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Original Article

Anti-inflammatory effect of *Moringa oleifera* Lam. leaf extract on UVB-irradiated human keratinocytes

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Abstract

Moringa oleifera Lam. has multiple biological properties that are applicable to developing cosmeceuticals. The objective of this study was to evaluate anti-inflammatory effects of *M. oleifera* on normal human keratinocytes. The anti-inflammatory effects of water extract and 50% ethanol extract of *M. oleifera* leaves, and of the bioactive compound astragalin, on UVB-irradiated human keratinocytes were investigated. The levels of inflammatory mediators IL-1α, IL-8, NO and PGE_2 released from keratinocytes were measured using ELISA and Griess assays. UVB irradiation increased the release of IL-1α, IL-8 and PGE_2 into cell culture media after 24 h. The effect was significantly decreased by treatments with astragalin and *M. oleifera* leaf extracts, of which the 50% ethanol extract showed stronger inhibition than the water extract. These results suggest that this extract is a potential ingredient in products for relieving UVB-induced skin inflammation. Astragalin could be used as a bioactive positive control in the quality control of the extract.

Keywords: *Moringa oleifera*, astragalin, anti-inflammatory activity, primary human skin cells**,** UVB

1. Introduction

Keratinocytes are the most abundant cell type in the epidermis, which is the outermost layer of the skin. The keratinocytes function as a barrier against environmental exposure to bacteria, viruses, chemicals and ultraviolet (UV) radiation. These cells are known to initiate skin inflammation. Because their position is at the interface between the body and the environment, keratinocytes receive signals from the environment and transmit them to other cells in the skin, by releasing a wide range of inflammatory mediators.

UVB (290-320 nm) radiation is absorbed by the epidermis and can cause skin inflammation. Keratinocytes are the main target of UVB, and play a crucial role in inflammatory response to UVB exposure through the release of pro-inflammatory cytokines such as interleukin 1 alpha (IL-

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1α) and interleukin 8 (IL-8) (Kondo, Kono, Sauder, & McKenzie, 1993; Kupper, Chua, Flood, McGuire, & Gubler, 1987). IL-1α has been mentioned as the primary cytokine for inflammation. It initiates a signaling cascade that induces expression and production of secondary mediators, such as adhesion molecules, other cytokines, and itself (Groves *et al.*, 1992; Larsen, Anderson, Oppenheim, & Matsushima, 1989; McKenzie & Sauder, 1990). IL-8 is a considerable chemotactic factor that may be especially relevant to the skin. IL-8 stimulates leukocyte infiltration from the vasculature into the skin (Barker *et al.*, 1991; Kemeny, Ruzicka, Dobozy, & Michel, 1994). In addition, UVB induces the release of important inflammatory mediators of erythema from keratinocytes, namely nitric oxide (NO) and prostaglandin E_2 (PGE_2) (Deliconstantinos, Villiotou, & Stravrides, 1995; Miller, Hale, & Pentland, 1994; Terazawa, Nakajima, Shingo, Niwano, & Imokawa, 2012).

Moringa oleifera Lam. (Moringaceae family) is commonly distributed in many tropical and subtropical countries. Its English common names include moringa, horseradish tree or drumstick tree. *M. oleifera* has been used in folk medicine for the treatment of disorders associated with inflammation such as bronchitis, glandular swelling and articular pain (Anwar, Latif, Ashraf, & Gilani, 2007). Astragalin (Figure 1), a major flavonoid in *M. oleifera* leaves, is one of the bioactive constituents related to anti-inflammatory actions (Han *et al.*, 2004; Li *et al.*, 2014; Luo *et al.*, 2015; Soromou *et al.*, 2012; Vongsak, Sithisarn, & Gritsanapan, 2014). It is also known to have antioxidant properties (Choi *et al.*, 2013;). Currently, skincare products that use *M. oleifera* extracts as active ingredients are available commercially. However, there have been only few studies concerning the effects of *M. oleifera* on keratinocytes. The aim of this study was to explore the anti-inflammatory effects of water extract, 50% ethanol extract, and astragalin on UVB-irradiated human keratinocytes *in vitro*. The levels of inflammatory mediators IL-1α, IL-8, NO and PGE_2 released from the cells were determined.

