

Unripe Fruit Extracts of *Mangifera indica* L. Protect against AGEs Formation, Melanogenesis and UVA-Induced Cell Damage

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Abstract

In this study, we explored the effects of unripe fruit extracts of *Mangifera indica* L. on the anti-aging activity in skin cells. *Mangifera indica* L. is a popular economical and medicinal plant with numerous health-beneficial properties. The aqueous extracts of unripe fruit of *Mangifera indica* L. were obtained and subjected to HPLC and NMR analyses for the identification of bioactive compounds. The anti-glycation effect of Mango unripe fruit extracts was monitored by *in vitro* model system of AGEs (Advanced glycation end products) formation. Mango unripe fruit extracts significantly inhibited the AGEs formation in a dose-dependent manner. Meanwhile, Mango unripe fruit extracts possessed a comparable efficiency to commercialized Kojic acids in the inhibition of melanogenesis in B16-F10 melanoma cells. The UVA-induced cell damages can be prevented and repaired by Mango unripe fruit extracts in skin fibroblast CCD-966SK. Compared to the untreated control, Mango unripe fruit extracts significantly increased the cell viability while being applied before (36%) or after (43%) UVA irradiation. These results verified the potential application of Mango unripe fruit extracts in the skin protection and recovery from UVA irradiation, as well as the suppression of AGEs formation and melanogenesis.

Keywords

Mangifera indica L., Anti-Glycation, Melanogenesis, UVA-Induced Cell Damage

1. Introduction

Mangifera indica L. (Mango) is a fruit crop with high economic values, culti-

vated throughout the tropical and subtropical areas. Mango is notable for its excellent quality of taste and nutrition, and particularly for a wide range of health protective properties. Mango fruit is rich in antioxidants, such as polyphenols, carotenoids and ascorbic acid, which have highlighted its applications in pharmaceutical, nutraceutical and cosmetic industries [1]. Polyphenols may scavenge the free radicals include reactive oxygen and nitrogen species produced as by-product during metabolic processes in the body [2] [3]. Polyphenols with great antioxidant potential can provide significant protections against development of several chronic diseases such as cancer, atherosclerosis, diabetes, infections, aging, asthma and so on [4] [5].

The major polyphenolic compounds found in Mango fruit include quercetin, kaempferol, tannin, Chlorogenic acid, gallic acid, vanillic acid and mangiferin. Among these polyphenolics, mangiferin is a major bioactive constituent of *M. indica*. Mangiferin is a xanthone glycoside possessing a wide range of pharmacological properties such as antioxidant, antiaging, anticancer, antimicrobial, antidiabetic and immunomodulatory [6]. Although Mangiferin has such multiple benefits as a therapeutic agent, the relevant studies on its potential role as cosmetics are limited. Mangiferin has been reported to inhibit the oxidative stress-induced activity of Matrix Metalloproteinase-1 (MMP-1) in human keratinocytes (HaCat), which was considered a potential mechanism of anti-aging of human skin by preventing the collagen degradation by MMPs [7].

Skin aging is mainly due to intrinsic and extrinsic aging. Intrinsic aging of the skin is an inevitable and natural consequence. The two greatest exogenous factors of skin aging are smoking and exposure to UV light [8] [9]. The appearance of photoaged skin is featured by collagen degradation, wrinkling, irregular pigmentation, dryness, elastosis, and so on [10]. It has been reported that oral administration of mango extract showed anti-photoaging activity in UVB-irradiated hairless mice, by improving the wrinkle formation, epidermal thickness, and increasing collagen bundles [11].

UV irradiation results in photo-aged skin and accumulation of Advanced glycation end products (AGEs). AGEs are generated via the nonenzymatic glycation or oxidation of proteins, lipids, and nucleic acids. UV-induced intracellular accumulation of AGEs generates reactive oxygen species (ROS) which triggers inflammation and causes dermal protein damages [12]. AGEs have been proven to promote melanogenesis by the coupling with receptor for AGEs (RAGE) and the regulation of Cyclic AMP response element-binding protein (CREB) and extracellular signal-regulated kinases (ERK) 1/2 [13]. Therefore, the AGEs formation is correlated to the melanin synthesis in melanocytes. Since both AGEs and UV irradiation are involved in the skin pigmentation, there might be a functional correlation between these two factors.

