



Effect of *Emblica officinalis* (fruit) against UVB-induced photo-aging in human skin fibroblasts

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ABSTRACT

Ethnopharmacological relevance: *Emblica officinalis* fruit (EO), commonly known as Amla is a reputed traditional medicine and functional food used in Indian subcontinent. It has long been used in Indian folk medicine to treat liver diseases, stomach ulcers, inflammatory diseases, metabolic disorders, geriatric complaints, skin disorders and beauty care.

Aim of the study: Recently, it has been shown to promote pro-collagen content and inhibit matrix metalloproteinase levels in skin fibroblast. The aim of the present study was to investigate the efficacy of EO to inhibit UVB-induced photo-aging in human skin fibroblasts.

Materials and methods: Mitochondrial activity of human skin fibroblasts was measured by MTT-assay. Quantifications of pro-collagen 1 and matrix metalloproteinase 1 (MMP-1) release were performed by immunoassay techniques. Hyaluronidase inhibition assay was studied *in vitro* using bovine testicular hyaluronidase and human umbilical cord hyaluronic acid. Cell cycle analysis was performed by flowcytometry using propidium iodide.

Results: EO stimulated, the otherwise UVB inhibited cellular proliferation and protected pro-collagen 1 against UVB-induced depletion via inhibition of UVB-induced MMP-1 in skin fibroblasts (10–40 µg/mL, $p > 0.001$). EO exhibited inhibitory activity of hyaluronidase (10–40 µg/mL, $p > 0.001$). Treatment with EO also prevented UVB disturbed cell cycle to normal phase.

Conclusion: The results of the present study suggests that EO effectively inhibits UVB-induced photo-aging in human skin fibroblast via its strong ROS scavenging ability and its therapeutic and cosmetic applications remain to be explored.

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1. Introduction

Human skin is constantly exposed to potentially harmful compounds and radiations because it serves as a protective barrier between environment and internal organs, thus making it liable to aging process (Rittie and Fisher, 2002; Ding and Wang, 2003).

UV irradiation has deleterious effects on human skin, including sunburn, immune suppression, cancer, and photo-aging (Jones et al., 1999; Offord et al., 2002; Moon et al., 2008). UVB, in particular, is the most hazardous environmental carcinogen known with regard to human health through generation of reactive oxygen species (ROS) (Katiyar et al., 2001; Fisher et al., 2002; Ding and Wang, 2003). The ROS results in the subsequent activation of complex signaling pathways, followed by matrix metalloproteinases (MMPs) induction in skin cells and degradation or synthesis inhibition of

collagenous extracellular matrix in connective tissues (Fisher et al., 1998; Oh et al., 2004; Ho et al., 2005) have commented that plant sources, since time immemorial have been known as source of natural bioactives. Natural products and related drugs are used to treat 87% of all categorized human diseases (Chin et al., 2006).

Emblica officinalis has long been used in Indian folk medicine to treat a broad spectrum of disorders, such as liver diseases, stomach ulcers, inflammatory diseases, to inhibit tumor growth, in diabetes, and geriatric complaints (Tasduq et al., 2005). *Emblica officinalis* has also been traditionally used for skin disorders and beauty care. It is one of the three constituents of 'myrobalan' an important group of three fruits named in Ayurveda as 'Triphala': the other two being *Terminalia chebula* and *Terminalia bellerica*.

The clinical efficacy of the fruit of *Emblica officinalis* is held in high esteem in Ayurveda and is referred to as 'Maharasayana'. It is reputed to promote health and longevity by increasing defense against disease, arresting the ageing process and revitalizing the body in debilitated conditions. *Emblica officinalis*, alone or as a part of many polyherbal formulations, currently are in use in India for

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skin and hair related disorders and beauty care. Recently *Emblica officinalis* has been shown to promote pro-collagen production and inhibit MMP-1 in human and mouse skin fibroblasts (Fujii et al., 2008; Chanvorachote et al., 2009). However, there is no report on the effect of *Emblica officinalis* against UVB-induced photo-aging in human skin fibroblasts. Therefore in the present study, we evaluated the protective effect of *Emblica officinalis* in human skin fibroblasts against UVB-induced photo-aging.

