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Inhibition of UV-induced ROS and collagen damage by *Phyllanthus emblica* extract in normal human dermal fibroblasts

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Synopsis

As a part of ongoing research for novel natural cosmeceutical actives from plant extracts, this study demonstrates that *Phyllanthus emblica* fruit extract has shown its efficacy in protection against ultraviolet-B (UVB) irradiation-induced reactive oxygen species (ROS) and collagen damage in normal human dermal fibroblasts. At a concentration of 0.5 mg/ml, emblica extract showed a significant response of 9.5 ± 0.28-fold protection from UVB induced-collagen damage as compared to untreated cells. A known active, ascorbic acid, at a concentration of 0.5 mg/ml, showed 3.7 ± 0.07-fold protection from UVB-induced collagen damage. While the untreated cells showed 84 ± 1.4% induction in ROS on UVB irradiation as compared to the non-irradiated cells, emblica extract treatment inhibited the induction of ROS to 15 ± 4% at a concentration of 0.5 mg/ml. Ascorbic acid inhibited the induction in ROS to 64 ± 2% at a concentration of 0.5 mg/ml. Emblica extract is a significantly better natural active, with promising cosmeceutical benefits against photoaging.

INTRODUCTION

In the 21st century natural cosmetics are emerging as new skin care concepts with a perfect synergy between nature and technology. *Phyllanthus emblica,* commonly known in India as Amla (Sanskrit name Amalaki) is used in Ayurveda for its cosmetic applications due to its antioxidant properties. For several decades, the emblica fruit had been claimed to be a rich source of ascorbic acid, and its high antioxidant potential has been attributed to the presence of ascorbic acid (1). However, recent studies have confirmed that only trace amounts of ascorbic acid are found in emblica extract and that the earlier reported antioxidant hydrolyzable tannins, emblicanins A and B, correspond to 1-O-galloy1-β-D-glucose (β-glucogallin) and mucic acid 1,4-lactone 5-O-gallate, respectively (2). The trace amount of free ascorbic acid in emblica extract suggests that the antioxidant effects exhibited by emblica fruits are due to gallic acid esters (2).

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Ultraviolet-B (UVB) irradiation is a major environmental factor responsible for a high incidence of premature skin aging, referred to as photoaging, as well as skin cancer and melanoma. The majority of UV energy is absorbed by unidentified photosensitizers in the cells that are postulated to generate reactive oxygen species (ROS). UV irradiation increases ROS production via PKCdelta signaling in primary murine fibroblasts (3). UVB irradiation has been demonstrated to produce ROS in the cells and skin, which induces the synthesis of matrix metalloproteinases (MMPs), causing skin photoaging (4). Photoaging is a process involving alteration of type I collagen, the major component of dermis on exposure to UV radiation. Long-term exposure to UV radiation induces various cutaneous changes that differ because of physiological aging, including structural destruction of dermal collagen fiber bundles, which comprise the major component of the dermis (5). Wrinkling, a representative change in skin surface associated with photoaging, is often seen at the corners of the eyes and in the space between the eyebrows (5). Type I collagen degradation is the major cause of wrinkle formation. Several factors promoting this degradation process have been identified, including UV radiation and ROS (6). Intracellular and extracellular oxidative stress initiated by ROS advance skin aging, which is characterized by wrinkles and atypical pigmentation. Because UV enhances ROS generation in cells, skin aging is usually discussed in relation to UV exposure. The use of antioxidants is an effective approach to prevent symptoms related to photo-induced aging of the skin (7). Strategies to prevent or at least minimize ROS-induced photoaging and intrinsic aging of the skin necessarily include protection against UV irradiation and antioxidant homeostasis (8).

