Original Article

Protective effect of purple corn silk extract against ultraviolet-B-induced cell damage in human keratinocyte cells

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Abstract

Ultraviolet-B (UVB) could lead to inflammation and cell death induction. Purple corn silk (PCS), part of female flower of corn has multiple pharmacological properties. This investigation focused on determining the preventive effects of PCS extract on human keratinocyte HaCaT cell damage induced by UVB irradiation. Cells were irradiated with 25 mJ/cm^2 UVB after pre-treated with PCS extract for 1 h. Then, the cells were then placed in culture medium followed by subsequent experiments. Cell survival was determined by MTT assay. The immunofluorescence, DCFH-DA, JC-1, and Hoeshst33342 staining assays were used to determine γ-H2AX, intracellular reactive oxygen species (ROS), membrane potential of mitochondria, and nuclear condensation, respectively. Western blot analysis was used to investigate the proteins expression. The statistically significant comparison was calculated by analysis of variance at $P < 0.05$. The fluorescence and protein band intensity were quantified by Image J densitometer. The results indicated cell survival was increased upon PCS extract pretreatment followed by UVB exposure. PCS extract decreased γ-H2AX expression, intracellular ROS overproduction, and nuclear condensation in cells induced by UVB. Furthermore, The PCS extract pretreatment attenuated apoptosis response through stabilized mitochondrial membrane potential, decreased apoptosis mediator proteins including Bax, Bak, cleaved-caspases, and cleaved-PARP, and increased Bcl-2 and Bcl-xL expression comparing to the UVB-treated control. This finding demonstrated that the PCS extract can reduce the deleterious effects from UVB exposure through decreased intracellular ROS, DNA damage, and apoptosis induction on HaCaT cells.

Key words: Apoptosis, keratinocyte, purple corn silk, ultraviolet-B-protection

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INTRODUCTION

Skin serves as a physical barrier to solar ultraviolet (UV) radiation of human body. The epidermis in skin layer mainly composed of keratinocyte cells which are always expose to solar UV.[1,2] UVB-exposed skin can trigger and activate the histone H2AX phosphorylation to generate γ-H2AX, a hallmark of DNA damage and induces the overproduction

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of reactive oxygen species (ROS) causing cell damages leads to inflammation and death on skin cells.[3-7]

Generally, the extensive cellular damage results in apoptosis.[8] UVB induces apoptosis through imbalanced Bcl-2 proteins family. The increased pro-apoptotic proteins lead to reduced mitochondrial permeability, the release of apoptosis-inducing factor and cytochrome c resulting in caspase9 then caspase3/7 activation. The cleaved-caspase3/7 subsequently inactivated PARP to cleaved-PARP, which indicating an irreversible apoptosis cell death.[9,10] Furthermore, UVB directly induces apoptosis via activating apoptosis-related surface molecule CD95 causing caspase8, then caspase3/7 activation.[11]

Purple corn silk (PCS), female flower of *Zea mays* L, which is rich in anthocyanin and flavonoid contents.[12-15] However, the preventive effect of PCS extract on HaCaT cell damage induced by UVB irradiation has not been determined. Therefore, this study we have an objective to examine the protective potential of hydroglycolic PCS extract on UVB-induced HaCaT cell damage.

MATERIALS AND METHODS

Plant extraction

PCS extract is purple stigma part of *Zea mays* L., obtained from Prof. Dr. Malyn Ungsurungsie, Mahidol University, Bangkok, Thailand. The specimen was harvested from Khonkaen province in March, 2014 and kept in the laboratory at Plant Breeding Research Centre for Sustainable Agriculture of Khonkaen University, Thailand (Voucher sample Number 050914T001). The extraction procedure was followed previously published.[16] Briefly, the fresh purple stigma was dried and ground, then immersed in 50% propylene glycol: water (10% W/V) for 2 days. The macerated material was primarily filtered through the cheesecloth and Whatman filter paper No. 4, then evaporated, and kept refrigerated until use.

Cell culture

Human keratinocyte cell line (HaCaT) was purchased from American Type Culture Collection (ATCC, VA). The Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Merck Corp., Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, NY) were used for HaCaT cell culture and maintained in at 37°C in a humidified atmosphere of 5% carbon dioxide.

Ultraviolet-B irradiation system

An UV incubator, BIO SUN UVB VILBER LOURMAT, FRANCE consisted of UVB light at 312 nm wavelength (2 × 30 W) was used as a UVB source. Before irradiation, cells were covered with phosphate buffered saline buffer. After that, cells were placed in medium.

