Research Article

Protective Effect of *Mangifera indica* Linn., *Cocos nucifera* Linn., and *Averrhoa carambola* Linn. Extracts against Ultraviolet B-Induced Damage in Human Keratinocytes

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This study was aimed at investigating the antioxidant activity of *Mangifera indica* Linn., *Cocos nucifera* Linn., and *Averrhoa carambola* Linn. and their biological effect on human keratinocytes affected by the ultraviolet B (UVB), a major cause of cell damage and skin cancer through induction of DNA damage, production of reactive oxygen species (ROS), and apoptosis. The richest antioxidant activity was found in ethanol fraction of *M. indica* (21.32 ± 0.66 mg QE/g dry weight), while the lowest one was found in aqueous fractions of *M. indica* and *C. nucifera* (1.76 ± 2.10 and 1.65 ± 0.38 mg QE/g dry weight, respectively). Ethanol and aqueous fractions of *A. carambola* (250 μg/mL) significantly reduced the number of apoptotic cells. The expression of cleaved caspase 3 in UVB-treated group was significantly greater than that in untreated group. Both fractions of *A. carambola* (50, 100, and 250 μg/mL) significantly decreased the expression of cleaved caspase 3. Regarding the induction of DNA repair, ethanol (100 and 250 μg/mL) and aqueous (50, 100 and 250 μg/mL) fractions of *A. carambola* significantly decreased the percentage of cyclobutane pyrimidine dimers (CPD). Taken together, our results suggest that both fractions of *A. carambola* may be potentially developed for dermal applications.

1. Introduction

Ultraviolet B (UVB) is a well-known risk factor playing a role in photoaging and skin cancer in epidermis through triggering DNA damage or generating reactive oxygen species (ROS). ROS are chemically reactive molecules containing oxygen and play the important roles in cell signaling and homeostasis. In environmental stress such as UV or heat exposure, ROS levels can dramatically increase causing cell structures and DNA damage and apoptosis [1, 2]. Prevention of UVB-induced damage in skin by lowering ROS production is an evidence-based strategy against photoaging and skin cancer.

Thailand is rich in fruits that are not only diversified but also inexpensive and delicious. Unfortunately, there have been a few researches with evidence-based findings that demonstrate the health benefits of these fruits. For example, resveratrol mostly found in grapes and red wine could exert photoprotective properties on UVB-irradiated cells. To reduce cell death in UVB-damaged skin, resveratrol reduced the production of ROS and attenuated the activation of caspase 3 and caspase 8 that play a major role in apoptosis [3]. Moreover, the extracts of *Elaeocarpus hygrophilus* (makoknum) and *Phyllanthus emblica* (makampom) had high antimicrobial and strong antioxidant activities [4].

Caspase 3 is an effector caspase protein frequently activated in mammalian cell apoptosis [5–7]. It is associated with the initiation of the death cascade. Pathways to caspase 3 activation are either extrinsic or intrinsic apoptotic pathways by interacting with caspase 8 and caspase 9, respectively. Besides apoptotic pathway, caspase 3 is essential for cell survival that converges on many events such as cell shrinkage, blebbing, chromatin condensation, and DNA fragmentation [7–10].
The other pathway through which UVB damages cells is DNA damage. UV induction of DNA damage is a factor that influences the normal life process of all organisms. Minor DNA damage is to allow effective repair, while more severe damage can induce apoptosis and cell cycle arrest. There are two types of UVB-induced DNA damage such as cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP). Two results (CPDs and 6-4PPs) are the transition of C to T and CC to TT. CPD contains a four-membered ring which is from the coupling of the double bonds (C=C) of pyrimidines. CPD is the major source of UV-induced mutations because these dimers interfere with base pairing during DNA replication. CPD is usually at a 5 to 10 folding higher frequency than 6-4PPs. In minor DNA damage, CPD is repaired by exogenous CPD photolyase [1, 2, 11].

Previous study provided the evidence to support the protective effect of Thailand native herb extracts on UVB-induced toxicity in human keratinocyte. It found that the extracts of turmeric and ginger could protect human keratinocyte from UVB-induced DNA damage and apoptosis through the attenuation of caspase 3 activity and CPD formation [12].

