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Protective Efficacy of Pomegranate *(Punica granatum* Linn., Punicaceae) Peel Ethanolic Extract on UVB-Irradiated Rat Skin

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Abstract: Pomegranate (*Punica granatum* Linn.) peel ethanolic extract (PPE) contained substantial phenolics and flavonoids of 451.96 ± 4.29 µg GAE/mg and 37.61 ± 1.43 µg CAE/mg, respectively. PPE possessed marked antioxidant activities evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl radical) with IC_{50} 51.51 ± 2.03 µg/ml, FRAP (ferric reducing antioxidant power) with IC_{50} 49.07 ± 1.53 µg/ml and FTC (ferric thiocyanate) with IC_{50} 61.43 ± 4.18 µg/ml. Its antioxidant capacity, using ascorbic acid (AA) equivalents, was similar to the known natural antioxidants, catechin (CA) and epigallocatechin-3-gallate (EGCG), but 2.9-3.5 fold less than the synthetic antioxidants, 3-*tert*-butyl-4-hydroxyanisole (BHA) and butylated hydroxyl toluene (BHT). PPE topically applied on hairless rat skin for 30 min prior to UVB irradiation at 3xMED (minimal erythema dose = 0.07-0.08 J/cm^2) for 24 hrs, twice a week for one month. Propylene glycol was the vehicle control. PPE remarkably lessened the UVB-induced lesions on the skin. Topical pretreatments of PPE and EGCG at 8 mg/cm^2 were able to protect the skin against 3xMED UVB-induced erythema, epidermal thickness, sunburn cells and DNA fragmentation. The erythema was reduced 2.5 fold and the epidermal thickness was decreased 1.7 fold of the vehicles. The sunburn cells were 7.4 fold less than the vehicles. DNA fragmentation was very slightly occurred. These findings indicate that topical PPE prior to UVB irradiation is very effective in prevention the skin lesions and DNA damages. This also makes use of and was value added to the pomegranate peel.

Key words: Pomegranate Peel Ethanolic Extract · Antioxidant Activity · UVB Irradiation · Erythema · DNA Fragmentation

INTRODUCTION

Overexposure to UV radiation is a great concern of inducing sunburn formation and skin cancer. UVA (320-400 nm) and UVB (290-320 nm) cause oxidative damages to skin cells [1, 2]. UVA induces a variety of reactive oxygen species (ROS) inducing DNA, lipid and protein damages. UVB is responsible for erythema, inflammation, DNA damage and skin cancer [3, 4]. Acute exposure to UVB affects on keratinocytes producing sunburn formation on epidermis and inducing apoptosis [5] and hyperproliferation of epidermal cells [6]. Long-term and recurrent exposure to UV causes gradual deterioration of skin structure and function. Apparently, accumulation of DNA damages as results of the recurrent and acute DNA injuries and the effect of chronic inflammation [7] could ultimately lead to the development of skin cancers [8]. Protecting skin actinic damages by UV using topical sun block produced from plant products is now of interest.

Pomegranate (*Punica granatum* Linn.) has been used in many countries as traditional medicines, such as treatment of intestinal parasites [9] and Gram- negative bacteria [10]. Pomegranate pericarp exhibits antiviral activity [11]. Pomegranate bark, leaves, fruits and fruit rind are mild astringent, used to treat diarrhea, dysentery, hemorrhage and some fevers [12]. Recently, chemical constituents of pomegranate have been identified and found containing antioxidant property [13, 14]. Few studies reported that pomegranate fruit extract was able to suppress UV-induced skin pigmentation, when topically applied [15] or orally administration [16]. It also suppressed human epidermal keratinocyte damage [17] and inhibited skin tumorgenesis in CD1 mice [18]. There

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are few evidences of pomegranate peel on skin protection against UV irradiation. This study illustrated the protective effects of pomegranate peel ethanolic extract (PPE) against UVB irradiation on rat skin. The knowledge findings here could be useful for application of pomegranate peel to protect and heal UV-induced human skin damages.