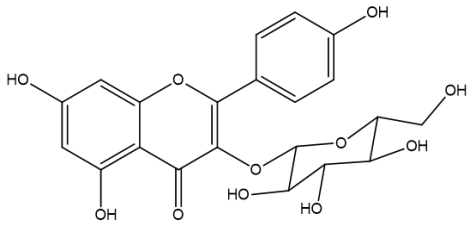

Figure 1. Structure of astragalin

2. Materials and Methods

2.1 Reagents

Keratinocyte Serum-free Medium (KSFM), recombinant epidermal growth factor (rEGF), bovine pituitary extract (BPE), penicillin-streptomycin (Pen Strep), dispase, trypsin-EDTA, fetal bovine serum (FBS), phosphate buffered saline (PBS) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Gibco, United States. Enzyme-linked immunosorbent assay (ELISA) kits for the human IL-1α and IL-8 were acquired from eBioscience, United States. Griess assay kit was obtained from Promega, United States. ELISA kit for the human PGE_2 was purchased from Abcam, United States. All solvents used were of analytical reagent grade and were acquired from RCI Labscan, Thailand.

2.2 Extract preparation

Leaves of *M. oleifera* were collected from Phitsanulok province, Thailand. The voucher specimen (Collection No. J. Engsuwan002) was deposited to the herbarium of Faculty of Science, Naresuan University. The plant was identified by Dr. Pranee Nangngam. The *M. oleifera* leaves were dried in a hot air oven at 50 °C. The dried leaves were ground into rough powder. The powdered leaves were extracted in two different solvents: water and 50% ethanol in water. For extraction by water, the powder was infused with water at 60 °C - 70 °C temperature, and shaken for 45 min. The resulting extract was filtered and the water was removed by freeze drying. For the 50% ethanol extract, the powder was macerated with 50% ethanol and shaken for 48 h and then filtered. The ethanol was then evaporated under reduced pressure and the residual water was removed by freeze drying. Extraction yields with 50% ethanol and water were 12% and 7.54%, respectively. The amount of astragalin in the water and the 50% ethanol extracts from *M. oleifera* leaves were 0.1 and 0.5% by dry weight, respectively, according to a previous study (Engsuwan, Waranuch, Limpeanchob, & Ingkaninan, 2017). The extracts were stored in a freezer (-20 °C) for further use.

2.3 Cell culture

The epidermal keratinocytes were isolated from foreskins obtained from the hospital with approval from the Naresuan University Institutional Review Board in accordance with the Helsinki Declaration, the Belmont Report, the CIOMS Guideline and the ICH-GCP Guideline (IRB No. 736/59). The foreskin was washed 3 times with PBS containing Pen Strep (100 units/ml penicillin and 100 µg/ml streptomycinin). After that, the foreskin was soaked in 5% dispase solution at 4 °C overnight to separate the epidermis from the dermis. The epidermal pieces were gently peeled off using forceps. Then, the epidermal pieces were trypsinized to single keratinocyte cells in 0.25% trypsin-EDTA solution and incubated for 5 min at 37 °C in 5% CO_2. Next, the trypsinization reaction was stopped by FBS. After that, the keratinocyte cells were cultured in KSFM supplemented with 5 ng/ml rEGF, 50 µg/ml BPE, and Pen Strep (100 units/ml penicillin and 100 µg/ml streptomycinin) in a humidified atmosphere with 5% CO_2 at 37 °C.