The level of procollagen type I protein in photoaged skin is lower than that in naturally aged skin. The level of matrix metalloproteinase-1 protein and the activity of matrix metalloproteinase-2 were higher in the dermis of photoaged skin than in naturally aged skin (9). Emblica extract has type I pro-collagen promoting and anti-collagenase activity that is attributed to its anti-aging potential (10). Emblica fruit had been claimed to be a rich source of ascorbic acid, and its high antioxidant potential was earlier attributed to the presence of ascorbic acid. Also, it has become obvious that the high ascorbic acid content could contribute to the anti-aging and anti-photoaging properties of emblica extract. However, recent studies have shown only trace amounts of ascorbic acid in emblica fruits (2). Therefore, we studied the comparative UVB protection efficacy of ascorbic acid and emblica extract. To investigate the effect of emblica extract on UVB-induced collagen damage, we initially studied the cell viability response to emblica treatment in normal human dermal fibroblast cells. In continuation with earlier studies on emblica extract, the objective of the present study was to investigate the protective effect of emblica extract in comparison to ascorbic acid against UVB-induced ROS and the collagen damage that results in photoaging.

MATERIALS AND METHODS

MATERIALS

Normal human dermal fibroblasts (from adult donors) and its ready-to-use growth medium were obtained from PromoCell GmbH, Heidelberg, Germany. A neutral red stain (NR) was procured from Himedia Laboratories, Mumbai, India. A human collagen type I ELISA kit was purchased from Cosmo Bio (Carlsbad, USA). Procollagen type-I goat

polyclonal antibody, collagen type-I goat polyclonal antibody, and FITC-coupled donkey anti-goat IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A Bio-Sun {R} Vilber Lourmat stimulator fitted with a UVB irradiation source composed of T20.M 312-nm Vilber Lourmat tubes, low-pressure mercury vapor tubes, hot cathodes, and a Vilber Lourmat RMX-365/312 radiometer with a microprocessor programmable in energy with time was procured from Marne la Vallee, France and was used as the source of the UVB irradiation.

EXTRACTION AND SAMPLE PREPARATION

The fresh fruits of *P. emblica* were procured locally from Bangalore, India, and freed from dust or other organic matter. The fruits were cut into small pieces and macerated with cold water at room temperature for 2 h with continuous stirring. The filtered juice was freeze-dried to obtain the dry powder form. Various samples of emblica extract were prepared by dissolving the dry emblica extract powder in deionized water at the indicated concentrations.

CELL VIABILITY ASSAY

Cell viability was determined by a neutral red uptake (NRU) colorimetric assay. Cells in 96-well plates were incubated with 30 μg/ml of neutral red prepared in a pre-warmed culture medium for 3 h at 37°C. The excess dye was then washed off with phosphate buffer saline. The dye was extracted in a 100 μl/well of developer solution consisting of 25 ml of water, 24.5 ml of ethanol, and 0.5 ml of glacial acetic acid at room temperature for 20 min. The optical density was measured at 492 nm using a microplate reader. The relative percentage of cell survival was calculated by dividing the absorbance of the treated cells by that of the control in each experiment.

IMMUNOCYTOCHEMISTRY

Twenty-four hour cultures of normal human dermal fibroblasts in a six-well plate, with or without exposure to UVB irradiation of 50 mJ/cm^2, were fixed and incubated with pro-collagen type-I goat polyclonal antibody or collagen type-I goat polyclonal antibody for 1 h at room temperature. After being washed with TBS, the cells were incubated with secondary antibody (FITC-coupled donkey anti-goat IgG) for 1 h with gentle rocking at room temperature. The cells were then washed, trypsinized, and resuspended in PBS. Two hundred microliters of each sample was transferred to a 96-well black microplate and the fluorescence intensity was measured by a fluorescence microplate reader set for excitation at 485 nm and emission detection at 535 nm (10,11).

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

After specific treatment of the cells with the sample and UVB irradiation of 50 mJ/cm^2, the culture medium was removed and the cells were detached using a cell scraper. The cells were then treated with 0.1 mg/ml of pepsin in 50 mM of acetic acid and incubated

overnight at 4°C with continuous shaking. After incubation, the samples were centrifuged at 10,000*g* for 10 min and the supernatant was neutralized with neutralization solution containing 200 mM of Tris and 150 mM of NaCl. The assay sample and the biotinylated anti-collagen antibody solution were mixed well in the ratio of 1:9. Then 50 µl of each of the samples was added to the wells of the collagen-coated microplate and incubated for 1 h at 28°C with moderate shaking. After washing with wash buffer, 50 µl of Avidin-horseradish peroxidase conjugate was added to each well and incubated for 1 h at 28°C with moderate shaking. After washing the wells, 50 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added and incubated for 15 min at 28°C without shaking. After incubation, 50 µl of a stop solution was added and the optical density was measured at 450 nm within 10 min. The assay is a competitive enzyme immunoassay and the optical density is proportional to the collagen content. The culture medium of the cells was also analyzed in a similar manner to estimate the collagen content. The assay was performed using a human collagen type I ELISA kit purchased from CosmoBio (Carlsbad, USA) as per the kit data sheet.