MATERIALS AND METHODS

Materials: Folin-Ciocalteau reagent and gallic acid were obtained from Fluka, Switzerland. Catechin (CA), epigallocatechin-3-gallate (EGCG), ascorbic acid (AA), 3-*tert*-butyl-4-hydroxyanisole (BHA), butylated hydroxyl toluene (BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and linoleic acid were purchased from Sigma-Aldrich, USA. Agarose was from Promega, Spain. Ethidium bromide was from Bio-Rad, USA. RNase A was purchased from Amresco®, USA. DNA ladder and Genomic DNA Extraction Kit were obtained from RBC Bioscience, USA. All other chemicals and solvents were reagent grade and purchased from Sigma-Aldrich, USA. A UV light source (285-350 nm), Waldmann UV 109B equipped with UV21 lamp and Variocontrol spectroradiometer were from Waldmann (Villingen-Schwenningen, Germany).

Pomegranate Collection and Peel Extraction: Pomegranate fruits were purchased from local farms in Nakhon Ratchasima, Thailand. The fruits were cleaned and peeled. The peel was dried and ground to powder. The pomegranate peel powder was extracted in 70% ethanol in a Soxhlet extraction apparatus. The pomegranate peel ethanolic extract (PPE) was evaporated, lyophilized and kept at -20°C. The PPE power was re-dissolved in its original solvents for all experiments.

Determination of Total Phenolic Compounds: Total phenolic content (TPC) was measured by Folin-Ciocalteu method [19]. One hundred microliters of sample was mixed with 2 ml of 2% sodium carbonate and 100 µl Folin-Ciocalteu reagent (Folin-Ciocalteu : methanol, 1:1, v/v) and incubated for 30 min. The absorbance was measured at 760 nm. Gallic acid was used as a standard. TPC content was expressed as mg of gallic acid equivalents per milligram of sample.

Determination of Flavonoid Content: Flavonoid content was quantified by a colorimetric method modified from Liu *et al.* [20]. Briefly, 250 µl of sample was mixed with 1.25 ml of dH_2O and 75 µl of 5% $NaNO_2$. After incubation for 6 min, 150 µl of 10% $AlCl_3.6H_2O$ was added and allowed to stand for 5 min. Five hundred microliters of 1 NaOH was added and the final volume was made up to 2.5 ml with dH_2O. The solution was well mixed and the absorbance was measured at 510 nm. The flavonoid content was calculated and expressed as micrograms of CA equivalents per milligram of the sample.

Free Radical Scavenging Activity: Antioxidant property was determined by free radical scavenging activity using DPPH (2,2-diphenyl-1-picrylhydrazyl radical) method [21]. Sample solution of 50 µl was mixed with 1.95 ml of DPPH reagent. The mixture was kept in the dark for 45 min and then the absorbance was measured at 515 nm. CA and EGCG were used as standard controls. The free radical scavenging value was calculated using AA equivalents per µg of sample. The antioxidant activity of sample was defined as the amount of antioxidants necessary to reduce the initial $DPPH^{\bullet}$ concentration by 50% (IC_{50}).

Ferric Reducing Antioxidant Power (FRAP) Assay: The ferric reducing antioxidant power (FRAP) was estimated according to the method of Benzie and Strain [22]. Briefly, sample solution of 100 µl was mixed with 2.9 ml of fresh FRAP reagent, containing 100 mM acetate buffer, pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) and 20 mM $FeCl_3.6H_2O$ solution. The reaction was incubated at 37°C for 30 min and the absorbance was measured at 593 nm. CA and EGCG were used standard controls. The FRAP value was calculated and expressed as IC_{50} using AA equivalents per µg of sample.

Ferric Thiocyanate (FTC) Assay: Inhibition of lipid peroxidation was measured by ferric thiocyanate (FTC) method as described by Huang *et al.* [23]. One milliliter of sample, diluted in 99.5% ethanol, was mixed with 1.5 ml of 2.51% linoleic acid in 99.5% ethanol, 2.5 ml of 0.05 M phosphate buffer, pH 7.0 and kept at 40°C in the dark. Then, to 50 µl of this mixture was added to 4.9 ml of 75% ethanol and 50 µl of 30% ammonium thiocyanate. Precisely 3 min after the addition of 50 µl of 20 mM $FeCl_2$ in 3.5% HCl to the reaction mixture, the absorbance of red color of $Fe(SCN)_3$ was measured at 500 nm every 24 hrs until the day after the absorbance of the control reached its maximum. EGCG and the synthetic antioxdidants, BHA (3-*tert*-butyl-4-hydroxyanisole) and BHT (butylated hydroxyl toluene), were used standard controls. The lipid peroxidation value was calculated using CA equivalents

per μg of sample. The lipid peroxidative inhibition of sample was defined as the amount of antioxidants necessary to reduce the initial lipid peroxide concentration by 50% (IC_{50}).

