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# Research Article
# Protective Effects of Diosgenin Against Ultraviolet B (UVB) Radiation-induced Inflammation in Human Dermal Fibroblasts

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## Abstract

**Background and objective:** Diosgenin is a steroidal sapogenin known for its pharmacological properties including anti-inflammatory effects. This study was aimed to evaluate the *in vitro* anti-photoaging and anti-inflammatory effects of diosgenin on ultraviolet B (UVB) radiation-induced damage on human dermal fibroblasts. **Materials and Methods:** The UVB-induced HDF cells were treated with diosgenin at concentrations 5 and 10 μM, respectively. Cytotoxicity induced by UVB radiation on pre-treated and post-treated HDF cells were tested. The levels of pro-inflammatory cytokines in HDF cells were determined using ELISA method. Western blot analysis was performed to determine the expressions of inflammatory mediators NF-kβ and MMPs. The concentrations of reduced glutathione (GSH) and malondialdehyde (MDA) were determined in the cell homogenates. **Results:** Diosgenin significantly ($p<0.01$) reduced the cytotoxicity induced by UVB on HDF cells. Pro-inflammatory cytokines and expressions of inflammatory mediators were significantly reversed ($p<0.01$) by diosgenin in a concentration dependent manner. Diosgenin was able to increase the level of GSH and prevent MDA formations. **Conclusion:** The results proved that diosgenin has protective effects on HDF cells against UVB-induced inflammation. Therefore, this active compound can be recommended for pharmaceutical formulations against UV induced skin inflammation.

**Key words:** Diosgenin, ultraviolet radiation, anti-photoaging, human dermal fibroblast, pro-inflammatory cytokines, skin inflammation

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**Competing Interest:** The authors have declared that no competing interest exists.

**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Photoaging is an extrinsic ageing skin condition in humans caused by exposure to ultraviolet (UV) radiation. There are three main types of UV radiation; UVA, UVB and UVC which are segregated according to wavelengths[1]. The UVB is in the medium range wavelength (290-320 nm) and the exposure that reaches earth is lesser than UVA, but the intensity of UVB in causing skin damage is higher either directly or indirectly. Human skin overexposed to UV, specifically to UVB radiation has shown direct detrimental effects such as pigmentation, skin reddening, sunburn, wrinkles and skin cancer[2]. Indirect effect of UVB on human skin includes DNA damage, accumulation of reactive oxygen species (ROS), apoptotic cell death, skin inflammation through activation of pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and activation of several transcription factors related to skin inflammation such as nuclear factor-kβ (NF-kβ) and overexpression of matrix metalloproteinases (MMPs)[3]. Accumulation of ROS in the skin layer constricts the activities of reduced glutathione (GSH), antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and increases the production of malondialdehyde (MDA) as a result of lipid peroxidation[4]. *In vitro* analysis on skin inflammation model due to UVB exposure was commonly done using human dermal fibroblasts (HDF)[5,6]. Natural drugs are opted for medication of UVB exposed skin inflammation since they have negligible side effects compared to synthetic drugs[7,8].

Diosgenin is a naturally found steroidal sapogenin, which has been reported for its pharmacological properties. Diosgenin is formed from the hydrolysis of dioscin, an active saponin that can be found in several plant species such as; *Trigonella*, *Smilax*, *Costus* and *Dioscorea*. Diosgenin is a bioactive compound which can be found in these plant species[9]. Anticollagenase and anti-inflammatory activities of the extract of Wild Yam plant (*Dioscorea villosa*) suggested the use of bioactive compounds in the extract against irritable skin problems and photoaging. Various pharmacological studies were successfully performed using diosgenin for numerous medical conditions. Antioxidant and anti-inflammatory properties of diosgenin prevailed in most of the studies which can be attributed to its therapeutic properties on diabetes, cerebral ailments, obesity, skin ageing, cardiovascular diseases, allergic and menopausal syndrome and also cancer[9,10]. Considering the fact that diosgenin has multiple health benefits and is safe for consumption, it was suggested for its anti-photoaging properties. Therefore, this research was conducted to evaluate the defensive effects of diosgenin on UVB radiation-induced inflammation on HDF cell lines *in vitro*.

## MATERIALS AND METHODS

**Materials:** The experimental protocols of this study were carried out for approximately 7 months beginning in February, 2018 in the laboratory of Dermatology Department, Shandong Provincial Hospital Affiliated to Shandong University. Diosgenin was purchased from Pure One Biotechnology Ltd. (Shanghai, China). Reagents and commercial kits for ELISA and western blot analysis were procured from Pierce Biotechnology Inc., US. Chemicals used in this experiment were of purest grade obtained from Sigma Aldrich Inc., US.