ROS INHIBITION ASSAY

ROS was estimated using DCFH-DA dye (12,13). After specific treatment, the cells were incubated with 0.002% DCFH-DA dye for 1 h at 37°C. The fluorescence intensity was measured by a fluorescence microplate reader set for excitation at 485 nm and emission detection at 520 nm. The increase in fluorescence is proportional to the ROS induced. The percentage of ROS induced is calculated with respect to the fluorescence intensity of non-irradiated control cells:

$$\% \text{ ROS enhanced} = \{[100/A] * B\} - 100$$

where A is the fluorescence of non-irradiated cells (control) and B is the fluorescence of UVB-irradiated cells with and without sample treatment.

RESULTS

EFFECT OF EMBLICA EXTRACT ON NORMAL HUMAN DERMAL FIBROBLAST VIABILITY

Cells were treated with varying concentrations of emblica extract (0.125, 0.25, 0.5, 1, and 2 mg/ml). Cell viability was determined after 24-h incubation by NRU assay. Treatment of cells with emblica extract did not have significant effect on cell viability at a concentration of 0.125–0.5 mg/ml. At higher concentrations, emblica extract had a toxic effect, as indicated by the decrease in cell viability to 67% and 42% at treated doses of 1 mg/ml and 2 mg/ml, respectively (Figure 1).

EFFECT OF EMBLICA EXTRACT ON UV-INDUCED COLLAGEN DAMAGE IN NORMAL HUMAN DERMAL FIBROBLAST CELLS

When 24-h cultures of normal human dermal fibroblasts were irradiated with 10, 20, 30, 50, and 100 mJ/cm^2 of UVB irradiation, a 50% inhibition in cell viability and a maximal enhancement in ROS generation up to 53% was observed at 50 mJ/cm^2. It was also

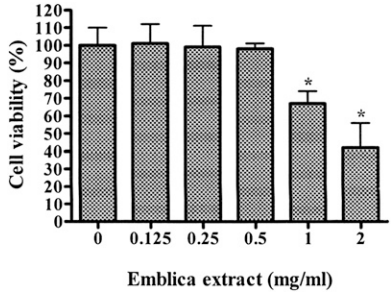

Figure 1. Effect of emblica extract on cell viability. Normal human dermal fibroblasts were treated with various concentrations (0, 0.125. 0.25, 0.5, 1, and 2 mg/ml) of emblica extract for 24 h. Cell viability was analyzed by NRU assay. The data are represented as percentage of cell viability compared with the untreated control. The experiments were performed independently in triplicate, and the number of cells was 50,000 cells in each sample. The data are represented as mean ± S.D. and were analyzed by Student's *t*-test. *Significant difference, $p < 0.05$, compared to untreated control.

observed by immunocytochemistry that there was significant damage to the procollagen (precursor of collagen) synthesized inside the cell and significant damage to the collagen synthesized (from the exocytosed procollagen) and deposited on the extracellular matrix (ECM) of the cell. There was a 64% reduction in fluorescence intensity due to UVB-induced collagen damage and a 43% reduction in fluorescence intensity due to UVB-induced procollagen damage as compared to the respective unexposed control cells.