In order to unravel the role of fruit extracts of *M. indica* in the anti-aging of skin, we chose the unripe fruit as materials to avoid excess solubilization of pectic polymers during fruit ripening. We identified the bioactive components from whole fruit extracts by HPLC and NMR analyses. Our investigation focused on

the elucidation of antiglycation, anti-melanogenesis and anti-UV irradiation potency of Mango unripe fruit extract. With these preliminary data, we provided useful information and further insights on the application of Mango fruit extracts in skin care.

2. Materials and Methods

2.1. Cell Lines and Chemicals

CCD-966SK (CRL-1881) and B16-F10 (CRL-6475) cell lines were derived from the American Type Culture Collection (Manassas, VA, USA). NMR: 1D and 2D NMR experiments were performed on Varian 400 MHz 400 FT-NMR spectrometer (Varian, Inc. Palo Alto, CA); Chemical shifts are reported in δ (ppm), and TMS was used as an internal standard. All chemicals used in HPLC and NMR were of analytical grade commercial preparations purchased from Merck (Merck Ltd. Taipei, Taiwan). All cell culture media and reagents including MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma-Aldrich (MO, USA) or Gibco (Thermo Fisher Scientific, Inc., CA, USA) and were of reagent grade or cell-culture grade.

2.2. Preparation of Extracts

The unripe fruit of *Mangifera indica* L. of size ranging from 3 to 7 cm in length was purchased from the local farmers in Pingtung, Taiwan. The whole fruit was homogenized and extracted in distilled water containing 0.5% citric acid at 85°C. The fruit extract composed of fruit homogenates and distilled water in a ratio of 1:3 (v/v). The supernatant of fruit extract was collected by centrifugation and filtered to obtain the filtrate. The total volume of filtrates was evaporated by vacuumed centrifugation at 50°C to the scale of Brix14 (One-degree Brix is 1 g of the dissolved solid content in 100 g of solution, for approximate calculation). The concentrates were directly proceeded to antiglycation assay, or proceeded to freeze drying to obtain extract powders that can be quantified and applied in content analysis and cell culture assays.

2.3. HPLC Conditions

The HPLC system consisted of a Hitachi L-2310 series pump (Hitachi Ltd., Tokyo, Japan) equipped with a Hitachi L-2420 UV-VIS detector. Absorbance was recorded at 254 nm. LC analyses were performed on a Mightysil RP-18 GP 250 C18 reversed-phase column (10 mm \times 250 mm, 5 μ m, Kanto Chemical Co, Inc., Tokyo, Japan) and the column temperature was set at 25°C. The mobile phase consisted of MilliQ water containing 0.1% formic acid (solvent A) and methanol containing 0.1% formic acid (solvent B). The flowing conditions of mobile phase were 1.0 ml/min flow rate and 20 μ l injection volume. The column was equilibrated (A:B; v/v) in 85:15 (5 min), and elution was carried out with the following steps; 85:15 (5 min), a linear gradient increasing from 15% B to 100% (20 min), and 100% B (5 min). Fractions of major peaks were collected for pre-

liminary bioactivity evaluations. Compounds with bioactivities were further identified by NMR spectroscopic analysis under the conditions as described in Section 2.1, and by comparing with the reported data in the literature to resolve the planar structures.

2.4. *In Vitro* Antiglycation Assay

The antiglycation activity was evaluated following the method proposed by Yamaguchi *et al.* [14] with modification. Prepare the sterile 60 mg/ml collagen (containing 0.06% sodium azide) and 1.5 M D-fructose in 200 mM potassium phosphate buffer (pH 7.4). Mix 200 μ l of collagen and 200 μ l of fructose with 200 μ l of Mango unripe fruit extract of different concentrations, or with 200 μ l of blank solvent as a control. The reaction mixture was incubated at 50°C for 24 h. Take 100 μ l of reaction mixtures before (0 h) and after reaction (24 h) for the measurement of glycated materials. The fluorescence from the glycated materials was measured at 360 nm excitation and 460 nm emission using a Hitachi F-2500 spectrofluorometer. The relative AGEs formation rate was calculated as: $[(\text{Abs of sample}_{24\text{h}} - \text{Abs of sample}_{0\text{h}})/(\text{Abs of control}_{24\text{h}} - \text{Abs of control}_{0\text{h}})] \times 100\%$.