2.4 Cell viability test

MTT assay was conducted in order to assess the effects of *M. oleifera* leaf extracts on the survival of keratinocytes. The mitochondrial reductase enzymes of living cells can reduce MTT reagent, which has a yellow color, to insoluble formazan which has a purple color, and that color then reflects the amount of viable cells present. The cells were seeded at a concentration of 2×10^4 cells/well in 96-well

plates containing KSFM supplemented with rEGF, BPE and Pen Strep in a humidified atmosphere of 5% CO_2 at 37 °C. After the cells completely adhered to the well plate, they were washed with PBS and then treated with the KSFM containing the water extract or the 50% ethanol extract at various concentrations, for 20 h. Then, the MTT solution was added to each well. Next, the plate was incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 4 h. The MTT solution was removed and dimethyl sulfoxide: ethanol (1:1) blend was added. After that, the absorbance was read at 595 nm by using a microplate reader. The absorbance of the control group (untreated cells) was considered as 100% cell viability, and the cell morphology was observed under an inverted microscope.

2.5 UVB irradiation

The keratinocytes were seeded at a concentration of 1×10^5 cells/well in 24-well plate containing KSFM supplemented with rEGF, BPE and Pen Strep in a humidified atmosphere of 5% CO2 at 37 °C for 20 h. The cells were washed once with PBS and were then covered with a thin layer of PBS. Then, the cells were exposed to UVB using UV test chamber BS-04 (Opsytec Dr. Groebel, Germany; wavelength 313 nm) with the doses of 35 mJ/cm^2 for IL-1α and IL-8 assay, and 50 mJ/cm^2 for PGE_2 assay. For NO assay, the doses of UVB exposure were varied from 35-100 mJ/cm^2. After UVB exposure, PBS was replaced with the KSFM containing *M. oleifera* extract, astragalin or diclofenac sodium, an anti-inflammatory drug, at various concentrations. Then, the cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 24 h. After that, the cell culture medium was collected for further experiments.

2.6 Determination of IL-1α and IL-8

IL-1α and IL-8 released from keratinocytes were determined by ELISA kits according to the protocols proposed by the manufacturer. Briefly, the antibody was pre-coated onto the well plate. The cell culture medium was added to the wells followed by incubation. The detection antibody was added and incubated for 2 h. Subsequently, sterptavidin-horseradish peroxidase (HRP) was added to each well for 1 h. Then unbound streptavidin-HRP was removed during a wash step and the substrate reactive with HRP was added to the well. The colored product was formed in proportion to the amount of IL-1α or IL-8 present in the sample. Standard curves for each cytokine were generated using the cytokine standards. The results were calculated in pg of cytokine/ml of cell culture medium.

2.7 Determination of NO

NO released from the keratinocytes was assessed by quantitation of nitrite using a Griess assay kit according to the supplier's protocol. The cell culture medium was added to the well plate, then sulfanilamide solution was added into each well. The plate was incubated for 10 min. Next, N-1-napthylethylenediamine dihydrochloride solution was added and the plate was incubated for 10 min again. A sample containing nitrite developed a magenta color that absorbs light at 543 nm. Standard nitrite at known concentrations was used to generate standard curve. Results were calculated as µM of nitrite (µmol of nitrite/l of cell culture medium).

2.8 Determination of PGE_2

PGE_2 release from keratinocytes was determined by ELISA kit according to protocol proposed by the manufacturer. Concisely, the antibody was pre-coated onto the well plate. The cell culture medium was then added to the wells. Next, the alkaline phosphatase tracer-conjugate PGE_2 was added and it bound to the remaining antibody sites. Following incubation, unbound alkaline phosphatase tracer-conjugate PGE_2 was removed during a wash step. The substrate reactive with alkaline phosphatase was added. The intensity of the color of product was inversely proportional to the concentration of PGE_2 in the sample. The results were converted to pg of PGE_2/ml of cell culture medium.

2.9 Statistical analysis

The mean and standard deviation were calculated for all the data collected. A one-way ANOVA and post hoc LSD test were used to compare between groups. A *p*-value less than 0.01 or 0.05 was considered significant on using IBM SPSS Statistics 17.0 software program.