### Methods

**Cell culture and UVB-exposure protocol:** Human dermal fibroblast cell lines were obtained from ATCC (Manasas, VA; USA) and cultured in DMEM medium was containing 100 U $mL^{-1}$ streptomycin-penicillin and 5% fetal bovine serum (FBS). The cells were sub-cultured, incubated and maintained at 37°C in humidified incubator supplied with 5% $CO_2$. Cells that attained confluence (80%) were seeded and passaged.

The cells were rinsed with PBS and exposed to UVB radiation at an energy dose of 100 mJ $cm^{-2}$ with a distance of 5 $cm^{-2}$ from the UVB light source to culture plates following the method of Guo *et al*.[5]. The UVB radiation source consists of UVB lamp at 280-320 nm range (peak intensity at 312 nm). The UVB doses were estimated using UVB meter (UVP, Upland, CA). Fresh medium was added upon irradiation and cell viability was measured by MTT analysis by measuring absorbance of the formazan crystals under Tecan microplate reader at 570 nm.

**Cell viability test:** The HDF cells were seeded in 96-well plates at a density of $1\times10^4$ cells/well in DMEM medium containing 100 U $mL^{-1}$ streptomycin-penicillin and 5% FBS allowed to attach for 24 h. The cells were rinsed with PBS and treated with diosgenin at concentrations of 1.25, 2.5, 5, 10, 20 and 40 μM in PBS. Then PBS was removed from the cells to be replaced with fresh medium. After 24 h, HDF cells were treated with 5 mg $mL^{-1}$ of MTT for 3 h at 37°C. The formazan crystals were dissolved in DMSO for 30 min and the absorbance was read at 570 nm using Tecan microplate reader. The protocols were done following the method of Guo *et al*.[5].

Table 1: Effects of diosgenin on pro-inflammatory cytokines concentrations on UVB-irradiated HDF cells

| Parameters | TNF-α (pg mL$^{-1}$) | IL-6 (pg mL$^{-1}$) | IL-1β (pg mL$^{-1}$) |
|---|---|---|---|
| Control | 120.79±18.35 | 139.91±7.57 | 288.94±6.32 |
| UVB | 444.35±27.44$^{###}$ | 282.62±4.18$^{###}$ | 1493.60±55.71$^{###}$ |
| Diosgenin 5 μM | 243.31±23.40*** | 183.41±8.78*** | 1213.18±97.05*** |
| Diosgenin 10 μM | 189.82±14.43*** | 160.48±6.10*** | 975.39±72.06*** |

Data presented as mean and standard deviation (n = 3). $^{###}$p<0.01 compared to untreated control cells, ***p<0.01 compared to cells exposed to UVB alone

**Determination of pro-inflammatory cytokines in UVB-induced cells:** The HDF cells were seeded in 6 well plates at a density of $5\times10^5$ cells/well in DMEM medium containing 100 U mL$^{-1}$ streptomycin-penicillin and 5% FBS allowed to attach for 24 h. The cells were rinsed with PBS and pretreated with diosgenin at concentrations of 5 and 10 μ M$^{-1}$ in PBS. The cells were consequently exposed to UVB-irradiation for 250 Sec. and then PBS was removed from the cells to be replaced with fresh medium. After 24 h of UVB radiation exposure, cells were harvested for western blot analysis. The cell culture supernatants was subjected to ELISA analysis (R and D Systems, Minneapolis, US) to determine the levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 following the protocols of manufacturer. The tests were performed in triplicates. The protocols were performed following the method of Santhanam *et al.*[6].

**Determination of MMPs and NF-kβ expressions using western blot analysis:** Harvested HDF cells were homogenized with RIPA buffer and centrifuged for 20 min at 12000 g at 4°C. Nanodrop (Thermo Scientific, US) was used for protein determination. Equal amounts of protein (20 μg) containing cell extracts were separated on 10% SDS-PAGE gel, transferred to polyvinylidene fluoride membrane and blocked for 1 h with 5% BSA at room temperature. The membrane was washed with PBST (PBS with 0.1% Tween 20) and incubated overnight with goat polyclonal antibodies against MMP-1 (1:500), mouse polyclonal antibodies against MMP-3 (1:500) and MMP-9 (1:500) at 4°C. The membranes were then washed thrice with PBST and incubated with horseradish peroxidase conjugated secondary antibodies for 1 h. The membrane was developed with enhanced chemiluminescence-substrate and the immune reactive proteins bands were detected with ECL western blotting detection system. The protocols were performed following the method of Chang *et al.*[1] with some modifications.