2. Materials and methods

2.1. Chemicals

The normal human newborn foreskin fibroblast cell line, HS68 cell (ATCC CRL 1635), was obtained from American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified eagle's media (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, trypsin–EDTA, 3-(4,5-dimethylthiazol-yl)-diphenyl tetrazolium bromide (MTT), propidium iodide, ribonuclease A (RNase A), hyaluronic acid (HA), hyaluronidase (ENZ) were obtained from Sigma–Aldrich Chemicals (St. Louis, MO). pro-Collagen type 1 C-peptide protein and matrix metalloproteinase-1 Elisa kits were procured from Takara, Japan; Cat. #MK101 and GE Healthcare; Code: RPN2610 respectively. All other biochemicals used were of high purity biochemistry grade.

2.2. EO preparation and standardization

Fresh fruits of *Emblica officinalis* were collected locally during the period March–April, fruits were identified by institute taxonomist, and a voucher specimen (0177) has been deposited in the institute herbarium. Finely cut pieces of fruit were minced and soaked in 50% ethanol and shaken at room temperature for 18 h. The mixture was centrifuged (4000 rpm × 30 min) and the supernatant was evaporated to dryness under reduced pressure at 40 °C in a rotary evaporator (Buchi B-480). The yield of dried residue was 3.9% and it contained 33% (w/w) tannin content. The extract (EO) was further standardized on the basis of two markers, ellagic acid and gallic acid by HPLC.

2.3. Chemo profiling

2.3.1. High-performance liquid chromatography (HPLC) analysis

The Water HPLC system comprising of two waters 515 HPLC pumps, automatic sampling unit (waters 717 plus auto sampler), column oven, photodiode array detector (waters 2996), Merck Rp-18 column (5 µm, 250 mm × 4.00 mm ID), temperature control module II and Waters Empower software was used for data analysis and data processing.

2.3.2. Sample preparation and quantification

The accurately weighed quantity of the dried extract (34.7 mg) was dissolved in 2.75 mL methanol:water (1:1, v/v) HPLC grade. The sample was centrifuged and filtered through Millipore micro-filter (0.45 µm) and was used for analysis. For markers, working solutions in the concentration range of 90–117.6 µg/mL were prepared by diluting with methanol. These working solutions of the marker compounds were mixed together in equal volumes and were injected in different concentrations. The calibration curve was plotted and linearity was observed in the concentration range of 0.22–1.47 µg/mL. The calibration curve of each marker in the mixture was also determined using five levels of concentration. The marker compounds in the extract were quantified using these calibration curves.

2.4. Cell culture

HS68 cells used for the experiments were between 10 and 25 passages. HS68 cells were plated in 175 cm² culture flasks and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Sigma, St. Louis, MO, USA). For treatment, cells were maintained in culture media without FBS overnight. The cells were pretreated with EO and ascorbic acid for 24 h, washed with phosphate buffer saline (PBS; Sigma, St. Louis, MO, USA) and irradiated under UVB radiation. After UVB irradiation, cells were again rinsed twice with PBS and immediately, incubated in fresh culture media without serum, in the presence of EO and ascorbic acid materials for further 24 h. All UVB irradiations were performed under a thin layer of PBS.

2.5. Ultraviolet irradiation

The source of UVB radiation was a band of four UVB lamps (Daavlin, UVA/UVB Research Irradiation Unit, Bryan, OH, USA) equipped with digital controller to regulate UV dosage at a fixed distance of 24 cm from the lamps to the surface of the cell culture plates. The majority of the resulting wavelengths were in the UVB (290–320 nm; above 90%) and UVA (less than 10%) range and the peak emission was recorded at 314 nm.

2.6. Cytotoxicity/cytoprotection

Cell viability was assessed as described by Moon et al. (2008), using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, MO, USA). For cytotoxicity experiments cells were treated with test materials at various concentrations and incubated for 48 h. For cytoprotection experiments, cells were pre- and post-treated with test materials at desired concentrations prior (24 h) and after (24 h) UVB irradiation. After the corresponding treatments the medium was removed and cell viability was evaluated by assaying for the ability of functional mitochondria to catalyze the reduction of MTT (0.5 mg/mL, at 37 °C for 3 h) to form formazan salt by mitochondrial dehydrogenases, as determined by ELISA reader at 565 nm (Multiskan Spectrum; Thermo Electron Corporation, USA).