2.5. Melanin Content Analysis

B16-F10 mouse melanoma cells were cultured in Dulbecco's modified Eagle's medium containing 1% Penicillin-streptomycin and 10% Fetal bovine serum. The B16-F10 cells were seeded at a density of 1.5×10^5 cells per well in 6-well culture plates, and incubated at 37°C under a 5% CO₂ atmosphere for 24 h. Renew the medium with fresh 3 ml of DMEM medium containing 0.25 mg/ml Kojic acid, or 0.25 mg/ml Mango unripe fruit extract, or blank solvent as control. Cells were harvested after incubation at 37°C for 48 h. Rinse the collected cells with $1 \times$ PBS twice and then resuspend in 200 μ l of $1 \times$ PBS. The cell lysates were obtained by freeze-thaw in liquid nitrogen for 10 min and room temperature for 30 minutes. Collect the pellets of cell lysates by centrifugation at $12,000 \times g$ (Thermo Scientific™ Heraeus™ Fresco™ 17 Microcentrifuge, Langensfeld, Germany) for 30 minutes. Suspend the pellets with 120 μ l of 1 N NaOH in 60°C dry bath for 1 hour. Take 100 μ l of samples to 96-well plate for spectrophotometric measurement at 405 nm. The amounts of melanin were calculated as: Relative melanin formation (%) = $(\text{OD}_{\text{sample}}/\text{OD}_{\text{control}}) \times 100\%$.

2.6. Cell Viability Assay

Human skin fibroblast CCD-966SK (5×10^3 /well) were seeded in 96-well plates and incubated at 37°C for 24 hours. For UVA protection, the medium was replaced with fresh medium alone or containing 0.5 mg/ml Mango unripe fruit extract, and then incubate cells at 37°C for 24 hours. Irradiate the sample plate with UVA by using ultraviolet radiation chamber under 12 J/cm² for 1 hour. This condition would cause LD50 (Lethal Dose, 50%), which presents the dose of ionizing radiation leading half percentages of cell death. Set up sample plates

without UVA irradiation as control. For UVA resistance, cells were irradiated before treatment of 0.5 mg/ml Mango unripe fruit extract. Cell viability was assessed through MTT assay. Briefly, 15 μ l of MTT (4 mg/ml) was added and the cells were incubated for an additional 4 h. Remove medium and add 50 μ l/well of DMSO to resolve formazan crystal. Place the plate on shaker and incubate for 10 min and measure the absorbance at 570 nm. Cells viability in response to treatment was calculated as: Cell viability (%) = $(OD_{\text{sample}}/OD_{\text{control}}) \times 100\%$.

2.7. Statistical Analysis

All values are expressed as mean \pm SD. The statistical significance of the differences between two sample populations was determined by unpaired two-tailed Student's t-test. Statistical significance was considered at P value < 0.05.

3. Results and Discussion

3.1. Gallic Acids, Mangiferin and PGG Were Identified in Unripe Fruit Extract of *Mangifera indica* L.

We identified three polyphenols from the aqueous extracts of Mango unripe fruit by HPLC and NMR analyses: Gallic acids, Mangiferin and penta-O-galloyl- β -d-glucose (PGG) (Figure 1). Under HPLC conditions, the retention times of Gallic acids, Mangiferin and PGG were 3.42, 6.49, and 16.57 min, respectively. These three compounds are major polyphenolic compounds found in Mango fruit with health-promoting effects. Mangiferin is a promising antioxidant.