UV Irradiation and Experimental Animals: UVB (290-320 nm) irradiation and its strength were set and determined on hairless skin surface to obtain minimal erythema dose (MED) to produce minimally perceptible erythema reaction. MED was 0.07-0.08 J/cm^2 at 13 cm height with an increment of 0.04 J/cm^2 in 24 hrs [24].

Female Wistar rats, 7-8 week old and 150-200 grams weight were obtained from the National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand. The rats were housed at the Animal Facility of Suranaree University of Technology at 25°C, 50-70% RH and fed *ad libitum*. The rat hair on its back was shaven off about 4×2 cm for all experiments. The rats were divided into 6 groups of 6 rats per group. Group I was normal control (-UV). Group II received non-vehicle (-VE). Group III received vehicle control (propylene glycol) (+VE). Group IV received 2 mg/cm^2 EGCG control (+EGCG). Group V received 2 mg/cm^2 PPE (2-mg PPE). Group VI received 8 mg/cm^2 PPE (8-mg PPE). Groups II – VI were topically applied the test samples for 30 min prior to UVB irradiation at 3xMED for 24 hrs. The rats were irradiated twice a week (every 3 or 4 days) for a month. The UV damages were observed by erythema using dermatoscope and photodocumentation and evaluated by the Draize score system [25, 26] on scale of 0 to 4 (0: none, 1: very slight, 2: well define, 3: moderate, 4: severe). The treated rats were sacrificed, the central exposed area of skin was removed and processed for histological and DNA preparations.

Histological Preparation and Histopathologic Analysis: The skin samples were fixed in 10% neutral-buffered formalin overnight and processed for paraffin sectioning by standard histological techniques. The sections at 3-5 μm thickness were stained with hematoxylin and eosin. Epidermal area and thickness were measured by computerized image analysis at 400 magnifications. Sunburn cells (SCs) were counted per square millimeter.

DNA Fragmentation: DNA was isolated from the treated skin using a genomic DNA extraction kit. RNase A (10 mg/ml) was added to the cell lysate and let stand at room temperature for 30 minutes. The DNA precipitate was centrifuged at 13,000 rpm for 3 minutes, washed in elution buffer and resuspended in TE buffer containing 10 mM Tris-HCl, pH 7.6 and 1 mM EDTA. The DNA, 4 μg, was electrophoresed on 2% agarose gel containing 0.5 μg/ml ethidium bromide in 45 mM Tris, 45 mM boric acid and 1 mM EDTA at 100 mVolts for 1.5 hrs. The DNA ladders were visualized under UV fluorescence and photographed.

Statistical Analysis: Data were analyzed by ANOVA, using the least significant test to determine the level of significant at $P \leq 0.05$ and 0.01. All data were expressed as mean ± standard error. For single comparisons, the different significance of means was determined by Student's *t*-test at significant level of $P \leq 0.01$.

RESULTS

Total Phenolic and Flavonoid Contents: It has been well known that plant phenolics possess high antioxidant activity. In this study, pomegranate peel ethanolic extract (PPE) contained total phenolic compounds (TPC) of 451.96 ± 4.29 μg GAE/mg extract and flavonoid content (FC) of 37.61 ± 1.43 μg CAE/mg extract (Table 1). The amount of TPC and FC could indicate the antioxidant activity of PPE.

Free Radical Scavenging Activity: The antioxidant activity of PPE, measured by DPPH method, was able to scavenge oxidants as concentration dependent manner. At 100 μg/ml PPE could inhibit DPPH$^\bullet$ radicals up to 79% (Table 2). The effectiveness of PPE, determined by IC_{50}, was similar to CA. The IC_{50} of PPE was 51.51 ± 2.03 μg/ml and of CA was 55.43 ± 0.83 μg/ml. However, the effectiveness of PPE was a half of EGCG, IC_{50} of 27.51 ± 1.32 μg/ml ($P \leq 0.01$).