This objective of this study was to evaluate the protective effect of three Thai fruit species, Mangifera indica Linn., Cocos nucifera Linn., and Averrhoa carambola Linn., on UVB-induced damage in human keratinocytes. All fruits selected in this investigation were evaluated for their antioxidant activities as potential mechanisms for antiapoptotic activity and induction of DNA repair in human keratinocyte cell line (HaCaT). Development of natural products for dermal applications is our future goal based on the findings of this work.

In traditional medicine, M. indica was used to clear digestion and acidity. It is antiuretic, antidiarrheal, antiemetic, and cardiac herb. Its fruits are known as a potential source of natural antioxidants containing phenolic compounds, ascorbate, and β-carotene [13].

The aqueous extract of C. nucifera was found to contain a free amino acid, L-arginine, which reduced the free radical generation. Moreover, vitamin C significantly reduced lipid peroxidation and increased antioxidant enzymes. C. nucifera could reduce lipid peroxidation content due to the high content of L-arginine. Besides, the high content of polyphenol could maintain the normal levels of lipid in tissue and serum. The aqueous extract of C. nucifera may be a new source of antineoplastic and antimultidrug resistance activities [14].

A. carambola or star fruits contain high polyphenol contents which were contributed significantly in ferric reducing capacity and radical scavenging capacity. Their antioxidant capacities were significantly increased with ripening and associated with flavonol, flavones, and hydrolysable tannins [15].

2. Materials and Methods

2.1. Chemicals and Reagents. All reagents used in this study were of analytical grade. Dimethyl sulfoxide (DMSO) and ethanol were purchased from Merck (Darmstadt, Germany). 1,4-Dithiothreitol (DTT) was purchased from Bio Basic Inc. (Ontario, Canada). Phenylmethyl sulphonyl fluoride (PMSF) was purchased from United States Biochemicals (Cleveland, OH, USA). Kodak processing chemicals for autoradiography films, Amersham ECL Select Western blotting detection reagent, and Hyperfilm ECL were purchased from GE Healthcare (Piscataway, NJ, USA). Dulbecco’s modified Eagle medium (DMEM)/high glucose were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and penicillin-streptomycin solution (10,000 units/mL of penicillin and 10,000 μg/mL of streptomycin) were purchased from HyClone (Logan, UT, USA).

A solution of 30% acrylamide/bis-acrylamide (37.5:1) was purchased from Biorad (Hercules, CA, USA). Ammonium persulfate (APS) was purchased from EMD Millipore (Billerica, MA, USA). The monoclonal rabbit anti-caspase 3 (8G10, cat#9665) and polyclonal rabbit anti-GAPDH (I4C10, cat#2118) were purchased from Cell Signaling Technology (Beverly, MA, USA). FITC Annexin V Apoptosis Detection kit with PI was purchased from Biologend (CA, USA). Oxiselect™ Cellular UV-Induced DNA Damage ELISA Kit (CPD) was purchased from Cell Biolabs (CA, USA).

Folin Ciocalteu’s phenol reagent, gallic acid, Aluminium Chloride (AlCl₃), Sodium Acetate (NaOAc), and Sodium Carbonate (Na₂CO₃) were purchased from Sigma Aldrich (USA).

2.2. Cell Line. HaCaT cells, an immortalized human epidermal keratinocyte cell line, were purchased from cell line service (Heidelberg, Germany). They were cultured in DMEM/high glucose containing 10% FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37°C in a humidified atmosphere at 5% CO₂.

2.3. Plant Materials. Thai fruits were collected from Pathumthani and Nakornpathom provinces. They were authenticated based on their characteristics by Professor Dr. Thaweesakdi Boonkerd (Department of Botany, Faculty of Science, Chulalongkorn University). The voucher specimens deposited at Professor Kasin Suvabhandhu Herbarium (Department of Botany, Faculty of Science, Chulalongkorn University) were A015246 (BCU), A015247 (BCU), and A015251 (BCU) for A. carambola, M. indica, and C. nucifera, respectively.

2.4. Thai Fruit Extraction. The dried fruits were extracted by maceration method using absolute ethanol (ratio 1:2) at 4°C for 48 h and filtered. For extraction using water, the mixture (ratio 1:2) was incubated at 100°C for 30 min and filtered. The residues were extracted twice. The two filtrates were combined and concentrated by evaporation at 45°C. The crude extracts were dissolved in DMSO or kept at −80°C until further investigation.