**Determination of reduced glutathione and lipid peroxidation levels:** Supernatants of treated HDF cell culture were subjected to biochemical analysis to determine the levels of reduced glutathione (GSH) and lipid peroxidation (LPO). The GSH levels were determined by the colorimetric analysis method by measuring the formation of 5-thiol-2-nitrobenzoic acid spectrophotometrically at 412 nm. Lipid peroxidation levels were determined by the colorimetric method of thiobarbituric acid (TBA) conjugate formation with malondialdehyde (MDA) measured spectrophotometrically at 535 nm. All experiments were conducted in triplicates. The protocols were done following the method of Santhanam *et al.*[6].

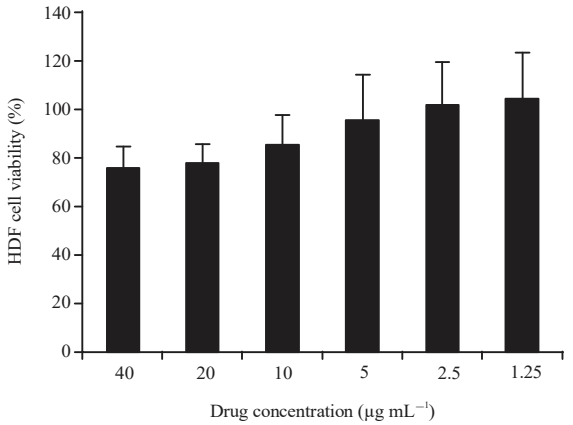


Fig. 1: Effects of diosgenin at different concentrations on cell viability of human dermal fibroblasts. Cell viability test was conducted using MTT assay. Data presented as mean and standard deviation (n = 5)

**Statistical analysis**: The statistical analysis was performed using GraphPad Prism (version 5) software. Mean difference between groups were analyzed using one-way analysis of variance (ANOVA) and all p-values less than 0.05 (p<0.05) were considered significant.

## RESULTS

**Cell viability and cytotoxicity analysis:** The cell viability of HDF cells were analyzed against different concentrations of diosgenin treatment using MTT assay. Figure 1 shows that diosgenin did not induce cytotoxicity to the cells at different concentrations.

**Protection of diosgenin against UVB-radiation induced inflammatory modulators:** Treated and non-treated normal HDF cells were subjected to ELISA analysis to assess the pro-

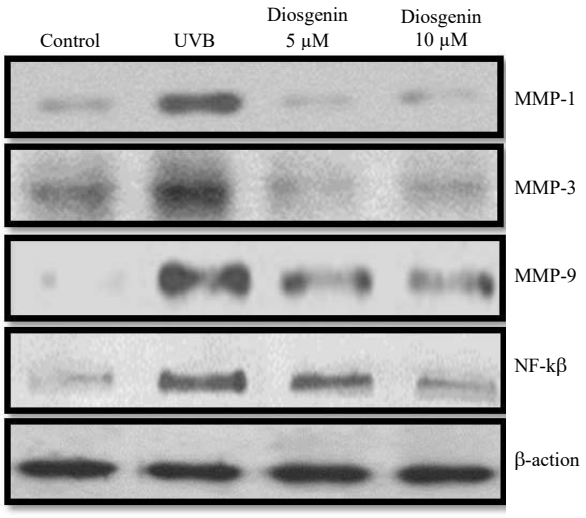


Fig. 2: Effects of diosgenin on MMPs and NF-kβ expressions in UVB-irradiated HDF cells. Western blot analysis was performed on the expressions of MMP-1, MMP-3, MMP-9 and NF-kβ in HDF cells. β-actin was used as loading control

Table 2: Effects of diosgenin on malondialdehyde (MDA) and reduced glutathione (GSH) concentrations on UVB-irradiated HDF cells

| Parameters | MDA (nmol mg protein$^{-1}$) | GSH (nmol mg protein$^{-1}$) |
|---|---|---|
| Control | 8.39±1.02 | 205.45±5.87 |
| UVB | 34.52±3.91$^{###}$ | 75.37±5.70$^{###}$ |
| Diosgenin 5 μM | 21.98±1.88*** | 105.09±6.19*** |
| Diosgenin 10 μM | 14.85±0.95*** | 165.89±6.38*** |

Data presented as mean and standard deviation (n = 3), $^{###}$p<0.01 compared to untreated control cells, ***p<0.01 compared to cells exposed to UVB alone

inflammatory cytokine levels. Table 1 shows that UVB-exposed HDF cells exhibited severe inflammatory reactions (p<0.01) with elevated levels of pro-inflammatory cytokine TNF-α, IL-1β and IL-6. Pretreatment of diosgenin showed significant preventive and protective effect (p<0.01) against UVB-induced inflammation on HDF cells.

