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Full Length Research Paper

In vitro protective effect of *Pandanus ordoratissimus* extract on ultraviolet B (UVB)-induced DNA damage

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Pandanus ordoratissimus **is an herb which has been widely used in Thai traditional medicines. A number of studies provided experimental evidence indicating that extracts from roots and leaves of** ***P. ordoratissimus*** **had powerful antioxidant and anti-inflammatory activities. However, the protective effects of extract from** ***P. ordoratissimus*** **flowers on ultraviolet B (UVB)-induced DNA damage have not yet been reported. This study focused on assessing the protective effect of** ***P. odoratissimus*** **extract (POE) against UVB by using human keratinocyte stem cells (KSCs) as an** ***in vitro*** **testing system. The keratinocytes were pre-treated with POE before being assayed for DNA damages caused by UVB (290 to 315 nm). Ascorbyl glucoside and DN-AGE®, reagents commonly used in cosmetic products to protect the skin against UV exposure due to their abilities to scavenge free radicals, were used as positive controls. The alkaline comet assay was used to quantify DNA damage. Photo-dependent cytogenetic lesions were assessed by the micronucleus test (MNT). It was found that POE effectively reduced the extent of DNA breakages and cytogenetic lesions upon exposure to UVB (erythemal ultraviolet (EUV); 17.09 mJ/cm^2). POE significantly decreased tail DNA (TD%), tail length (TL) and micronucleus frequencies (MNFs) which is similar to the protective effects provided by ascorbyl glucoside and DN-AGE®.**

Key words: *Pandanus odoratissimus*, comet assay, micronucleus test.

INTRODUCTION

Pandanus odoratissimus is a kind of herb found in abundance in Thailand. It was shown that methanol extract from *P. odoratissimus* exhibited high antioxidative activity (Jong and Chau, 1998). Analysis of chemical components from the root parts of *P. odoratissimus* led to the isolation of two phenolic compounds, four lignan type compounds, and a new benzofuran derivative (Jong and Chau, 1998). The antioxidant activity of POE has been studied based on its ability to scavenge DPPH, nitric acid, superoxide radicals and hydroxyl radicals (Londonkar and Kamble, 2009).

UVB component of natural sunlight has been regarded as the major cause of human skin cancer. It is well established that UVB induced DNA damage by photoisomerization, resulting in the formation of the 6-4 photoproducts (Clingen et al., 1995) or lesions in DNA among adjacent pyrimidines in the form of dimers (Matsumura and Ananthaswamy, 2004). These dimers are cyclobutane dimmers (CPDs) between adjacent thymine (T) or cytosine (C) residues. If not repaired, UVB-induced DNA lesions can lead to mutations in the DNA sequences. In this study, we focused on the protective effect of POE on UVB-induced DNA damage using an *in vitro* system of human keratinocyte stem cells (KSCs).

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Abbreviations: POE, *Pandanus ordoratissimus* extract; **KSCs,** keratinocyte stem cells; **AAG,** ascorbyl glucoside.

MATERIALS AND METHODS

Cell culture

KSCs were provided from Anatomy Laboratory, Faculty of Science, Mahidol University, Thailand. KSCs were used in all experiments.

KSCs were cultivated in DMEM-F12 supplemented with 10% FBS, 100 U/ml streptomycin, and 0.1 mg/ml penicillin in a humidified incubator supplied with 5% CO_2 and incubated at 37°C. To evaluate DNA damage by comet assay and MNT, 1×10^5 cells/ml were seeded in 6-well plate and 35 mm Petri dishes and cultivated for 24 h.

Treatment of the cells and irradiation procedure

The flower extract of *P. odoratissimus* in methanol was obtained from International Laboratories Corp., Ltd, Bangkok, Thailand. The extract has an IC_{50}, by DPPH method, of 0.015 mg/ml. Two ingredients commonly used in the manufacture of cosmetics, namely, ascorbyl glucoside (AAG) and DN-AGE® at concentrations of 0.200 mg/ml and 1%(v/v), were use as positive controls. Twenty four hours prior to UVB irradiation, 0.015, 0.030 and 0.060 mg/ml of POE, or AAG or DN-AGE®, was added to the medium. Preliminary cytotoxicity studies were carried out to ensure that no toxic effects could be observed at these concentrations. Before exposing to UVB light, the media containing these compounds was discarded and the cell cultures were washed twice with PBS. For UVB irradiation, cells were irradiated in comet slides and 35 mm Petri dishes containing 2 ml of PBS. TL 20W/01 RS SLV fluorescent tubes (Philips) served as UVB source and irradiation was applied at an irradiance of EUV 5.70, 11.40 and 17.09 mJ/cm^2, respectively.