Ferric Reducing Antioxidant Power (FRAP): The FRAP of PPE reduced ferric-2,4,6-tri(2-pyridyl)-s-triazine [Fe(III)-TPTZ] complex to its ferrous-2,4,6-tri(2-pyridyl)-s-triazine [Fe(II)-TPTZ] with IC_{50} of 49.07 ± 1.53 μg/ml (Table 2). The FRAP value of PPE was slightly higher than that of CA, IC_{50} of 58.36 ± 4.02 μg/ml and 1.5 fold lower than that of EGCG, IC_{50} of 33.16 ± 1.09 μg/ml ($P \leq 0.01$).

Ferric Thiocyanate (FTC): FTC assay demonstrated that PPE was able to inhibit the peroxidation of linoleic acid in a dose-dependent manner. The antioxidant effectiveness of PPE as IC_{50} value was 61.43 μg/ml (Table 2). The FTC of PPE was approximately 2.9-3.5 fold lower than those of BHA and BHT, the synthetic oxidants and of EGCG, the natural antioxidant.

Table 1: Total phenolic compounds content and flavonoid contents of pomegranate peel ethanolic extract. Data were mean ± SE, n = 4.

Extract	Total Phenolics μgGAE/mg	Total Flavonoids μgCAE/mg
PPE	451.96 ± 4.29	37.61 ± 1.44

PPE, pomegranate peel ethanolic extract; GAE, gallic acid equivalent; CAE, catechin equivalent.

Table 2: Antioxidant capacity of pomegranate peel ethanolic extract assayed by DPPH, FRAP and FTC methods. Data were expressed as mean ± SE, n = 4.

Antioxidant power by	Sample	Concentration (μg/ml)	Activity (%)	IC_{50} (μg/ml)
DPPH	PPE	25	21.99 ± 2.40	51.51 ± 2.03
		50	51.32 ± 2.03	
		100	97.65 ± 2.94	
	CA	50	50.02 ± 1.99	55.43 ± 0.83
	EGCG	50	74.36 ± 0.94	27.51 ± 1.32*
FRAP	PPE	25	25.84 ± 0.83	49.07 ± 1.53
		50	53.17 ± 1.97	
		100	97.37 ± 0.38	
	CA	50	43.53 ± 2.91	58.36 ± 4.02
	EGCG	50	75.87 ± 2.62	33.16 ± 1.09*
LPI	PPE	10	21.50 ± 0.65	61.43 ± 4.18
		50	46.81 ± 5.87	
		100	78.88 ± 5.81	
	BHA	10	48.13 ± 7.34	19.84 ± 7.60*
	BHT	10	54.69 ± 8.38	17.71 ± 5.21*
	EGCG	10	40.25 ± 1.03	21.25 ± 4.99*

DPPH, 2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity; FRAP, ferric reducing antioxidant power; LPI, lipid peroxidative inhibition; PPE, pomegranate peel ethanolic extract; CA, catechin; EGCG, epigallocatechin-3-gallate; BHA, 3-*tert*-butyl-4-hydroxyanisole; BHT, butylated hydroxyl toluene; IC_{50}, median inhibitory concentration. * $P \leq 0.01$.

Erythema Reduction: Topical application of PPE on hairless rat skin prior to UBV irradiation at 3xMED was able to reduce the skin erythema. The UVB irradiation on non-vehicle (-VE) and vehicle (+VE) pretreated controls induced erythema at 3.69 ± 0.13 and 3.57 ± 0.20 scores, respectively. While, topical application of 2- and 8-mg/cm^2 PPE significantly lowered erythema at 1.78 ± 0.15 and 1.40 ± 0.24 scores, respectively ($P \leq 0.01$) (Figure 1). The erythema symptom induced by the UVB irradiation was illustrated in Figure 2. Apparently, the erythema reduction by 8-mg/cm^2 PPE and EGCG pretreatment was about 2.5 fold of the vehicle controls. This clearly demonstrated that PPE well prevented the rat skin from UVB-induced erythema.