2.5. Antioxidant Determination by Folin Ciocalteu Phenol Assay and Total Flavonoid of Determination

2.5.1. Folin Ciocalteu Phenol Assay (FCP). Thai fruit extracts (50 μL) and 10% Folin Ciocalteu phenol reagent (50 μL) were mixed and incubated in the dark at room temperature for 30 min. Na₂CO₃ solution (35 μL) was added, mixed, and
incubated in the dark at room temperature for 20 min. The absorbance of reaction was measured with a spectrophotometer at 750 nm. Gallic acid was used as a standard. The amount of phenolic compound is in gallic acid equivalent (GE) mg/g of dry weight.

2.5.2. Detection of Total Flavonoid. Thai fruit extracts (50 μL) were mixed with the solution (150 μL of 100% ethanol, 10 μL of 1 M NaOAc, and 10 μL of AlCl₃). The mixture was incubated in the dark at room temperature for 40 min and the absorbance was measured with a spectrophotometer at 415 nm. Quercetin was used as a standard. The content of flavonoid is in Quercetin equivalent (QE) mg/g of dry weight.

2.6. The Effect of Thai Fruits Extracts on Cell Viability by MTT Assay. Cells were seeded at 10,000 cells/well in 96-well plates and incubated at 37°C for 24 hours. Cells were treated with Thai fruit extracts at different concentrations ranging from 0 to 500 μg/mL for 48 hours. MTT working solution was added at 20 μL/well and incubated at 37°C for 4 hours. In this step, formazan product was formed. The cytotoxicity was detected by removing media carefully and dissolving formazan product with 150 μL of 100% DMSO. Supernatant was collected by centrifuge and transferred to a new 96-well plate and the absorbance was measured at 550 nm. The percentage of cell viability was calculated by using the formula

\[
\% \text{ cell viability} = \left( \frac{\text{Abs}_{\text{treated cells}} - \text{Abs}_{\text{blank}}}{\text{Abs}_{\text{untreated cells}} - \text{Abs}_{\text{blank}}} \right) \times 100. \quad (1)
\]

2.7. The Effect of UVB on Cell Viability by Trypan Blue Exclusion. Cells were seeded in 6-well plates at 200,000 cells/well and cultured for 24 hours. Media were removed and cells were treated with UVB at different doses (0, 200, 400, 800, and 1600 mJ/cm²). After UVB treatment, media were added and cells were cultured for 24 hours. To determine cell viability Trypan blue dye (ratio 1:10) was used.

2.8. The effect of Thai Fruit Extracts on Cell Apoptosis by Flow Cytometry. Cells were seeded at 200,000 cells/well in 6-well plates and incubated at 37°C for 24 hours. Having been incubated, media were removed and cells were treated with UVB 200 mJ/cm². After treatment, cells were treated with Thai fruit extracts at the concentrations of 50, 100, and 250 μg/mL for 24 hours. Having been treated, media were removed and cold PBS was added to wash cells twice. Cells were harvested and centrifuged at 400 g for 5 min. The supernatant was discarded and cells were resuspended in 100 μL of 1x annexin-binding buffer. Cell suspension was added with 2.5 μL of Alexa Fluor 488 annexin V and 5 μL of PI and incubated at room temperature for 15 min. Having been incubated, 400 μL of 1x annexin-binding buffer was added and cells were kept on ice. Cell apoptosis was analyzed by flow cytometry.

2.9. The Effect of Thai Fruit Extracts on Caspase 3 Protein Expression by Western Blotting. Cells were seeded at 200,000 cells/well in 6-well plates and incubated at 37°C for 24 hours. Having been incubated, media were removed and cells were treated with UVB 200 mJ/cm². After treatment, Thai fruit extracts at the concentrations of 50, 100, and 250 μg/mL were treated for 24 hours. In the following day, protein extraction was carried out using 1 mM of DTT and 1 mM of PMSF in NP-40 lysis buffer. Total protein (20 μg) was mixed with Laemmlı buffer (ratio 1:1) and boiled for 5 min. Protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% nonfat milk either for 1 hour at room temperature or overnight at 4°C. Membranes were incubated with caspase 3 and GAPDH primary antibodies for 1 hour at room temperature or overnight at 4°C. After incubation, membranes were washed by 1x TBS-Tween 20 (TBST) for 15 min 3 times, incubated with secondary antibodies (anti-rabbit IgG, HRP-linked antibody) for 45 min at room temperature, and washed by TBST for 15 min 3 times. Protein bands were visualized by adding the enhanced chemiluminescence detection reagent and visualized by using Amersham Hyperfilm ECL and Kodak processing chemicals for autoradiography films. Each band was normalized against GAPDH as an internal control.