Micronucleus test

The test was carried out as described by Botta et al. (2008) with slight modifications. Twenty-four hours before the experiment, a total of 2×10^5 KSCs were plated on cover slips in 35 mm Petri dishes and kept at 37°C in a humidified atmosphere containing 5% CO_2. For irradiation, culture medium was replaced by PBS and the POE pre-treated cells were irradiated by the UVB. After irradiation, PBS was removed and cells were allowed to divide in the dark at 37°C in a 5% CO_2 atmosphere in culture medium with cytochalasin B (2 µg/ml) and the incubation was extended for an additional 24 h. Cytokinesis-blocked slides were fixed with 95% ethanol and stained with Wright rapid stain set (Biotec), containing eosin solution and polychrome methylene blue solution. Five-hundred binucleated KSCs were examined and micronuclei were identified according to the morphological criteria defined by Kirsch-Volders et al. (2003).

The index of binuclearity (IBIN), considered as a measure of cytotoxicity, was determined by scoring the number of binucleated cells among 1000 stained cells with well-preserved cytoplasm. It was expressed as described by Botta et al. (2008) as the following:

IBIN = [(mononucleated cells) + 2 (binucleated cells) + 3 (cells with at least 2 nuclei)] / 1000

Comet assay

The method was carried out as described by Singh et al. (1988) with slight modifications. Aliquots of 60 µl with 1×10^6 cells/ml were combined with 120 µl of 0.75% (w/v) low melting point (LMP) agarose in PBS and 60 µl of the mixture was pipetted onto a slide precoated with 1.8% (w/v) normal melting point agarose in PBS, followed by a third layer of 0.75% (w/v) LMP agarose. The slides were then exposed to UVB. Subsequently, the solidified gels were immersed for 12 h at 4°C in cold lysing solution consisting of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 1% N-lauryl-sarcosinate at pH 10, 1% Triton-X-100 and 10% DMS. The slides were placed in an electrophoresis buffer consisting of 300 mM NaOH, and 1 mM EDTA, pH 13. An electrophoresis was carried out at 25 V with a resulting current of 300 mA for 30 min. After the electrophoresis was done, the slides were placed horizontally in cold neutralizing buffer of 0.3 M Tris–HCl, pH 7.5 for 5 min (3x), fixed in absolute methanol for 15 min and allowed to air-dry before storing in a sealed container at 4°C. The gels were immersed in ethidium bromide solution (20 µg/ml) for 15 min and examined under a fluorescence microscope (Olympus) at 400x magnifications. Fifty randomly-selected cells per slide were scored from a total of 100 cells per treatment. DNA damage of KSCs was visually analyzed using Lucia (4.51) image analysis software. The parameters assessed included TD% and TL (measured from the end of the head to the end of the tail).

Statistical analysis

All data were analyzed statistically using analysis of variance, while mean comparisons were assessed by Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

POE protection against the increase of UVB-induced micronucleus

UVB radiation used in this experiment was EUV at three levels, namely, 5.70, 11.40 and 17.09 mJ/cm^2. These levels are higher than the maximum of the EUV recorded at monitoring stations in four provinces of Thailand which showed levels ranging from 4.09 to 5.16 kJ/m^2-day (Buntoung and Janjai, 2006). The results show that UVB radiation reduced rates of cell division (IBIN) having percentages lower than the untreated non-irradiated cells. However, there are no significant differences among the pre-treated cells receiving different UVB doses (Figure 1). Dose-response relationship of IBIN (%) versus UVB irradiation of KSCs had an R^2 of 0.8954 (Figure 1). Other reports also showed that UVB inhibited cell division. For example, Petrocelli and Slingerland (2000) showed that human melanoma cell line (WM35) irradiated with UVB at 9 mJ/cm^2 for 12 h decreased the number of cells at S phase from 33 to 5% and to 2% if radiation continued for 24 h and increased to 15% at 36 h of irradiation. The finding suggested that UVB radiation inhibited cell division during G_1 phase.

