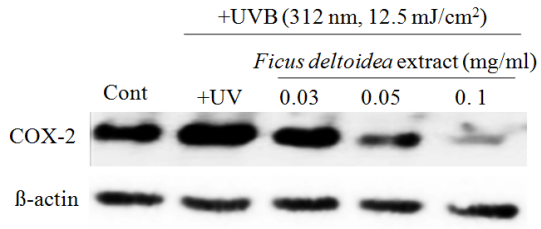

Fig. 5 Attenuation of UVB-induced COX-2 expression by *F. deltoidea* extract. HaCaT keratinocytes after exposure to UVB irradiation of 12.5 mJ/cm2 at 312 nm were treated with *F. deltoidea* at concentration of 0.05, 0.08 and 0.1 mg/ml for 48 h. After treatment, the cells were harvested and analyzed by western blot. Normalization was carried out with β-actin

IV. Discussion

Continuous exposure to ultraviolet (UV) irradiation leads to a variety of skin damage, such as sunburn, pigmentation, premature ageing, and photocarcinogenesis. Therefore, various studies have been identified distinctive solutions, such as anti-melanogenic and antioxidant materials, that work efficiently against photodamage of the skin. A *F. deltoidea* (Mas cotek) water extract has been widely used for women's health in Malaysia. In a previous study from this lab, the *F. deltoidea* extract exhibited strong anti-melanogenic effects towards cultured B16F1 melanoma cells [14]. Additional studies were intended to evaluate the effects of the *F. deltoidea* extract on anti-inflammation activity using cultured immortalized human keratinocytes (HaCaT).

F. deltoidea has been reported to exhibit a high antioxidant activity; this species also contain a variety of phytochemicals such as flavonoids, tannins, triterpenoids, proanthocyanins, and phenolics [9], [13]. Furthermore, Abdullah et al. have indicated that *F. deltoidea* leaf extracts exhibit anti-inflammatory properties in three models in *in vitro* assays: lipoxygenase (LOX), hyaluronidase (HAase), and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced ear oedema [15]. The LOX pathway generates hydroperoxy-eicosatetraenoic acids and leukotrienes, which are important mediators in a variety of inflammatory events. HAase hydrolyses glycosaminoglycans, including hyaluronan, in the extracellular matrix during tissue remodelling. Hyaluronidase activity increases in chronic inflammatory conditions; therefore, a study of hyaluronidase inhibition would investigate the ability of a plant extract to function as an anti-inflammatory agent. The TPA-induced mouse ear oedema inhibitory assay was used to investigate the anti-inflammatory activity. *F. deltoidea* extracts were observed to moderately inhibit the three models during the *in vitro* assay for the anti-inflammatory analysis.

However, the investigation of the extract towards protection from UVB-induced inflammatory effects on human skin cells has not yet been reported. Such a study is important because the UVB irradiation of skin cells has stimulated the production of pro-inflammatory cytokines such as TNF-α, IL-1α, and IL-6, and various second messengers, such as prostaglandin, which are synthesized by cyclooxygenase-2 (COX-2) [3], [4]. Moreover, skin ageing is strongly related to the inflammatory response in acute UV-exposed skin. Hence, the inhibition of inflammation signalling pathways, such as those involving TNF-α, IL-1α, IL-6, and COX-2, are the primary options for inflammation prevention. The results of this study demonstrate that UVB irradiation resulted in a more than three-fold increase in the levels of TNF-α and IL-1α, whereas the levels of IL-6 increased up to 1-fold in the HaCaT cells (Figs. 2-4). Under identical irradiation conditions, treatment with 100 µg/ml *F. deltoidea* extract strongly decreased the production of IL-1α and IL-6 by 70% and 60%, respectively, compared with the TNF-α activity, which decreased by only 30%. In UVB-induced IL-6 production, treatment with dexamethasone as an inflammatory drug resulted in a less than 20% reduction. Reduction of IL-6 expression is important because several reports have indicated that the increase in the IL-6 levels correlates well with increases in the occurrence of hyperalgesia and oedema [5], [6]. These results suggest that *F. deltoidea* may have a potential use as a novel anti-inflammatory drug for UVB-induced tissue damage. This suggestion is strongly supported by the results of the COX-2 expression analysis (Fig. 5), which are consistent with previous data. The inhibition of COX-2 expression may reduce ROS generation in keratinocytes after UVB irradiation, which would lead to a decrease in the expression of pro-inflammatory cytokines.

In conclusion, findings of the present study demonstrate the efficacy of the *F. deltoidea* extract towards alleviating UVB-induced inflammation in the skin by modulating the expression of pro-inflammatory cytokines and COX-2. Therefore, the *F. deltoidea* extract is a potential immunomodulatory agent for the treatment of and protection against skin inflammation diseases.

Acknowledgement

We thank the Ministry of Education and Universiti Teknologi Malaysia (Vote Number 09J88 and 00K75) for financial support.

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She has dedicated her career to the concepts of skin health and the development of aromatherapy techniques and formulations that help in restoring skin to its original health and beauty. She is currently IBD's Senior lecturer, having been actively involved in R&D and commercialization of aromatherapy and cosmeceutical products for the past 14 years. She has participated as an advisor of Bakawali SPA, formerly known as CEPP Aromatherapy Centre.

She is an Associate Member of Advancing Chemical Engineering Worldwide (IChemE). She has conducted lectures and courses all over Malaysia and educates the technique and philosophy to hundreds of aromatherapy practitioners. There are several recognitions and awards have been granted to Dr Rosnani including Gold Medal Award for the project entitled *Ficus deltoidea*'s Natural Regenerating Treatment Serum For Skin Lightening and Anti-aging at Seoul International Invention Fair on Dec 2013 at Seoul Korea; and Biotechnology Innovation of The Year 2013 Award at BioInnovation and BioMalaysia Awards 2013.