3. Results and Discussion

3.1 Effects of *M. oleifera* leaf extracts and astragalin on normal cell viability

Prior to the analyses of anti-inflammatory properties of *M. oleifera* leaf extracts on normal human keratinocyte cells, it was necessary to evaluate the effects of the extracts on viability of the cells. The results from MTT assay showed that neither water nor 50% ethanol extract at concentrations ranging within 8-125 µg/ml affected cell viability compared to the control (untreated cells) (Figure 2), and also morphology of the cells did not change. Therefore, extracts at the concentrations 10, 50 and 100 µg/ml were chosen for the anti-inflammatory studies.

Astragalin at concentrations of 0.5, 5 and 50 µg/ml and diclofenac sodium at concentrations of 2 and 4 µg/ml were used in the anti-inflammatory test. At all concentration levels tested, astragalin and diclofenac sodium did not affect the viability or morphology of the human keratinocyte cells.

3.2 *M. oleifera* leaf extracts and astragalin reduced IL-1α secretion induced by UVB irradiation

The effect of *M. oleifera* leaf extracts and astragalin on IL-1α secretion after UVB irradiation of human keratinocytes is shown in Figure 3. IL-1α level significantly increased from 15 to 28 pg/ml (~2-fold) when exposed to UVB. The water extract did not affect the IL-1α release induced by UVB exposure whereas the treatment with the 50% ethanol extract at 50 and 100 µg/ml and with astragalin at 5 and 50 µg/ml significantly decreased IL-1α level. Interestingly, 100 µg/ml of the 50% ethanol extract contained about 0.5 µg/ml of astragalin but the treatment with astragalin

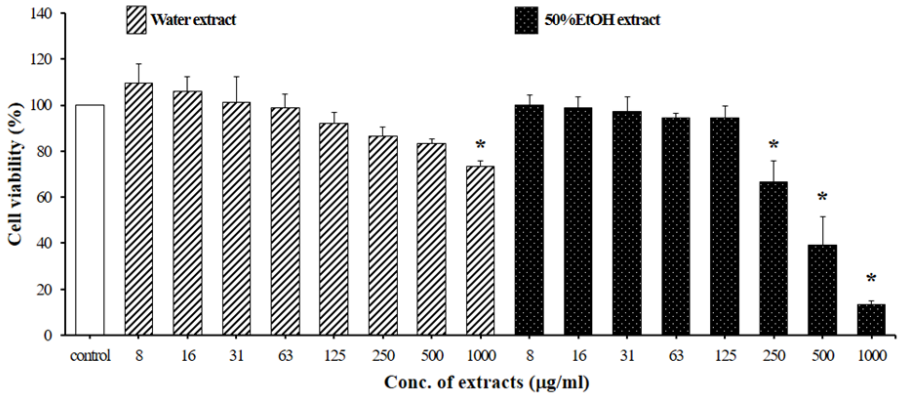

Figure 2. Cell viability of normal human keratinocytes when treated with various concentrations of water extract or of 50% ethanol extract from *M. oleifera* leaves. Values are given as mean ± S.E. of triplicate experiments. * indicates that $p < 0.05$ with significant difference from the control group.