Our results indicate that the highest irradiation dose of UVB used at 17.09 mJ/cm^2 induced chromosome breakages. This can be seen in the formation of micro-nucleus at the rate of 9.00 ± 1.00 per 500 binucleated cells. It was an increase of 3.86 times when compared with the control. While pre-treatment of KSCs with POE significantly decreased MNFs 3-3.37 times when compared with untreated irradiated cells (Table 1). It was reported that the narrow-band UVB (311 nm) caused chromosome breakages without the loss of the centromere of chromosome (Keulers et al., 1998). Other investigators also found that irradiating skin fibroblasts

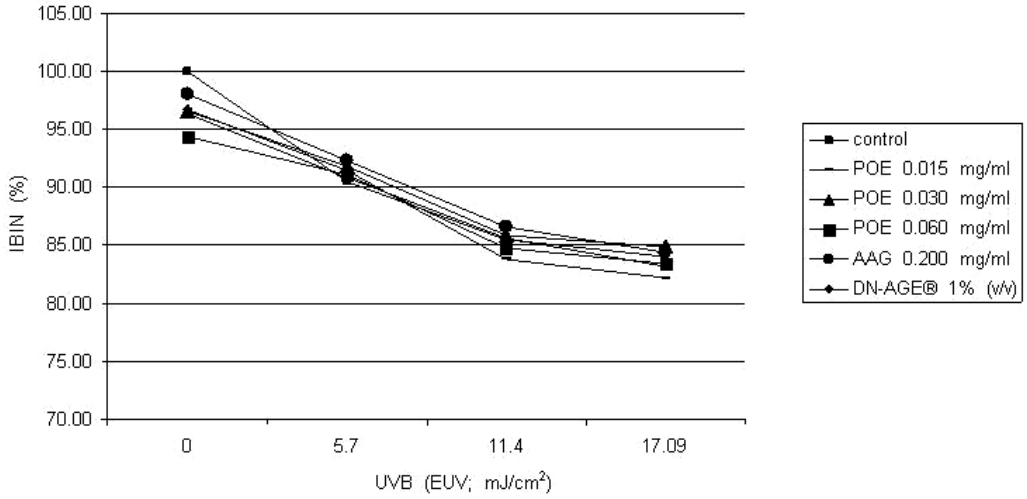

Figure 1. Percentage of IBIN on pre-treated KSCs after receiving irradiation at different UVB-doses (EUV; 0-17.09 mJ/cm^2).

Table 1. Micronuclei frequency in pre-treated KSCs after receiving irradiation at different UVB-doses.

Treatment	UVB (EUV; mJ/cm^2)			
	0	5.70	11.40	17.09
Control	2.33±0.33^{ab}	1.67±0.67^{ab}	3.33±0.67^{b}	9.00±1.00^{c}
POE 0.015 mg/ml	2.00±0.58^{ab}	2.33±0.33^{ab}	1.67±0.67^{ab}	3.00±1.00^{ab}
POE 0.030 mg/ml	1.67±1.20^{ab}	2.00±0.00^{ab}	1.67±0.67^{ab}	3.00±0.58^{ab}
POE 0.060 mg/ml	2.00±0.58^{ab}	1.67±0.33^{ab}	2.00±1.00^{ab}	2.67±1.20^{ab}
AGG 0.200 mg/ml	1.33±0.33^{ab}	1.33±0.33^{ab}	1.00±0.58^{a}	2.67±0.33^{ab}
DN-AGE® 1% v/v	2.00±0.00^{ab}	1.67±0.33^{ab}	1.33±0.33^{ab}	2.67±0.33^{ab}

[a]Means within a column and row not sharing the same letter are significantly different at $P<0.05$ by DMRT.

with UVB at 20 kJ/m^2, before culturing those melanocyte and fibroblasts for 4 or 8 days could induce MNFs at 1.27 ± 0.24 and 1.1 ± 0.24%, respectively (Emri et al., 2000). POE was shown to contain phenolic compounds and also have antioxidative activity (Jong and Chau, 1998) where the POE exhibited IC_{50} of 0.015 mg/ml by the DPPH method. Another study showed that free radical scavenging efficiency of POE was higher than the extract from stem of *Arcangelisia flava*, having an IC_{50} of 0.026 mg/ml (Keawpradub et al., 2005).

The ability of POE to alleviate chromosome breakages was not significantly different from those of AGG and DN-AGE®. The latter two ingredients are well accepted for use in the manufacture of cosmetics. AAG is known to function as an antioxidant, and the effect of the antioxidant is good when used at concentrations less than 1 mM (Cemeli et al., 2009). DN-AGE® is an extract from leaves of the candle tree. It provides a complete protection of cellular DNA and has been widely used in anti-aging sun care products.