2.10. The Effect of Thai Fruit Extracts on UV-Induced DNA Damage. Cells were seeded at 200,000 cells/well in 6-well plates and incubated at 37°C for 24 hours. Having been incubated, media were removed and cells were treated with UVB 200 mJ/cm². After treatment, cells were treated with Thai fruit extracts at the concentrations of 50, 100, and 250 μg/mL for 24 hours.

2.10.1. Fixation and Denaturation. Media were removed and 100 μL of 75% methanol/25% acetic acid was added. Cells were incubated at room temperature for 30 min. Wells were aspirated and 100 μL of 70% ethanol was added and then they were incubated at room temperature for 30 min. Wells were aspirated and 100 μL of Denaturation Solution A was added and then they were incubated at room temperature for 5 min. Cells were gently washed with 200 μL of Dulbecco’s Phosphate-Buffered Saline (DPBS) containing magnesium and calcium. After aspirating wells, 100 μL of Denaturation Solution B was added and then they were incubated at room temperature for 10 min. Wells were aspirated and 200 μL of Assay Diluent was added and they were incubated at room temperature for 30 min.

2.10.2. CPD Detection. 100 μL of diluted anti-CPD antibody was added to wells and they were incubated at room temperature for 1 hour on the orbital shaker. Wells were washed with 1x wash buffer.

2.11. Statistical Analysis. Data were presented as the mean ± standard error (SD). Means were from three or more independent experiments. Data were analyzed by one-way analysis of variance (one-way ANOVA), followed by post hoc Dunnett’s test (P value < 0.05).
3. Results

3.1. Total Phenol and Flavonoid Contents of M. indica, C. nucifera, and A. carambola Extracts. Results of phenol and flavonoid of M. indica, C. nucifera, and A. carambola extracts were shown in Table 1. In all assays, the richest antioxidant activity was found in ethanol fraction of M. indica (21.32 ± 0.66 mg QE/g dry weight by total flavonoid determination). The lowest antioxidant activities were found in aqueous fractions of both M. indica and C. nucifera (1.76 ± 2.10 and 1.65 ± 0.38 mg QE/g dry weight, resp.).

3.2. The Effect of M. indica, C. nucifera, and A. carambola Extracts on Cell Viability. To evaluate the effect of Thai fruit extracts on HaCaT cell viability, MTT assay was employed. Cell was treated with the different concentrations of extracts (0–500 μg/mL). Results of cytotoxicity of all extracts were shown in Figure 1. The aqueous extract of A. carambola at the concentration of 500 μg/mL could significantly decrease cell viability (73.42% ± 3.66, 𝑃< 0.05). Therefore, three concentrations of all extracts used in this study were 50, 100, and 250 μg/mL.

3.3. The Effect of UVB Intensity on Cell Viability. Evaluating the effect of UVB intensity on cell viability was employed by Trypan blue assay. Cell viability values shown as mean ± SD were derived from 3 independent experiments. The cell viability in UVB-treated groups (200–1,600 mJ/cm²) significantly decreased when comparing to that in UVB-untreated group (0 mJ/cm²).

3.4. The Protective Effect of All Fractions of M. indica, C. nucifera, and A. carambola Extracts on UVB-Induced Apoptosis by Flow Cytometry. Results of the protective effect of all fractions on UVB-induced apoptosis were shown in Figures 3(a) and 3(b). Ethanol and aqueous fractions of A. carambola (250 μg/mL) could significantly decrease the number of apoptotic cells in comparison with the number of apoptotic cells in the UVB-treated group (𝑃< 0.05).