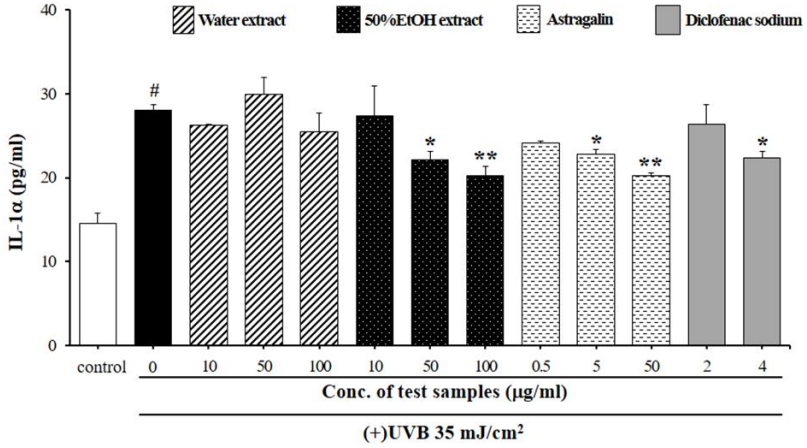

Figure 3. Inhibition of UVB-induced IL-1α secretion by *M. oleifera* leaf extract, astragalin and diclofenac sodium. Human keratinocytes after exposure to UVB irradiation of 35 mJ/cm^2 were treated with water extract, 50% ethanol extract, astragalin or diclofenac sodium for 24 h. The levels of IL-1α in medium were determined. Values are expressed as mean ± S.E. of triplicate experiments. UVB irradiation significantly increased IL-1α release compared to non-irradiated control ($^{\#}$ for $p < 0.01$). A significant difference in IL-1α secretion compared to UVB-irradiated control is indicated by * for $p < 0.05$ or ** for $p < 0.01$.

at 0.5 µg/ml did not significantly reduce IL-1α release (Engsuwan, Waranuch, Limpeanchob, & Ingkaninan, 2017). Other compounds including isothiocyanates might also contribute to this anti-inflammatory action (Waterman *et al.*, 2014). The result is in agreement with an earlier report that the treatment with the 50% ethanol extract from *M. oleifera* leaves significantly decreased serum levels of IL-1α and also TNF-α in atherogenic diet induced hyperlipidemic rat model (Rajanandh, Satishkumar, Elango, & Suresh, 2012).

3.3 *M. oleifera* leaf extract and astragalin reduced IL-8 secretion induced by UVB irradiation

UVB irradiation increased the release of IL-8 from human keratinocytes into the cell culture media after 24 h from 9 to 74 pg/ml (~8-fold). The effect was significantly dose-dependent when the cells were treated with *M. oleifera* leaf extracts (Figure 4A). With 100 µg/ml of water extract, the IL-8 level was decreased to 49 pg/ml ($p < 0.05$). In the 50% ethanol extract treated groups, the IL-8 level was decreased at all concentrations. The largest decrease of IL-8 level (to 2 pg/ml; $p < 0.01$) was observed when cells were treated with 100 µg/ml of 50% ethanol extract. Kooltheat *et al.* reported that *M. oleifera* decreased production of TNF-α, IL-1β and IL-8 in response to both lipopolysaccharide and cigarette smoke treated groups of human macrophage cells. These effects resulted from inhibiting expression of an inflammatory gene, *RelA*, implicated in the NF-κB p65 signaling in inflammation (Kooltheat *et al.*, 2014).

Then, the effect of astragalin on IL-8 release was determined. Similar to the previous experiment, UVB irradiation increased the release of IL-8 into the cell culture media after 24 h. However, the IL-8 levels of all groups, including the non-UVB irradiated group, were higher than in the previous experiment. The inconsistency of the inflammatory response might be due to different skin tissue donors. The level of IL-8 was significantly increased from 36 to 252 pg/ml (~7-fold), whereas the level decreased ($p < 0.01$) when cells were treated with astragalin in a dose-dependent manner; and diclofenac sodium was used as a positive anti-inflammatory drug control (Figure 4B). IL-8 level in cells treated with astragalin at 0.5 µg/ml was close to that obtained

for cells treated with diclofenac sodium at 2 µg/ml (181 vs 175 pg/ml).