POE protection against UVB-induced DNA single strand breaks

The levels of DNA damages were analyzed using TD% and TL parameter. Results show that UVB (EUV; 5.70, 11.40 and 17.09 mJ/cm^2) radiation induced DNA strand break formation in a dose-dependent manner, R^2_{TD} = 0.7657 and R^2_{TL} = 0.9822 (Tables 2 and 3). The results of UVB-induced DNA damage revealed by comet assay were the same as those obtained from micronucleus test at 17.09 mJ/cm^2. This highest level of the UVB dose caused significant DNA damage ($P<0.05$). The pre-treatment of KSCs with POE, AAG and DN-AGE® prevented DNA strand-break formation. All concentrations of POE showed significant reduction in DNA damage with the decreases of 0.63 to 0.69 and 0.51 to 0.64 times as analyzed by TD% and TL when compared with the untreated irradiated cells. According to Lehmann et al. (1998), detection of DNA damage on irradiated HaCaT keratinocytes (0.80 to 22 mJ/cm^2) by

Table 2. Comet parameter: TD% in pre-treated KSCs after receiving irradiation at different UVB-doses.

Treatment	UVB (EUV; mJ/cm^2)			
	0	5.70	11.40	17.09
Control	6.76±1.01^{ab}	6.41±1.85^{ab}	8.31±1.80^{ab}	13.18±1.84^{c}
POE 0.015 mg/ml	5.86±0.92^{ab}	7.61±1.96^{ab}	7.69±1.53^{ab}	8.36±0.94^{ab}
POE 0.030 mg/ml	7.46±0.68^{ab}	4.75±0.59^{a}	8.02±1.87^{b}	9.10±1.07^{ab}
POE 0.060 mg/ml	6.50±0.75^{ab}	8.10±0.65^{ab}	6.79±0.96^{ab}	8.71±1.51^{ab}
AGG 0.200 mg/ml	4.81±1.02^{ab}	6.55±0.60^{ab}	7.45±0.91^{ab}	6.40±1.21^{ab}
DN-AGE® 1% v/v	9.06±0.83^{ab}	6.80±0.56^{ab}	6.22±1.77^{ab}	9.12±0.83^{b}

[a]Means within a column and row not sharing the same letter are significantly different at P<0.05 by DMRT.

Table 3. Comet parameter: TL (µm) in pre-treated KSCs after receiving irradiation at different UVB-doses.

Treatment	UVB (EUV; mJ/cm^2)			
	0	5.70	11.40	17.09
Control	21.58±2.72^{abc}	27.70±10.24^{abcd}	35.62±9.33^{de}	46.70±3.71^{e}
POE 0.015 mg/ml	19.55±2.75^{abc}	22.05±4.83^{abcd}	21.96±3.65^{abcd}	23.77±2.15^{abcd}
POE 0.030 mg/ml	18.23±0.96^{ab}	15.28±1.53^{a}	19.22±4.03^{abc}	25.82±2.80^{abcd}
POE 0.060 mg/ml	17.87±2.33^{ab}	23.20±1.66^{abcd}	24.23±4.09^{abcd}	29.85±4.06^{bcd}
AGG 0.200 mg/ml	13.99±1.51^{a}	19.06±2.08^{abc}	21.78±3.73^{abcd}	18.52±3.50^{ab}
DN-AGE® 1% v/v	26.64±2.20^{abcd}	20.14±1.18^{abc}	22.19±5.74^{abcd}	32.85±2.03^{cd}

[a]Means within a column and row not sharing the same letter are significantly different at *P*<0.05 by DMRT.

comet assay technique showed a dose-dependent increase in the level of DNA damage.

UVB is absorbed maximally by DNA (Tornaletti and Pfeifer, 1996) and resulted in the formation of the CPDs and 6-4 photoproducts (6-4 PPs) (Clingen et al., 1995), thus, could result in the inhibition of the DNA polymerases. Therefore, these DNA lesions if unrepaired may interfere with DNA transcription and replication, thus can lead to misreading of the genetic code and cause mutations and death (Sinha and Hader, 2002). In addition, UVB radiation can affect DNA indirectly by the production of reactive oxygen and nitrogen species (RONS) and induced DNA oxidative damage (8-hydro-xyde oxyguanosine (8-OHdG) (Vayalil et al., 2004).

We can conclude from our study that POE efficiently reduces the extent of DNA breakages and cytogenetic lesions, induced by UVB, in a similar manner to those of AGG and DN-AGE®.

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