Cytotoxicity assay

The MTT assay was carried out to determine toxicity of PCS extract. Briefly, HaCaT cells were seeded at 0.7×10^4 cells/well, then treated with PCS extract for 24 h. The cytotoxicity of UVB on cells was analyzed by seeding 30×10^4 cells/well for overnight, followed by UVB exposure and placed in medium for 24 h. To evaluate the effect of PCS extract on UVB-induced cell death, the cells were treated with PCS extract for 1 h before 25 mJ/cm^2 UVB exposure, then placed in medium for 24 h. After treatment, 0.5 mg/ml MTT was added and incubated for 4 h. Then, DMSO was added to dissolve formazan crystal and measured the absorbance at 570 nm using a microplate reader (UV/visible spectrophotometer, Biotek Epoch). Cells survival was assessed as the cell viability (%).

Immunofluorescence staining

After cell seeding, the cells were pre-treated with PCS extract, UVB irradiated, and placed in medium for 24 h. Then, cells were fixed with methanol and incubated with anti-γ-H2AX serine139 (1:250) (Merck Corp., Germany) at 4 °C overnight. After that, cells were incubated with secondary antibody conjugated with FITC (1:500) (Cell Signaling, MA) and counterstained with Hoechst33342, and observed under the fluorescence microscope (DP73+IX71 Olympus, Japan). The relative green-fluorescence intensity was quantified by using Image J densitometer.

Measurement of reactive oxygen species

After UVB irradiation, cells were incubated with 20 μM of DCFH-DA (Merck Corp., Germany) for 30 min and observed under the fluorescent microscope (DP73+IX71 Olympus, Japan) and relative of DCF-fluorescence intensity was quantified.

Hoechst33342 staining

After 24 h of treatment, cells were stained with 5 μg/ml of Hoechst33342 dye (Invitrogen, USA) for 30 min before observed under a fluorescent microscope (DP73+IX71 Olympus, Japan).

Western blot analysis

Cells were harvested and lysed using RIPA buffer containing Complete Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Germany). The protein samples were resolved by SDS-PAGE, transferred onto polyvinylidene fluoride membranes (Pall Corp., USA), blocked with 5% non-fat milk, and incubated with primary antibodies including anti-γ-H2AX (Sre139) and β-actin (Merck Corp., Germany), PARP, caspase8,-9, and-7, Bax, Bak, Bcl-2, and Bcl-xL (Cell Signaling, USA) (1:1000) at 4°C overnight followed by secondary antibody conjugated with horseradish peroxidase (Cell Signaling, USA). The immunoreactive bands were detected by using enhanced chemiluminescent reagent (Merck Corp., Germany) and quantified using Image J densitometer.

Mitochondrial membrane potential *(ΔΨm)* detection

After 24 h of treatment, cells were stained with 5 µg/ml of JC-1 dye (Invitrogen, USA) and observed under a fluorescent microscope (DP73 + IX71 Olympus, Japan). The relative red-fluorescence intensity was quantified.

Statistical analysis

All the data represented three independent experiments ($n = 3$) as mean ± standard deviation. SPSS version 17.0 (SPSS, Inc.,Chicago, IL, USA) was used for statistical analysis. The statistical significance comparisons were calculated using one-way analysis of variance using Tukey's *post hoc* test at $P < 0.05$. The fluorescence and protein band intensity were quantified by Image J densitometer.

RESULTS

Comparison of cell viability

The result demonstrated that 1 mg/ml of PCS extract provided 94.0% ± 0.41% cell viability [Figure 1a]. For optimization of UVB irradiation, a serial UVB energy irradiation HaCaT cells was conducted [Figure 1b]. Form these results, 25 mJ/cm^2 UVB and 0.3, 0.5, and 1 mg/ml of PCS extract used for further experiments. The study on