3.4 Effect of UVB on NO secretion

Cultured human keratinocytes were separately irradiated with UVB at 35, 50, 75 and 100 mJ/cm^2. The secretion of NO was indirectly measured after irradiation at 6, 12 and 24 h by determination of nitrite content. Human keratinocytes produced NO, but the amount of NO was very low (less than 1 µM). No statistically significant differences were found in NO secretion between the non-irradiated controls and the UVB irradiated groups (data not shown). The results, however, did not correlate with the results by Deliconstantinos *et al.*, in which treatment of human keratinocytes with UVB radiation resulted in a release of NO. The difference may possibly be caused by the differences in cell source and condition: human keratinocyte cells were derived from a normal epidermal foreskin used in the current study, whereas human keratinocyte cells were derived from an epidermal squamous cell carcinoma cell line in the prior study (Deliconstantinos *et al.*, 1995). However, our results are similar to a study reported by Seo *et al.*, in which the cell conditions and source of cells are similar to ours. They reported that NO production did not significantly increase in human keratinocyte cells 48 h after UVB irradiation, whereas HaCaT and PAM212 cells had significantly increased NO releases after UVB irradiation for 48 and 12 h, respectively (Seo, Choi, Chung, & Hong, 2002). Therefore, we did not further study the effects of *M. oleifera* leaf extracts on NO secretion by keratinocytes.

3.5 *M. oleifera* leaf extracts and astragalin reduced PGE_2 secretion induced by UVB irradiation

UVB irradiation increased the release of PGE_2 into cell culture media after 24 h. from 42 to 99 pg/ml (~2.5-fold). The effect was highly decreased by treatment with *M. oleifera* leaf extracts or with astragalin ($p < 0.01$) at most of the concentrations tested (Figure 5). This is consistent with a recent study by Fard *et al.*, which found that the 90% ethanol leaf extract of *M. oleifera* significantly inhibited the secretion

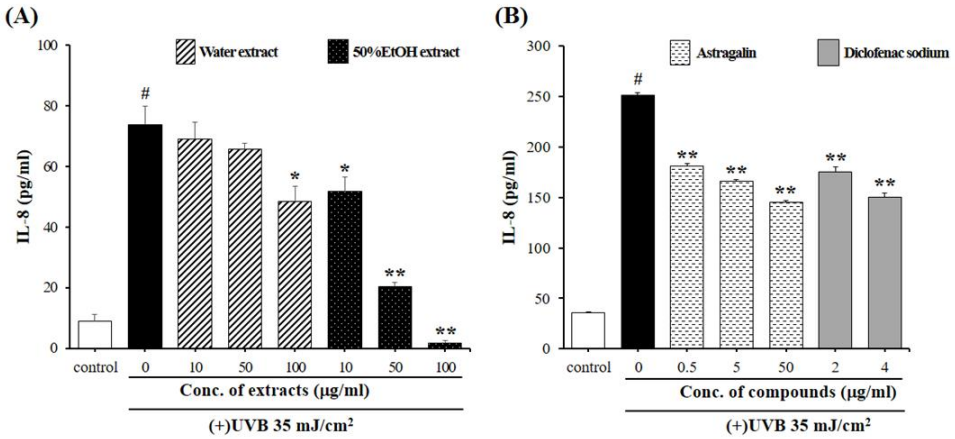

Figure 4. Inhibition of UVB-induced IL-8 secretion by (A) *M. oleifera* leaf extracts, and (B) astragalin and diclofenac sodium. Human keratinocytes after exposure to UVB irradiation of 35 mJ/cm^2 were treated with the test samples for 24 h. The levels of IL-8 in medium were determined. Values are the means ± S.E. of triplicate experiments. UVB irradiation significantly increased IL-8 release compared to non-irradiated control ($^{\#}$ for $p < 0.01$). A significant difference in IL-8 secretion compared to UVB irradiated control is indicated by * for $p < 0.05$ or ** for $p < 0.01$.