**Effects of diosgenin on the expression of MMPs and NF-kβ:** The expressions of MMPs and NF-kβ on treated and normal HDF cells were analyzed through western blot analysis. Figure 2 shows that MMP-1, MMP-3, MMP-9 and NF-kβ were highly expressed in the UVB-radiation induced HDF cells as compared to normal untreated cells. In contrast, diosgenin treatment significantly prevented the expressions of the proteins in both doses.

**Effects of diosgenin on lipid peroxidation and reduced glutathione levels:** The levels of lipid peroxidation and GSH in normal and treated HDF cells were determined using colorimetric methods. Table 2 shows that MDA levels were significantly elevated (p<0.01) in HDF cells due to UVB radiation as compared to the normal control cells. However, treatment with diosgenin was able to reduce MDA levels (p<0.01) in UVB-exposed HDF cells. Table 2 also shows that the levels of antioxidant GSH were markedly reduced (p<0.01) in the HDF cells exposed to UVB-radiation compared to normal cells. Conversely, the levels of GSH were elevated remarkably (p<0.01) in a dose dependent manner in diosgenin treated UVB-exposed HDF cells.

## DISCUSSION

Skin inflammation, photoaging and carcinogenesis are well known effects of exposure to UVB radiation. Reactive oxygen species free radicals are induced in the dermal cells upon exposure to UVB radiation thus leading to a state of oxidative stress[11]. Natural compounds are generally known to have no adverse effects on skin if used in a proper concentration[12]. Diosgenin was reported to possess multiple therapeutic potentials including anti-inflammatory activity, which can be related to the findings in this study[9,10].

Oxidative stress is the basis of skin ageing, inflammation and other pathological conditions[13]. Lipid peroxidation occurs during excessive free radical formation within a cell which is undergoing oxidative stress[14]. From the results obtained, it was clear that oxidative stress was induced by UVB radiation on HDF cells due to a sharp increase in the levels of lipid peroxidation as compared to normal control cells. Moreover, the level of GSH in HDF cells was reduced severely as a cause of UVB-radiation compared to normal control cells. Diosgenin was able to overcome oxidative stress caused by UVB radiation by replenishing the GSH activities and preventing lipid peroxidation within HDF cells. These results were complementing the findings from previous literature[7,15].

In this study, the levels of pro-inflammatory cytokines were highly elevated in UVB radiation exposed HDF cells against the normal concentrations in the control cells. Diosgenin successfully prevented the inflammatory process by reducing the levels of pro-inflammatory cytokines in a dose-dependent manner. This was in agreement with previous reports with similar findings[16,17]. This proved the progress of skin inflammatory process due to UVB radiation and the mechanisms involved. Pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 are important inflammatory mediators in an inflammatory process. The activation of inflammatory

process is also caused by free radicals or oxidative stress and lipid peroxidation[18]. TNF-α is primarily involved in inflammatory reaction where it is secreted by multiple immune cells such as monocytes and lymphocytes to signal and attract multiple inflammatory mediators to the site of inflammation[2,19].

The anti-inflammatory effect of diosgenin was also evidenced through western blot analysis that showed reduced expressions of NF-kβ and MMPs in UVB exposed HDF cells. Several signaling molecules within cells such as the NF-kβ and MMPs mediate adverse skin reactions caused by UVB exposure[20]. MMP-1, MMP-3 and MMP-9 are reported to be highly expressed in human skin layer upon exposure to UV radiation due to activation of several signaling pathways[17]. Activation of NF-kβ is directly involved in the increased expression of MMPs[1]. The result of this study supported this statement since the NF-kβ and MMPs were highly expressed in HDF cells exposed to UVB radiation as compared to normal cells. These results were also similarly in agreement with past literature[1,6]. The protective effect of diosgenin on HDF cells against UVB radiation proves that it has anti-photoaging properties.

## CONCLUSION

In this study, the anti-inflammatory and photoprotective effects of diosgenin against UVB-exposed HDF cells were evidenced through prevention of oxidative stress, reduced levels of pro-inflammatory mediators and inhibition of NF-kβ and MMPs expressions. The findings can supported the use of diosgenin as an active ingredient in sunscreens and skin lotion to protect against UV exposure. Further pre-clinical tests are needed to ascertain the extent of skin protection that can be provided by diosgenin against photoaging and skin inflammation prior to be formulated into a skin protection product.

## SIGNIFICANCE STATEMENT

This study discovered the anti-photoaging and anti-inflammatory effects of diosgenin that can be beneficial for the treatment of UVB-induced skin inflammation. This study will help researchers to uncover the critical areas in using natural compounds to treat UVB-induced photoaging and skin inflammation that are yet to be explored. Thus a new finding in the pharmaceutical formulation for skin protection may be attained.

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