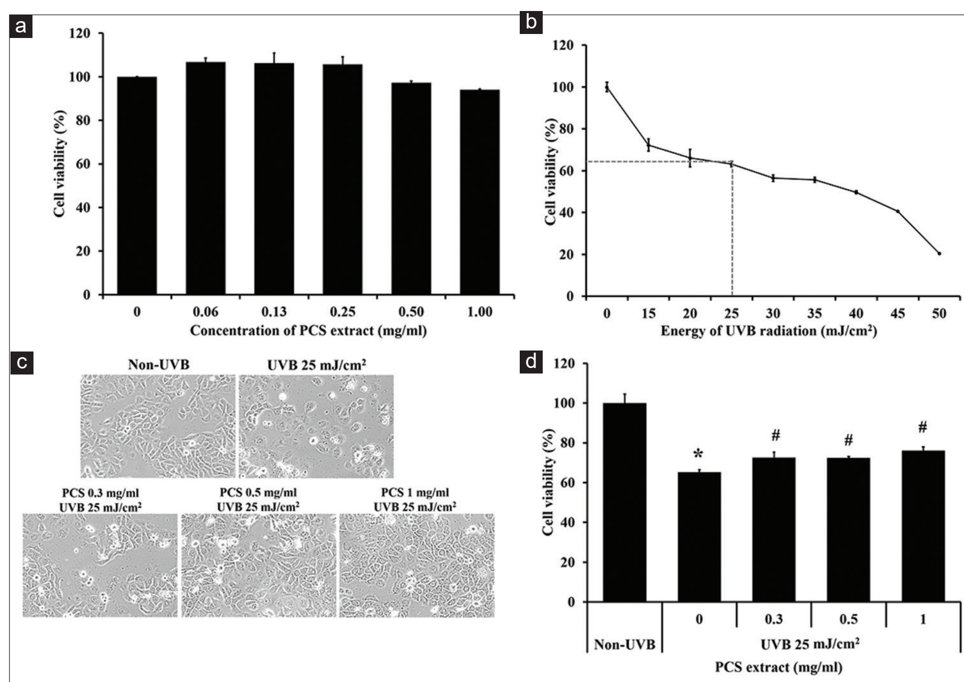

Figure 1: Cytotoxicity of purple corn silk extract on HaCaT cells (a) at various concentrations of purple corn silk extract. (b) At various ultraviolet-B -energy levels. (c) Images of HaCaT cells treated with purple corn silk and ultraviolet-B at ×20. (d) Histogram show percentage of cell viability treated with purple corn silk and ultraviolet-B. Results are the mean values ± standard deviation of three independent experiments. *$P < 0.05$ versus non- ultraviolet-B group and #$P < 0.05$ versus the ultraviolet-B-control group

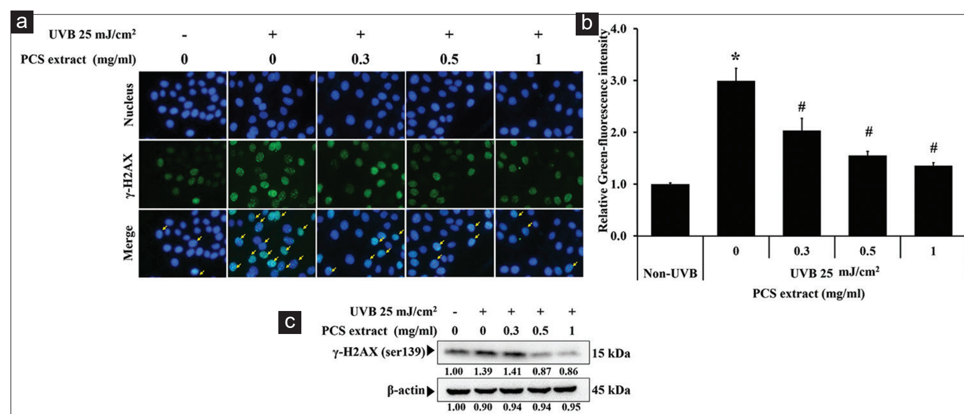

Figure 2: Effect of purple corn silk extract on γ-H2AX (ser139) levels in HaCaT cells. (a) Images of γ-H2AX (ser139) accumulation (green) in nuclei (blue) at 20. (b) Histogram of relative green fluorescence intensity. (c) Protein expression. Results are the mean values ± standard deviation of three independent experiments. *$P < 0.05$ versus non- ultraviolet-B group and #$P < 0.05$ versus the ultraviolet-B-control group. The arrows show the γ-H2AX-positive nucleus