Epidermal Thickness: UVB irradiation at 3xMED increased the epidermal thickness of the vehicle control rats to 69.71 ± 1.07 μm as compared with the non-treated normal control (18.47 ± 2.25 μm) as shown in Figures 3 and 4 A, C. That was UVB increased the skin thickness of irradiated rats about 3.8 fold of non-treated normal control. Topical pretreatment of PPE at 2 and 8 mg/cm^2 effectively reduced the epidermal thickness of UVB-irradiated skin to 50.34 ± 1.88 and 40.34 ± 1.12 μm, respectively ($P \leq 0.01$) (Figures 3 and 4 D, E). PPE and EGCG at 8 mg/cm^2 topical treatments prior to UVB irradiation equally reduced skin thickness (Figure 4 E, F), which was 1.7 fold of the vehicle. It is noticeable that topical pretreatment of PPE did not reduce the epidermal thickness down to the normal control level and it was about 2.2-2.7 fold thicker.

Sunburn Cells and DNA Fragmentation: UV radiation caused sunburn cells and damaged DNA, which lead to cell death. UVB irradiation significantly induced sunburn cells (SCs) in the epidermis of the vehicle control (+VE) approximately 17.86 ± 3.43 cells/mm^2 (Figure 5). Topical pretreatments of PPE at 2 and 8 mg/cm^2 were able to reduce sunburn cells down to 5.00 ± 1.01 and 2.40 ± 0.24 cells/mm^2, respectively (Figures 4 D, E and 5). The effects of PPE and EGCG at 8 mg/cm^2 were nearly equal and about 7.4 fold less than the vehicle control. In addition, the UVB irradiation induced DNA fragmentation in the non-vehicle and the vehicle controls, presented as long DNA ladder in Figure 6, lane 3 and lane 4. Topical pretreatments of PPE at 8 mg/cm^2, (lane 6) more greatly protected DNA from being fragmented by UVB than 2 mg/cm^2, (lane 5).

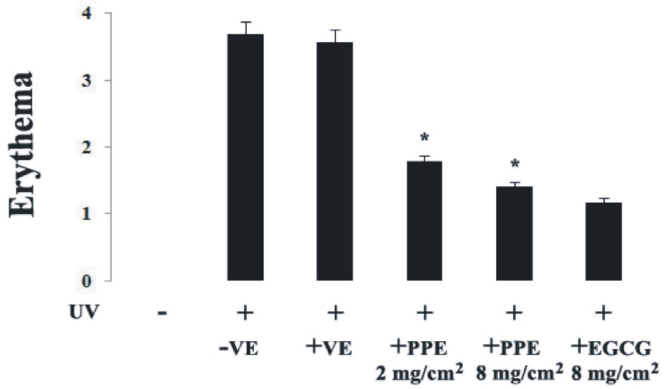

Fig. 1: The inhibitory effect of pomegranate peel ethanolic extract (PPE) on UVB-induced erythema on rat skin. The test skin was pretreated with PPE, epigallocatechin-3-gallate (EGCG) and vehicle controls (+UV, +VE) before irradiation with 3xMED for 24 h, twice a week for a month. Data were shown as mean score of erythema ± SE, n = 6, * $P \leq 0.01$.

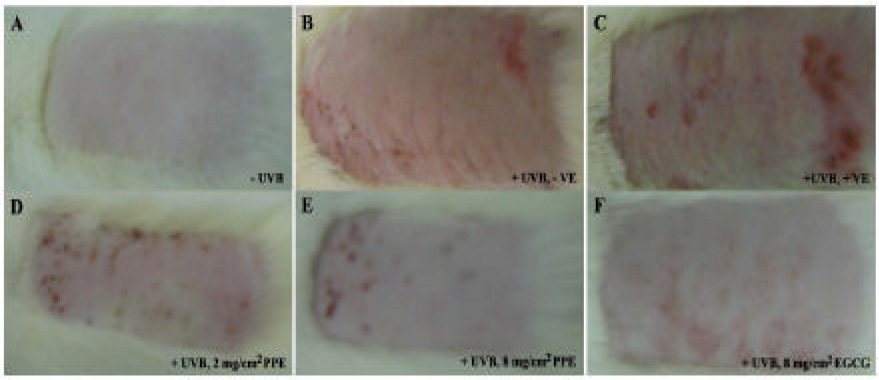

Fig. 2: Skin features demonstrated the erythema induced by UVB irradiation after topical pretreatment of pomegranate peel ethanolic extract (PPE) on hairless area of rat skin. The skin received 3xMED UVB for 24 h., twice a week for a month. A, normal control rat skin; B, UVB and non-vehicle control (-VE); C, UVB and vehicle control (+VE); D, UVB and 2 mg/cm^2 PPE; E, UVB and 8 mg/cm^2 PPE; F, UVB and 8 mg/cm^2 EGCG.