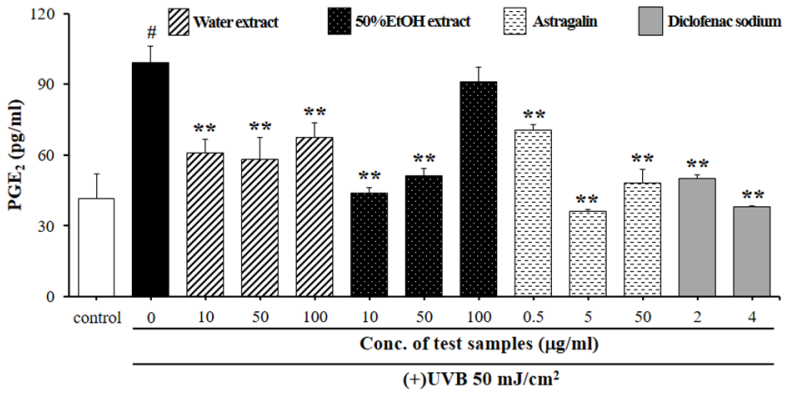

Figure 5. Inhibition of UVB-induced PGE_2 secretion by *M. oleifera* leaf extracts, astragalin and diclofenac sodium. Normal human keratinocytes after exposure to UVB irradiation of 50 mJ/cm^2 were treated with water extract, 50% ethanol extract, astragalin or diclofenac sodium for 24 h. The levels of PGE_2 in medium were determined. Values are expressed as mean ± S.E. of triplicate experiments. UVB irradiation significantly increased PGE_2 release compared to non-irradiated control ($^{\#}$ for $p < 0.01$). A significant difference in PGE_2 secretion compared to UVB irradiated control is indicated by * for $p < 0.05$ or ** for $p < 0.01$.

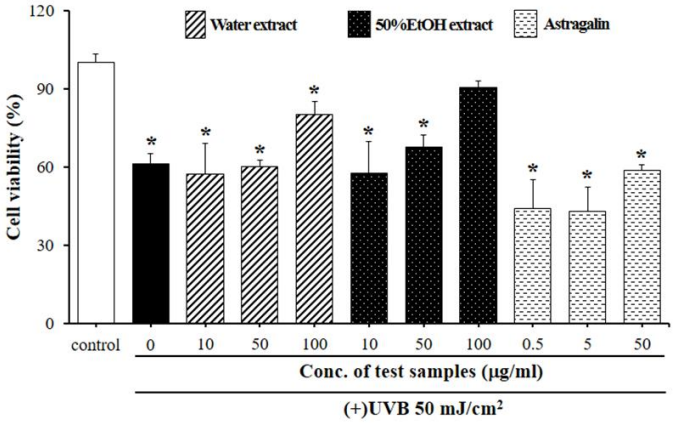

Figure 6. The effects of *M. oleifera* leaf extracts and of astragalin on viability of keratinocyte cells after UVB exposure (50 mJ/cm^2). Values are given as mean ± S.E. of triplicate experiments. * indicates that $p < 0.05$ for significant difference from non-UVB irradiated group.

of PGE_2 via blocking cyclooxygenase-2 (COX-2) protein expression in LPS-stimulated murine macrophages model (Fard, Arulselvan, Karthivashan, Adam, & Fakurazi, 2015).

However, it is noted that 50% ethanol extracts at 100 µg/ml did not affect the level of PGE_2 in UVB irradiated keratinocytes in comparison with the control group. One possible explanation is that the extract at this concentration enhanced viability of the UVB irradiated keratinocytes (Figure 6). A larger number of cells led to larger production of PGE_2 when compared with other concentrations and treatments. Another possible reason is that some compound(s) in the extract may increase PGE_2 levels in keratinocytes.

4. Conclusions

The present study provided evidence of anti-inflammatory properties of water extract and 50% ethanol extract and the major flavonoid constituent astragalin of the *M. oleifera* leaves, when tested on human keratinocytes. UVB irradiation significantly increased the secretion of inflammatory mediators IL-1 α, IL-8 and PGE_2 from the keratinocytes, and this effect was significantly decreased by treatments with astragalin or with 50% ethanol extract of *M. oleifera* leaf. These results support the use of this extract in cosmetic products to treat or inhibit skin inflammation, for example from sunburn. Astragalin could be used in the quality control of *M. oleifera* extracts.

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