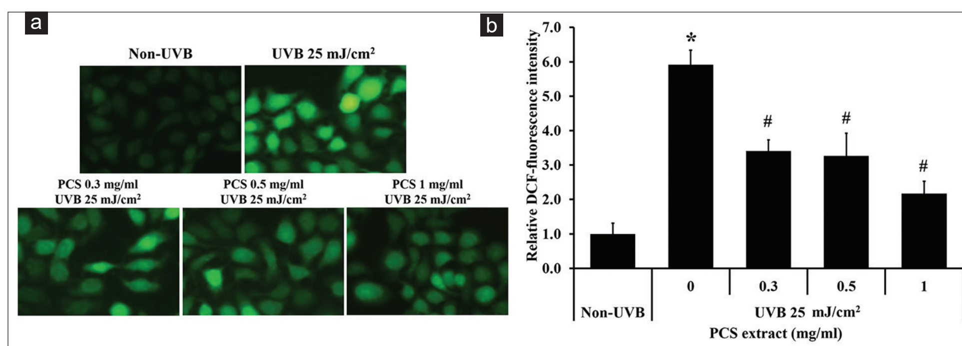

Figure 3: Effect of purple corn silk extract on reactive oxygen species production in HaCaT cells (a) (at ×20). (b) Histogram represents relative DCF-fluorescence intensity. Results are the mean values ± standard deviation of three independent experiments. *$P < 0.05$ versus the non- ultraviolet-B group and #$P < 0.05$ versus the ultraviolet-B-control group

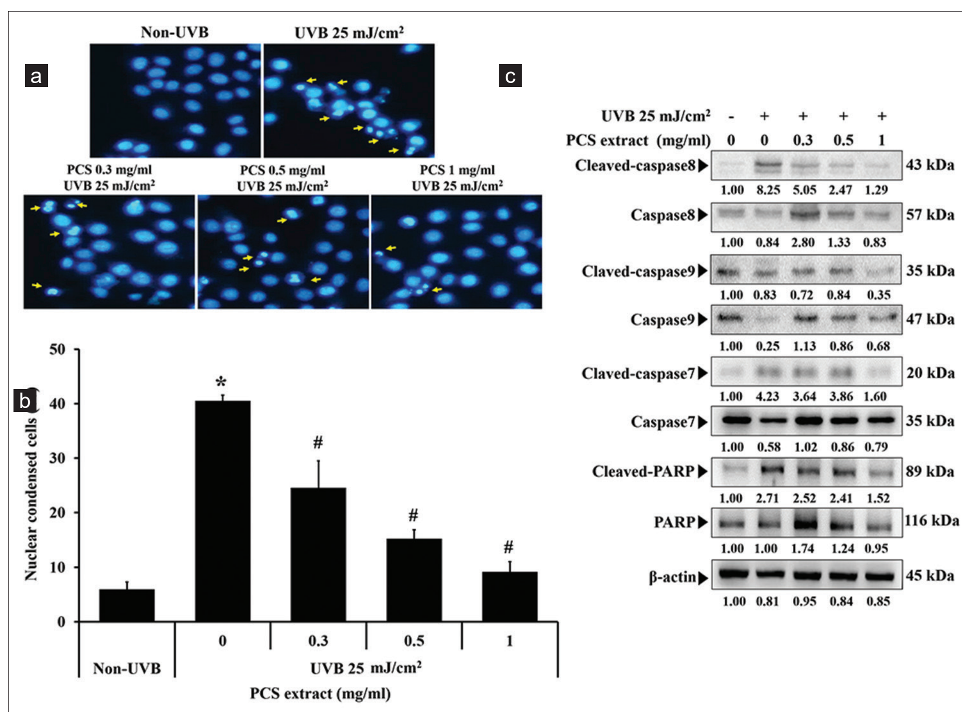

Figure 4: Effects of purple corn silk extract on ultraviolet-B-mediated apoptosis. (a) Nuclear changes of the cells stained with Hoechst33342 dye at ×20. (b) Histogram represents nuclear condensed cells. The arrows show nuclear-condensed cells. (c) The expression of apoptotic mediator proteins. Results are the mean values ± standard deviation of three independent experiments. *$P < 0.05$ versus the non- ultraviolet-B group and #$P < 0.05$ versus the ultraviolet-B-control group

morphological changes upon PCS-pretreatment revealed less cell shrinkage and membrane blebbing [Figure 1c] but significant increased cell viability compared to the UVB-control group [Figure 1d].

Purple corn silk extract attenuates DNA damage induced by ultraviolet-B

The effect on UVB-induced DNA damage was monitor by phospho-H2AX (Ser139) localization and protein expression. The results that PCS-pretreatment demonstrated decreased γ-H2AX (Ser139) nuclei accumulation as compared to the UVB-control group [Figure 2a and b] correlating with Western blot analysis [Figure 2c].

Purple corn silk extract decreases ultraviolet-B-induced intracellular reactive oxygen species

The results demonstrated that the UVB-control group showed the overproduction of ROS compared to non-UVB. While, cells pre-treated with PCS extract decreased ROS production as compared to the UVB-control group [Figure 3a and b].