2.7. pro-Collagen 1 and matrix metalloproteinase-1 immunoassay

The levels of type 1 pro-collagen (Takara, Japan) protein and matrix metalloproteinase-1 (GE Healthcare) in cell-free supernatants were determined by ELISA. The supernatants of cultured fibroblasts after indicated treatments were collected and stored at –80 °C until used. The assays were performed according to manufacturer's instructions as follows.

2.8. Hyaluronidase inhibition assay

Hyaluronidase (HA) was assayed as described by Sumantran et al. (2007), based on precipitation of HA with cetylpyridinium chloride. Enzyme (800 U/mL) and HA substrate (0.40 mg/mL) were incubated at 37 °C for 1 h. Enzyme activity was measured by monitoring the percentage of undigested HA substrate in the cetylpyridinium chloride precipitate at absorbance 415 nm (A 415 nm) after the enzyme reaction.

2.9. Cell cycle analysis

Cell cycle was analyzed as described by Yang et al. (2007). Briefly, non-treated and treated skin fibroblast cells were harvested by trypsinization, centrifuged at 1500 × g for 5 min, washed with PBS,

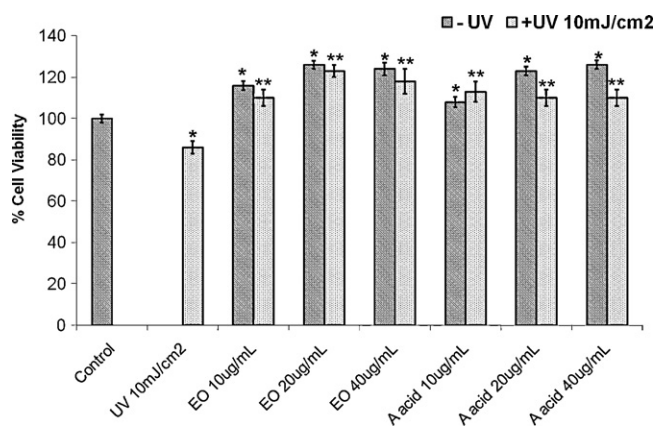


Fig. 1. Effect of UVB irradiation on cell viability of human skin fibroblasts and protection by *Emblca officinalis* (EO) and ascorbic acid (A. acid) in cultures. Each value is presented as mean \pm S.D. for each set of four wells. * $p < 0.001$, significantly different when compared with the control value and ¹ $p < 0.003$ and ** $p < 0.001$, when compared with UVB irradiated cells only (Student's *t*-test).

and fixed in 70% ethanol at 4 °C overnight. Fixed cells were washed twice with PBS and incubated in PBS containing 1.5 mg/L RNase A for 1 h at 37 °C, followed by staining with 5 μ L PI (1 mmol/L stock) for 20 min on ice. The cells were analyzed for DNA content using BD FACSCalibur cytometer using blue (488 nm) excitation from argon laser. Data were collected in list mode on 10,000 events for FL2-A vs FL2-W.

2.10. Statistical analysis

Results are expressed as mean \pm S.D. Data was analyzed for significant difference from the untreated control group by Student's *t*-test and *p* values < 0.05 were considered statistically significant.

3. Results

3.1. Quantification of gallic acid and ellagic acid in extract

The retention times (Rts) of marker compounds gallic acid was Rt-6.346 and ellagic acid was Rt-17.406. Quantity of gallic acid and ellagic acid as marker compounds estimated using standard curve was 9.207% at a retention time (Rt) of 3.198 min and 0.951% at a retention time (Rt) of 16.277 min respectively.