Thereafter, the effect of emblica extract on UVB-induced collagen damage was determined using the CosmoBio human type 1 collagen estimation ELISA kit, which is specific for type 1 collagen estimation. Normal human dermal fibroblasts were grown for 24 h and then treated with varying concentrations of emblica extract and exposed to UVB irradiation of 50 mJ/cm^2. After exposure, the medium was replaced with fresh medium and incubated for 24 h in a CO_2 incubator. After incubation, the collagen content in the ECM of the cells was determined by ELISA. Usually collagen is also secreted in the culture medium; however, under our experimental conditions, significant collagen was not detected in the culture medium. Hence, collagen was estimated in the ECM of the cells by ELISA. The collagen estimated in non-irradiated cells was 3.23 μg/ml. The collagen estimated in untreated UVB-irradiated cells was 0.285 μg/ml, whereas the collagen detected in emblica-treated UVB-irradiated cells was 2.72 μg/ml and the collagen detected in ascorbic acid-treated UVB-irradiated cells was 1.05 μg/ml (Figure 2). Treatment with emblica extract significantly provided protection from collagen damage in normal human dermal fibroblasts in a dose-dependent manner (at concentrations ranging from 0.125 to 0.5 mg/ml), with the maximum response of 9.5 ± 0.28-fold protection from collagen damage at a concentration of 0.5 mg/ml. Since ascorbic acid is reported to provide UV protection, we used ascorbic acid for comparison. Our results indicated that ascorbic acid showed only 3.7 ± 0.07-fold protection from collagen damage at a concentration of 0.5 mg/ml.

ROS INHIBITION OF EMBLICA EXTRACT

Reactive oxygen species (ROS) are generated by a variety of sources from the environment (e.g., photo-oxidations and emissions) and normal cellular functions (e.g., mitochondrial metabolism and neutrophil activation). ROS include free radicals (e.g., superoxide and

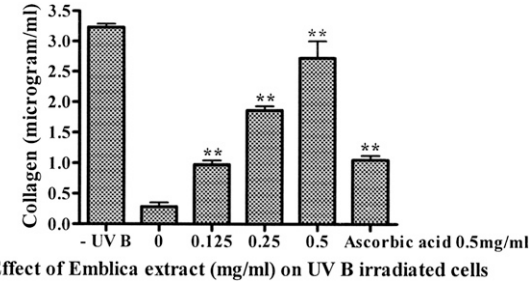

Figure 2. Effect of emblica extract and ascorbic acid on UVB-induced collagen damage. Normal human dermal fibroblasts were treated with various concentrations (0, 0.125. 0.25, and 0.5 mg/ml) of emblica extract and 0.5 mg/ml of ascorbic acid and irradiated with a UVB dosage of 50 mJ/cm^2. Followed by irradiation and 24-h incubation, the collagen content was estimated using the CosmoBio human collagen type 1 ELISA kit. The experiments were performed independently in triplicate, and the number of cells was 100,000 cells in each sample. The data are represented as mean ± S.D. and were analyzed by Student's *t*-test. **Significant difference, p <0.05, compared to untreated UVB-irradiated cells.

hydroxyl radicals), nonradical oxygen species (e.g., hydrogen peroxide and peroxynitrite), and reactive lipids and carbohydrates (e.g., ketoaldehydes and hydroxynonenal) (14). ROS is the main cause for photoaging. As shown in Figure 3, we found that emblica extract significantly inhibited ROS induced by UVB exposure in normal human dermal fibroblasts in a dose-dependent manner. Untreated UVB-irradiated cells showed 84 ± 1.4% induction in ROS as compared to the non-irradiated cells. Emblica extract treatment lowered the induction of ROS in UVB-irradiated cells in a dose-dependent manner, with maximal reduction of ROS induced to 15 ± 4% at a concentration of 0.5 mg/ml, while ascorbic acid reduced the induction in ROS to 64 ± 2% at a concentration of 0.5 mg/ml (Figure 3).

DISCUSSION

UV irradiation is a major environmental factor responsible for a high incidence of premature skin aging, referred to as photoaging, as well as skin cancer and melanoma. UV irradiation