Purple corn silk extract reduces ultraviolet-B-induced nuclear condensation

After cells staining, the result showed that PCS extract pre-treated cells significantly inhibited UVB-induced nuclear condensation comparing to the UVB-control group [Figure 4a and b].

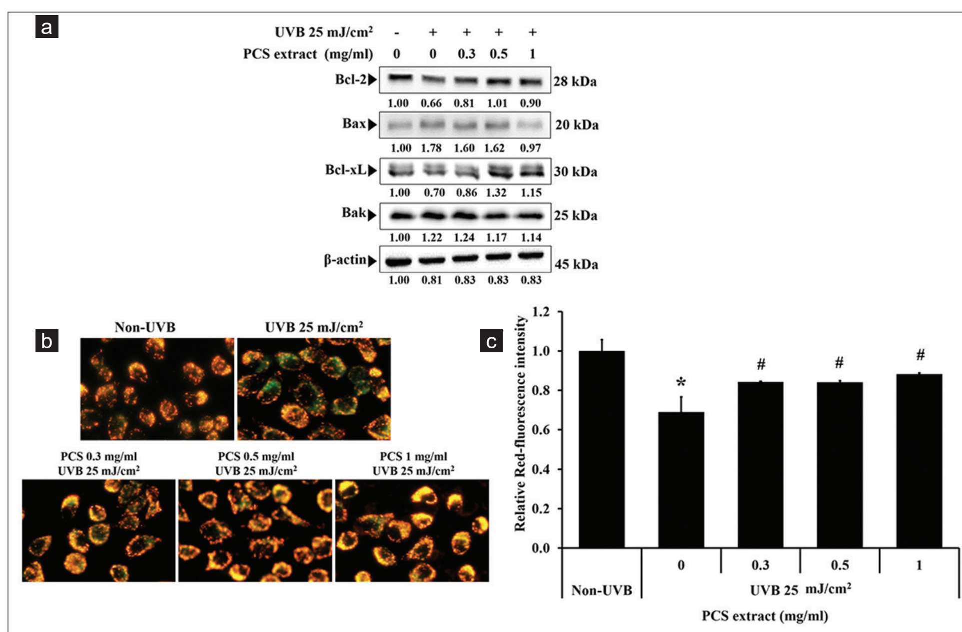

Figure 5: Effects of purple corn silk extract on ultraviolet-B - induced mitochondrial apoptotic pathway. (a) Expression of Bcl-2 family proteins. (b) The loss $\Delta\Psi m$ (at ×20). (c) Histogram of relative red-fluorescence intensity. Results are the mean values ± standard deviation of three independent experiments. $^{*}P < 0.05$ versus the non- ultraviolet-B group and $^{\#}P < 0.05$ versus the ultraviolet-B-control group

Purple corn silk extract prevents ultraviolet-B-induced apoptosis through apoptotic mediator proteins

We hypothesized that PCS might prevent UVB-mediated apoptosis. Thus, the activation of caspases and PARP was conducted by Western blot analysis. The result showed that the cleavage of proteins was decreased in HaCaT cells pre-treated with PCS extract compared with the UVB-control group [Figure 4c].

Purple corn silk extract attenuates ultraviolet-B-induced mitochondrial apoptotic pathway via Bcl-2 family proteins

To understand the apoptosis response of PCS extract in HaCaT cell induced by UVB, the expression of Bcl-2 family proteins was evaluated. This result demonstrated that PCS pretreated cells before UVB irradiation increased Bcl-2 and Bcl-xL, and decreased Bax and Bak proteins level [Figure 5a]. Overproduction of ROS and the imbalanced Bcl2 family protein lead to mitochondrial membrane dysfunction which was determined by JC-1 staining. We found that cells pre-treated with PCS extract showed significantly higher red-fluorescence intensity than the UVB-control group [Figure 5b and c]. These data suggest that PCS extract protects UVB-induced mitochondrial apoptotic pathway.