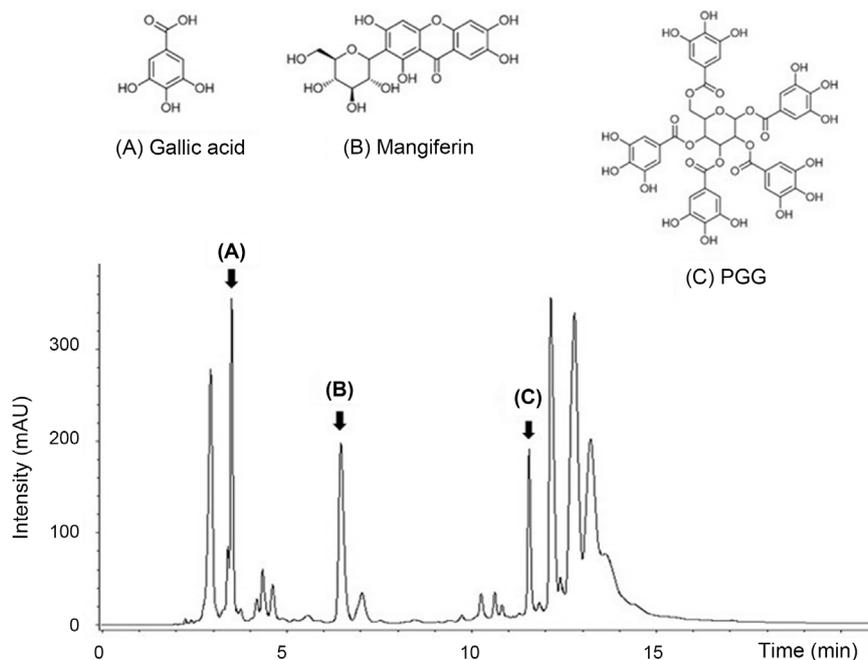


Figure 1. HPLC fingerprints and the common peaks of the aqueous extracts of *Mangifera indica* L. Gallic acids (A), Mangiferin (B) and penta-O-galloyl- β -d-glucose (PGG, C) were identified in unripe fruit extracts. The chemical structures of Gallic acids, Mangiferin and PGG were shown above.

However, whole Mango extracts are more potent than pure isolated mangiferin, which may be owing to the synergism between mangiferin and other Mango polyphenols [15]. In this study, we chose to obtain the Mango extract from unripe fruit to avoid excess solubilization of pectic polymers during Mango fruit ripening and also to acquire a higher potency of polyphenols.

3.2. Unripe Fruit Extracts of Mango Inhibit AGEs Formation in a Dose-Dependent Manner

High levels of AGEs contribute to impaired dermal regeneration, reduced collagen solubility and wrinkles of aging skin [13]. To test the potential of Mango unripe fruit extracts (MUE) in suppression of glycation, we applied 100%, 50%, 10% and 1% of extracts to *in vitro* model system of AGEs. Compared to the control, the AGEs formation rate was 3.67% for 100% MUE, 9.67% for 50% MUE, 39.67% for 10% MUE, and 59.33% for 1% MUE (Figure 2). Mango unripe fruit extracts exhibited a dose-dependent antiglycation effect using *in vitro* model system. The present reports on antiglycation activity of mango extracts focus on the relationship with diabetes [16] [17], rather than with skin aging. Thus, it is worth studying on the antiglycation activity in skin aging to provide a potential application of Mango extracts in skin care.

3.3. Unripe Fruit Extracts of Mango Inhibit Melanin Formation in Melanocytes

Although Mango fruit contains high amounts of polyphenols, whether the whole fruit extracts exhibit the potential of anti-melanogenic activity remain uncertain [18] [19]. We compared the melanin formation rate in B16-F10 melanocytes under applying 0.25 mg/ml of Mango unripe fruit extracts (MUE) and a commercial Kojic acid, a depigmenting agent. In MUE-treated cells, the melanin

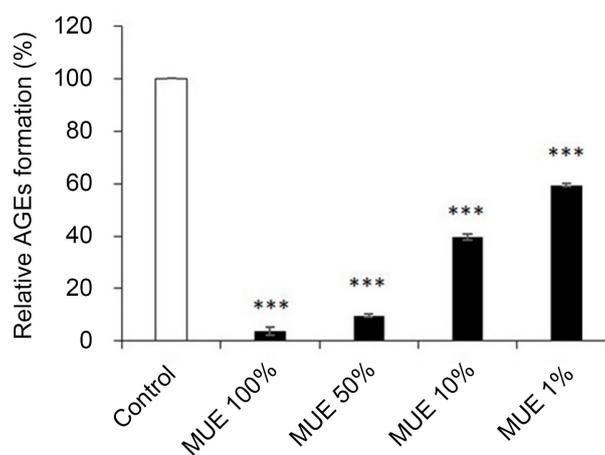


Figure 2. Unripe fruit extracts of Mango inhibit AGEs formation in a dose-dependent manner. The glycation was determined by *in vitro* model system of AGEs. The collagen and fructose mixtures were treated with or without Mango unripe fruit extracts at different concentrations (MUE, 100%, 50%, 10%, 1%) for 24 h. The relative AGEs formation after MUE treatment was calculated. *** $p < 0.001$ versus control.

formation rate was 82% relative to the untreated control, which was comparable to that of Kojic acid (78%) (**Figure 3**). Therefore, Mango unripe fruit extracts exhibited the anti-melanogenic activity.