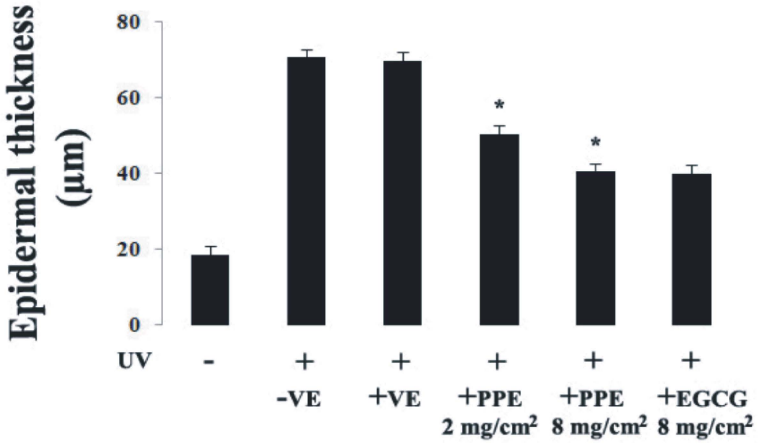

Fig. 3: Inhibitory effect of pomegranate peel ethanolic extract on UVB-induced epidermal thickness of rat skin. The test skin was topically pretreated with PPE, vehicle and non-vehicle control and then irradiated with 3xMED UVB for 24 h, twice a week for a month. Data were expressed as mean ± SE, n = 6, $^{*}P < 0.01$.

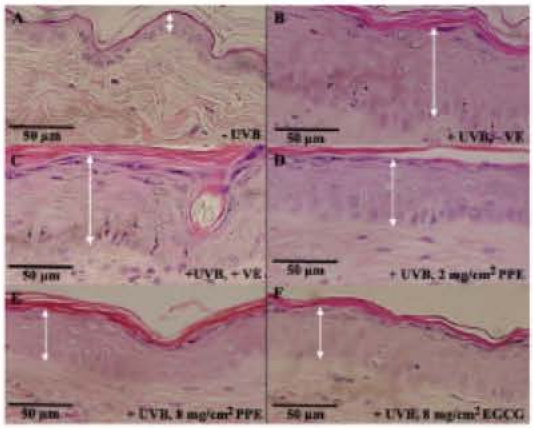

Fig. 4: Histographs of 3xMED UVB-irradiated rat skin illustrated the epidermal thickness (double head arrows) and sunburn cells (arrows) at 400x magnification. A, normal control; B, UVB and non-control (-VE); C, UVB and vehicle control (+VE); D, UVB and 2 mg/cm^2 PPE; E, UVB and 8 mg/cm^2 PPE; F, UVB and 8 mg/cm^2 EGCG.

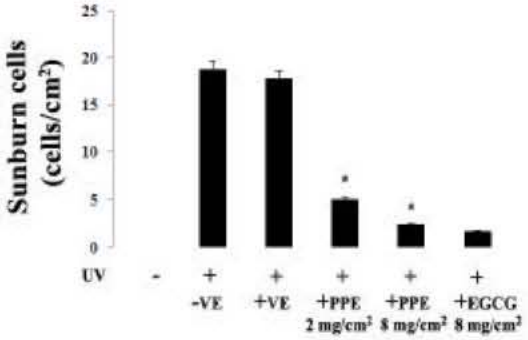

Fig. 5: Effect of PPE on UVB-induced sunburn cells (SCs) in rat skin. UVB at 3xMED irradiated on hairless skin for 24 h, twice a week for a month. Data were presented as mean ± SE, n = 6, $^*P \leq 0.01$.