DISCUSSION

Natural plants are known as rich sources of antioxidant phytonutrients. The ethanol and water extract of purple corn and corn silk are rich in phenolic content and present antioxidant power.[12,13,17-19] Recently, hydroglycolic extraction is popular for cosmetics applications. Nithitanakool *et al*. showed that the hydroglycolic extract of *Etlingera elatior* presented high total phenolic content and anti-oxidant activities.[16] We found that hydroglycolic PCS extract demonstrated antioxidant activity through DPPH scavenging in a dose-dependent manner [Additional Material; Figure S1]. Unfortunately, the phytochemicals of hydroglycolic PCS extracts have not yet been reported up to now. We have hypothesized that the phytochemicals of hydroglycolic PCS extract may be rich in anthocyanin content, thus we determine the presence of anthocyanin in PCS extract based on visible color changes of anthocyanin toward different pH values. The PCS extract was added with 2 N hydrochloric acid appeared pink-red color that turns deep-green after addition of ammonia [Additional Material; Figure S2], indicating PCS extract may be rich in anthocyanin content. However, the phytochemicals of hydroglycolic PCS extract needs to be further confirmed.

UVB radiation has been reported to damage skin cell death. Our study is the first to report the biological effect of hydroglycolic PCS extract on UVB-irradiated HaCaT cells through decreased apoptosis cell death. Previous study demonstrated that UVB-induced accumulation of γ-H2AX in nucleus resulted in apoptosis induction.[5] Interestingly, our results revealed that PCS pre-treatment decreased γ-H2AX Protein expression and nuclear accumulation correlated with the studies of cyanidin-3-galactoside (C3G), showing decreased γ-H2AX and DNA photoproducts in skin cells induced by UVB.[20]

Many studies have reported that anthocyanin prevented cell damage from UVB by decreased hydrogen peroxide

and enhanced antioxidant enzyme to eliminate free radicals in cells.[21-23] We found that ROS generation by UVB-treated HaCaT cells was inhibited by PCS pre-treatment before UVB irradiation. Studies showed that UVB induced nuclear changes, condensed chromatin, and DNA fragmentation on skin resulted in apoptosis.[24,25] Our result demonstrated that PCS extract can prevent cells from UVB-induced nuclear condensation. In addition, the increase of pro-apoptotic proteins and overproduction of ROS induces the opening of large pores in mitochondrial membranes and loss permeability. This event leads to depolarized mitochondrial membrane potential, impaired mitochondrial oxygen uptake and ATP production resulting in the efflux of apoptogenic proteins subsequently caspase cascade and apoptotic responses.[24,25] Interestingly, topical application of polyphenol compounds demonstrated the preventive effects on UVB-induced cell damages in human keratinocyte through decreased ROS, maintained mitochondrial membrane potential, decreased inflammatory cytokines and attenuated apoptosis via down-regulated apoptotic proteins *in vitro* and *in vivo*.[23-26] Correlated with our study, we suggested that PCS extract prevented apoptosis induction in UVB-irradiated cells via inhibited cleaved-caspases, suppressed pro-apoptotic proteins, and maintained mitochondrial membrane potential.

CONCLUSIONS

Our results demonstrated that UVB radiation is the cause of human keratinocyte cell damage which was attenuated by PCS extract through decreased DNA damage, intracellular ROS, and apoptosis induction. Hence, PCS extract may be used as a new anti-UVB agent in cosmetic products.

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Conflicts of interest

There are no conflicts of interest.

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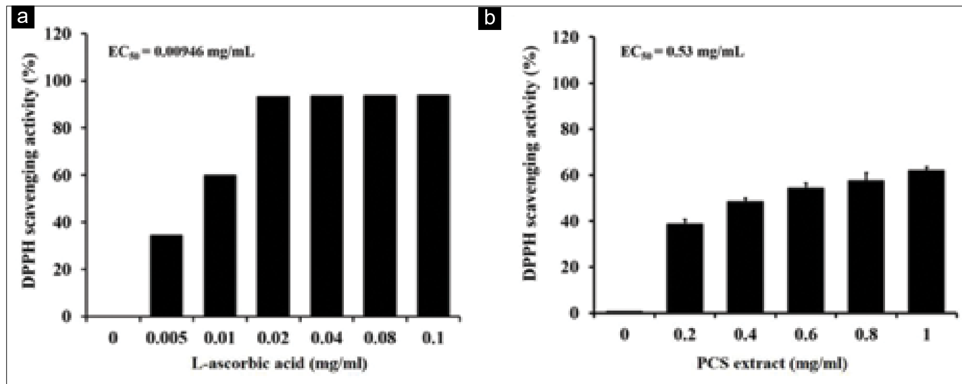

Additional Material Figure S1: DPPH-free radical scavenging (%) (a) L-ascorbic acid was used as a positive control. (b) the PCS extract

Additional Material Figure S2: Anthocyanins of PCS extract in acid-base condition. Red-pink color of PCS extract was developed by adding 2 N HCl (left). Deep-green of the PCS extract was by adding ammonia (right)