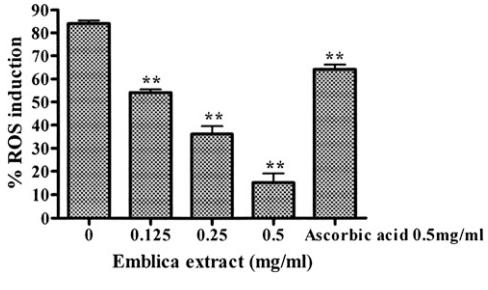

Figure 3. Effect of emblica extract and ascorbic acid on UVB-induced ROS generation. Normal human dermal fibroblasts were treated with various concentrations (0, 0.125. 0.25, and 0.5 mg/ml) of emblica extract and 0.5 mg/ml of ascorbic acid and irradiated with a UVB dosage of 50 mJ/cm^2. Followed by irradiation and 24-h incubation, the ROS induction was estimated. The data are represented as percentage of ROS induction compared with the non-irradiated control cells. The experiments were performed independently in triplicate, and the number of cells was 100,000 cells in each sample. The data are represented as mean ± S.D. and were analyzed by Student's *t*-test. **Significant difference, p <0.05, compared to untreated UVB-irradiated cells.

increases ROS production, which induces the synthesis of matrix metalloproteinases (MMPs) that degrade collagen, causing skin photoaging. Collagen fiber is primarily synthesized by fibroblasts as a pro-collagen protein, which is secreted and further processed to be a collagen fiber in the extracellular matrix (15,16). Among collagens, type I is the most abundant and comprises between 85% and 90% of the total collagen in skin (9). Type 1 collagen damage by UVB irradiation results in photoaging. Strategies to prevent or at least minimize ROS-induced photoaging and intrinsic aging of the skin necessarily include protection against UV irradiation and antioxidant homeostasis (8).

Due to their potent antioxidant activity, emblica extracts appear to have benefits as anti-aging actives. The level of procollagen type I protein in photoaged skin is lower than that in naturally aged skin. The level of matrix metalloproteinase-1 protein and the activity of matrix metalloproteinase-2 were higher in the dermis of photoaged skin than in naturally aged skin (10). Emblica extract stimulated proliferation of fibroblasts and also induced production of procollagen in human skin fibroblasts (17).

It is known that ascorbic acid increases the photostability of collagen and that the collagen becomes less sensitive to UV radiation (18). The present study found that emblica extract significantly protected normal human dermal fibroblasts from UVB-induced ROS generation and subsequent collagen damage. In addition, our results show that emblica has a greater potency than ascorbic acid. Moreover, only trace amounts of ascorbic acid have been recently reported in emblica fruits, suggesting that the antioxidant effects exhibited by emblica fruits are due to gallic acid esters (2). Our results suggest that the efficacy of emblica extract in preventing UV-induced ROS generation and collagen damage may not be associated with its ascorbic acid content.

The UV-protection efficacy of a product depends significantly either on its UV absorbance/blocking efficacy or its efficacy in inhibiting UV-induced adversaries (ROS generation, MMP generation, etc.) or both. Also, not all the antioxidants or MMP inhibitors are effective sunscreens and vice-versa. For example, octyl methoxycinnamate, zinc oxide, titanium dioxide, etc., are potential sunscreens but not potential antioxidants. Similarly, green tea (containing flavonoid antioxidants), butylated hydroxytoluene, trolox, etc., are potential antioxidants but are not used as sunscreens. This may be because many of the antioxidants may not be stable under UV exposure to provide UV protection benefits.

Ascorbic acid and emblica extract are potential antioxidants and are also used as sunscreens. Although there is enough literature on the antioxidant, MMP-inhibitory, and collagen-promoting properties of emblica extract that contribute to its UV-protection efficacy, there was no significant emphasis on the active constituents that contribute to its unique UVB-protection efficacy. Earlier, it was thought that emblica fruits were rich in ascorbic acid (1), and it is obvious that ascorbic acid could have contributed significantly to the UVB-protection efficacy of emblica fruit extract. However, recent work on the characterization of the emblica fruit extract indicates only trace amounts of ascorbic acid in the extract (2), and our work on its UVB-protection efficacy in comparison to ascorbic acid indicates that the combination of various gallates (1-O-galloyl-β-D-glucose (β-glucogallin)) and mucic acid (1,4-lactone 5-O-gallate in the emblica extract) contributes to its UVB-protection efficacy, making it a natural cosmetic, superior to a much-used cosmetic active, ascorbic acid. This composition is more stable under UV exposure and hence it could render its antioxidant and anti-inflammatory benefits against UV-induced adversaries, unlike other antioxidants that may not be stable under UV exposure to provide UV-protection benefits.

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