DISCUSSION

The importance of antioxidant activities of phenolic compounds and their possible usage in medicine and processed foods as a natural antioxidant have reached a new high of human health interest recently. Pomegranate was reported that it contained various phytochemicals, mainly phenolic hydroxyl groups, flavonoids tannin and unsaturated fatty acids with bioactivities [27] and antioxidant activities [14, 28, 29]. Our study demonstrated that PPE contained substantial amount of phenolic compounds and expressed marked antioxidant capacity as equivalent to AA and comparable to natural antioxidants

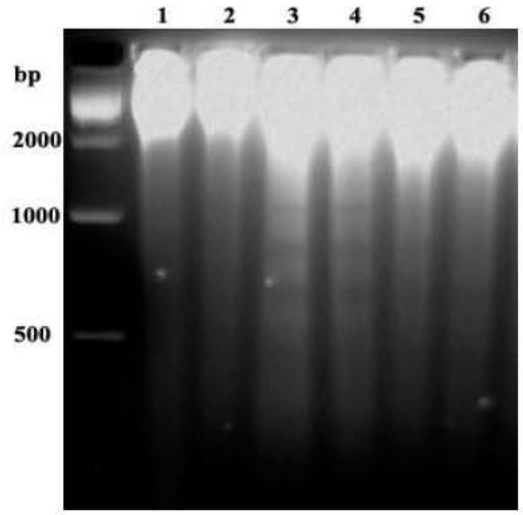

Fig. 6: Effects of 3xMED UVB irradiation on DNA fragmentation of rat epidermal cells: DNA, 4 µg, was electrophoresed on 2% agarose gel. Lane 1, normal control; Lane 2, 8 mg/cm^2 EGCG; Lane 3, non-vehicle control (-VE); Lane 4, vehicle control (+VE); Lane 5, 2 mg/cm^2 PPE; Lane 6, 8 mg/cm^2 PPE.

of CA and EGCG. This was in agreement with pomegranate peel methanolic extract [30]. However, PPE antioxidant capacity was only a half of BHA and BHT, which are synthetic antioxidants used for increasing shelf life or stabilizing lipid containing food products [31]. PPE was effective in reducing formation rate of secondary oxidant products, thiobarbituric acid reactive substances (TBARS) in sunflower oil storage [32]. On the contrary, acetone and methanolic extracts of pomegranate peel by sonication possessed higher antioxidant activity than BHA and BHT [33]. Therefore, it is obvious that extraction systems for pomegranate peel produce a variety of constituents and bioactivities.

This study demonstrated that topical pretreatment of PPE was able to protect rat skin from UVB irradiation. PPE was as effective as EGCG in prevention of epidermal erythema and cell injury and in reduction of epidermal thickness. There was evidence that dietary ellagic acid rich in berries and pomegranate reduced UV-induced epidermal thickness in mouse skin [34]. Similarly, some reports revealed that green tea could reduce erythema, sunburn cells, skin thickness and DNA damages in human skin [35-37]. A number of antioxidants (EGCG, vitamins C and E, CoQ10, lycopene, silybin, resveratrol, genistein and pycnogenol) and plant extracts (green tea, grape seeds,

pomegranate and coffee) were used in skin care formulations [38]. Oral ingestion of ellagic acid extracted from pomegranate rind could reduce erythema and inhibited pigmentation in human skin [39]. Ellagic acid presented in berries and pomegranate was also demonstrated to prevent collagen destruction and inflammatory responses in human fibroblasts and keratinocytes caused by UVB irradiation [34, 40]. Pomegranate was reported to down regulate the UVB-induced proliferating cell nuclear antigen (PCNA) and increase p21 and p53 leading to cell cycle arrests and apoptosis in human artificial skin EpiDerm [41-44]. These definitely supported our study that topical pretreatment of PPE attenuated the skin symptom, reduced sunburn cells and inhibited epidermal cell proliferation and induced epidermal cell apoptosis caused by UVB irradiation.

In conclusion, pomegranate peel was rich in antioxidants, played remarkable roles in prevention skin lesion from UVB irradiation by alleviation of erythema, sunburn cells and DNA damages. These findings provide useful knowledge of pomegranate peel for further researches on its potential in chemoprevention of skin cancer induced by UVB radiation and in agro-industry. This could make use of the pomegranate by-products.

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