3.5. The Effect of M. indica, C. nucifera, and A. carambola Extracts on Caspase 3 Expression by Western Blot. Since caspase 3 plays a major role in caspase-dependent apoptosis, the effect of Thai fruit extracts on the reduction of cleaved caspase 3 expression was investigated in this investigation. Using Western blot analysis, the aqueous extract (Figure 4(a))
Figure 3: Continued.
Figure 3: The effect of *M. indica*, *C. nucifera*, and *A. carambola* extracts on apoptosis of UVB-treated HaCaT cells. HaCaT cells were treated with UVB intensity at 200 mJ/cm$^2$ and Thai fruit extracts at the concentration of 50, 100, and 250 μg/mL for 24 h. Apoptotic cell images were shown as histogram (a) and apoptotic values were shown as mean ± SD derived from 3 independent experiments (b). * The extracts of both fractions of *A. carambola* (250 μg/mL) could significantly decrease the number of apoptotic cells in comparison with the number of apoptotic cells in the UVB-treated group.
(a) The effect of aqueous extract of A. carambola on cleaved caspase 3 expression

(b) The effect of ethanol extract of A. carambola on cleaved caspase 3 expression

Figure 4: The effect of both aqueous (a) and ethanol (b) extracts of A. carambola on cleaved caspase 3 expression. Cleaved caspase 3 expression was increased when cells were treated with UVB (200 mJ/cm²). After UVB stimulation, cells were treated with extracts for 24 h. Both extracts of A. carambola could significantly decrease cleaved caspase 3 expression.

and the ethanol extract (Figure 4(b)) of A. carambola could decrease the cleavage of caspase 3 expression after 24 hours of extract treatment. Vitamin C was used as a standard.

3.6. The Effect of M. indica, C. nucifera, and A. carambola Extracts on the Induction of DNA Repair by Cyclobutane Pyrimidine Dimers (CPD) Detection. CPD is the product of UVB-induced DNA lesions. In this study, UVB (200 mJ/cm²) treatment could significantly increase CPD expression. After the treatment of Thai fruit extracts, the result showed that ethanol (100 and 250 μg/mL) and aqueous (50, 100, and 250 μg/mL) fractions of A. carambola could significantly decrease the percentage of CPD (P < 0.05). The results were shown in Figure 5.

4. Discussion

UVB is a major cause of cell damage and skin cancer through inducing DNA damage and apoptosis. There are two pathways that decrease UVB-induced cell damage such as antiapoptosis and DNA damage repair. The effect of these extracts on apoptosis was detected by flow cytometry and Western blot analysis.

According to the flow cytometric data, the percentage of apoptotic cells in the untreated group was significantly different from that in the UVB-treated group, suggesting that UVB at 200 mJ/cm² could lead to the increase in apoptotic cells. In addition, both ethanol and aqueous extracts of A. carambola at the concentration of 250 μg/mL could significantly decrease the percentage of the number of apoptotic cells (P < 0.05).

To confirm the effect of both extracts on protecting UV-induced apoptosis, the expression of caspase 3 was detected by Western blot. Many studies indicated that caspase 3 (35 kDa) in UVB-treated cells was cleaved. Cleaved caspase 3 (17 and 19 kDa) is an important factor which plays a role in the induction of cell apoptosis through apoptotic pathway [7, 16–21].

It was recently reported that vitamin C exerted antiapoptotic activity by attenuating caspase 3 expression [22, 23]. Therefore, vitamin C was used as a control in this study.

Results of the expression of cleaved caspase 3 decreasing in both ethanol and aqueous fractions of A. carambola-treated and vitamin C-treated cells implied that the attenuation of cleaved caspase 3 was involved in cell survival after UVB irradiation [20, 24, 25].

The results showed that the level of CPD expression was increased when treated with UVB. After UVB radiation, CPD level in A. carambola-treated group was significantly decreased (P < 0.05).
The extract of *A. carambola* has been used in the traditional medicine for treating many diseases such as diabetes and diabetic nephropathy. Many studies indicated that it could inhibit apoptotic pathway by attenuating the activation of caspase 3, caspase 8, and caspase 9 [26, 27]. Level of active caspase 3 can affect the formation of DNA fragmentation, since caspase 3 is a primary activator which induces the cleavage of DNA fragmentation factor (DFF) complex. Cleaved DFF causes DNA damage and cell death [9, 28]. To date, there are not many studies to investigate the effect of *A. carambola* extract on DNA damage and cytotoxicity.

Collectively, our results showed that both ethanol and aqueous fractions of *A. carambola* could attenuate UVB-induced damage in human keratinocytes by inhibiting the cleavage of caspase 3 and CPD formation in the HaCaT keratinocyte cell line. The present study is the first to provide the evidence of potent protective effect of *A. carambola* extract against ultraviolet B-induced damage in human keratinocytes. The extracts of *A. carambola* may be developed as the agent for the protection of UVB-induced damage in skin.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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