3.2. Effect of EO on UVB-induced fibroblast photo-toxicity

Incubation of fibroblasts with various concentrations of EO and ascorbic acid (0–40 μ g/mL), for 48 h, resulted in enhanced cell viability in a concentration dependent manner. EO treatment resulted in increased viability by 16–24% compared to non-treated cells (control) ($p < 0.001$). Ascorbic acid at the same concentrations resulted the enhancement by 8–26% ($p < 0.001$). UVB irradiation at a dose of 10 mJ/cm² resulted in a decreased cell viability of 14% ($p < 0.001$) compared to untreated control. This UVB dose was selected to photo-toxicity in skin fibroblasts to evaluate the photo-protective effect of EO in further experiments. Treatment with EO at 10, 20 and 40 μ g/mL showed a dose dependent protective (21–30%, $p < 0.001$) effect against UVB irradiation induced loss of cell viability at 10 mJ/cm². Treatment of ascorbic acid at the same concentrations resulted in an enhancement of cell viability by 21–24% ($p < 0.001$; Fig. 1).

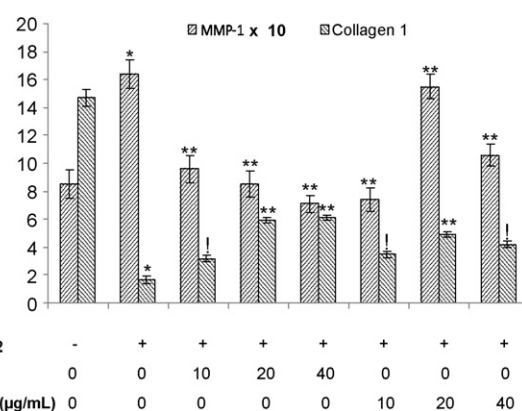


Fig. 2. Effect of EO and ascorbic acid on UVB irradiation-induced depletion of type 1 pro-collagen and MMP-1 in human skin fibroblast. Each column represents the mean \pm S.D. of three determinations. * $p < 0.001$, significantly different when compared with control value and ¹ $p < 0.003$ and ** $p < 0.001$, when compared with UVB irradiated cells only (Student's *t*-test).

3.3. Effect of EO on pro-collagen 1 content and pro-matrix metalloproteinase 1 (pro-MMP-1) levels

Treatment of skin fibroblast with 10 mJ/cm² of UVB irradiation resulted in 8.7-fold decrease in pro-collagen 1 levels. This shows the intense effect of UVB irradiation on pro-collagen 1 content in skin fibroblasts. This decrease in pro-collagen 1 content was restored by EO treatment at 10, 20 and 40 μ g/mL in a range of 47–72% ($p < 0.001$). Ascorbic acid at 10, 20 and 40 μ g/mL increased the UVB depleted levels of pro-collagen 1 by 52–65% ($p < 0.001$). pro-MMP-1 levels were enhanced by 19-fold due to exposure of skin fibroblasts to UVB irradiation at 10 mJ/cm². Treatment of skin fibroblast with EO and Ascorbic acid at 10, 20 and 40 μ g/mL caused a decrease in these levels by about 94% ($p < 0.001$; Fig. 2).

3.4. Inhibition of hyaluronidase by EO

Hyaluronidase enzyme (ENZ) at 800 U/mL caused 87.5% ($p < 0.001$) digestion hyaluronic acid (HA) (0.40 mg/mL) when incubated at 37 °C for 1 h. Incubation of ENZ and HA in the presence of EO at 10, 20 and 40 μ g/mL resulted in strong inhibition of digestion of HA by 44, 66 and 71% respectively ($p < 0.001$). However, ascorbic acid showed no effect in this assay (Fig. 3).

Inhibition of hyaluronidase by E.O.

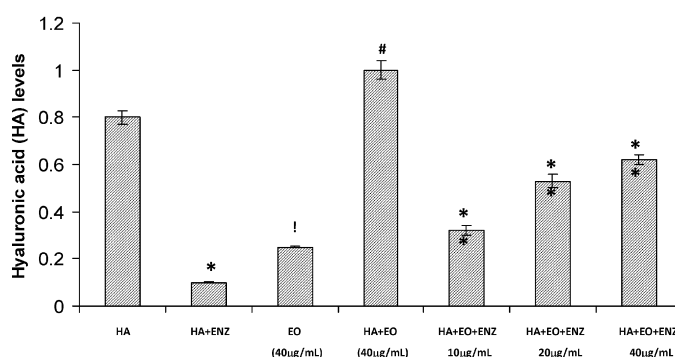


Fig. 3. Hyaluronidase inhibition by EO. Axis Y shows levels of hyaluronic acid (HA), measured by the spectrophotometer (A 415 nm) in each sample. The X-axis shows the contents of each sample. Each column represents the mean \pm S.D. of six determinations. * $p < 0.001$, significantly different when compared with HA alone; ¹ $p < 0.003$ when compared with HA + enzyme (ENZ); # $p < 0.003$ when compared with HA alone and ** $p < 0.001$, when compared with HA + ENZ (Student's *t*-test).