3.4. Unripe Fruit Extracts of Mango Possess the Ability to Protect and Recover the Fibroblast from the Damage of UVA Exposure

Mango is a potential source of polyphenolic compounds that are responsible for high antioxidant activities that protect the body against oxidative damages [1]. We examined the protective role of Mango unripe fruit extracts (MUE) against UVA irradiation on cells. The CCD-966SK fibroblast cells were treated with 0.5 mg/ml of MUE before UVA irradiation and subjected to cell viability calculation. Relative to irradiated cells without MUE pretreatment, the cell viability increased 23% by MUE pretreatment (**Figure 4(A)**). We also examined the potential of MUE on cell recovery ability against UVA irradiation. On the treatment of 0.5 mg/ml of MUE after UVA irradiation, the cell viability increased 36% in CCD-966SK cells, compared with that of untreated irradiated cells (**Figure 4(B)**). Based on these results, we suggested that MUE possessed the ability to promote fibroblast regeneration and can be applied in advance of or after UVA irradiation.

4. Conclusion

In conclusion, we evaluated the antiaging activity of Mango unripe fruit extracts on skin cells. We demonstrated that Mango unripe fruit extracts protected dermal fibroblast from UVA irradiation and repair the damages after UVA exposure,

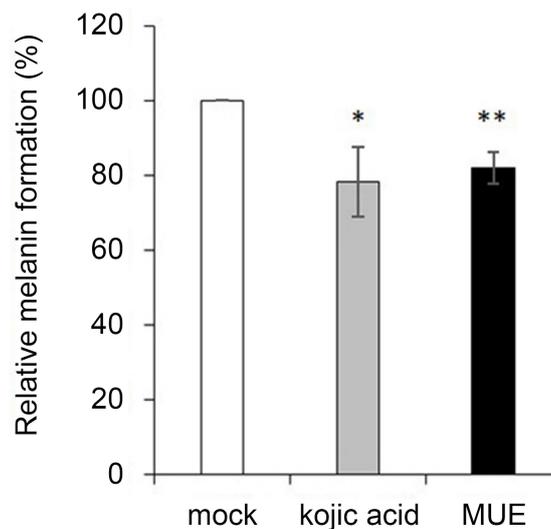


Figure 3. Unripe fruit extracts of Mango inhibit melanin formation in melanocytes B16-F10, with a comparable effect to Kojic acid. B16-F10 cells were treated with 0.25 mg/ml of Kojic acid or MUE. Cells without either treatment were set as mock. Melanin content in cells was reduced by both Kojic acid and MUE. There was no difference in melanin formation rate between Kojic acid and MUE treatments. * $p < 0.05$, ** $p < 0.01$ versus mock.

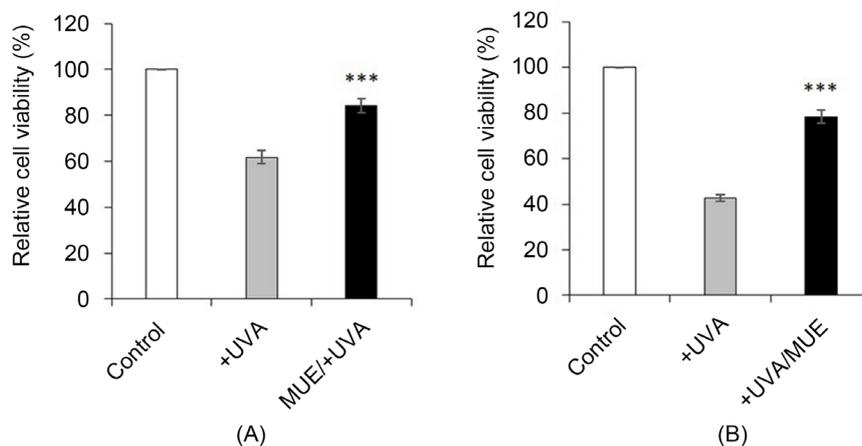


Figure 4. Unripe fruit extracts of Mango possess the ability to protect (A) and recover (B) the fibroblast cells CCD-966SK from the damages of UVA exposure. CCD-966SK were treated with or without 0.5 mg/ml of MUE followed by UVA irradiation to determine the protective activity of MUE (A). Cells were treated with UVA irradiation followed by MUE treatment or not to determine the recovery activity of MUE (B). Both activities were evaluated by the cell viability determined by MTT assay. The cells treated with neither UVA nor MUE were set as blank control. *** $p < 0.001$ versus +UVA only.

which might exert on the basis of antiglycation and anti-melanogenic activities of the extracts. Our preliminary studies demonstrated a novel potentiality of Mango unripe fruit extracts for promoting skin-whitening, fibroblast regeneration and anti-aging of skin.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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