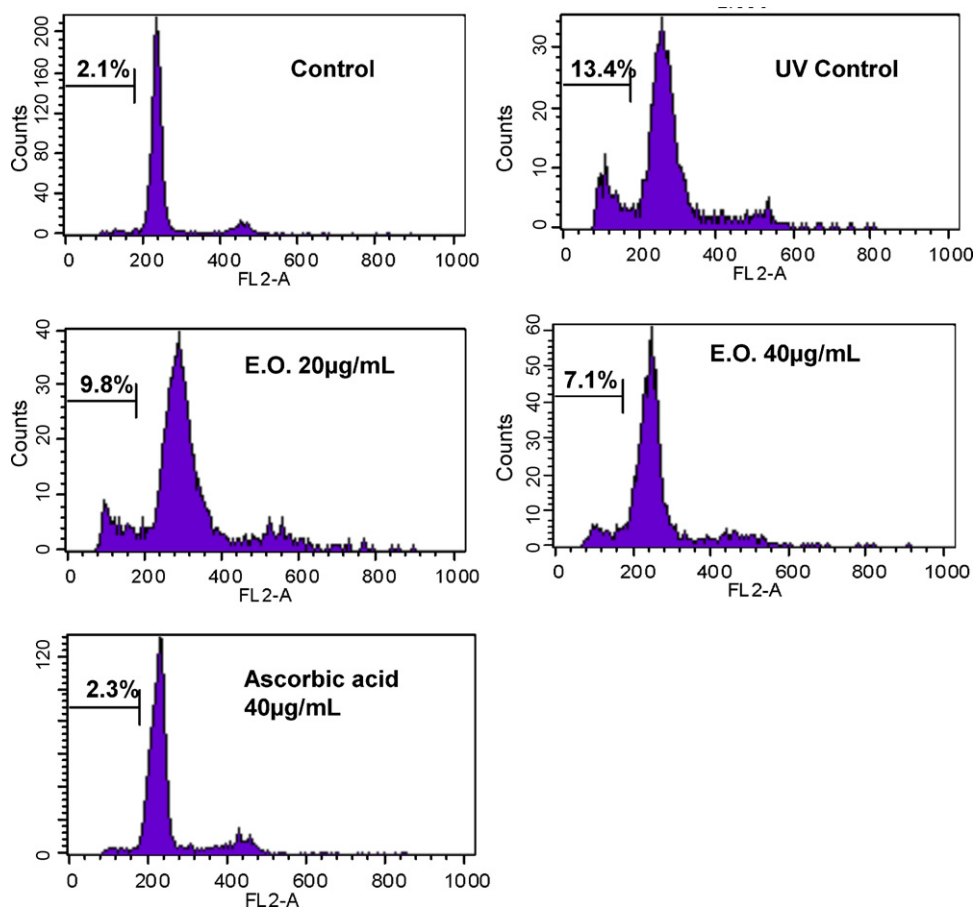


Fig. 4. Effect of EO and ascorbic acid against UVB (10 mJ/cm²)-induced cell cycle arrest. Representative histograms and percentage of cells in the sub-G₀/G₁ fraction (M1 zone, hypodiploid area) are shown.

3.5. Effect of UVB on cell cycle and amelioration by EO

In cell cycle analysis, UVB irradiation at 10 mJ/cm², significantly increased the apoptotic cells (13.4%) compared to normal untreated cells (2.1%). Treatment with EO at 20 and 40 µg/mL, resulted in decrease in apoptotic cells to 9.8 and 7.1% respectively. Ascorbic acid at 40 µg/mL restored the apoptotic cells to 2.3% (Fig. 4).

4. Discussion

It has been established that UVB irradiation of human skin fibroblast induces the expression of MMPs which degrades the extracellular matrix (ECM) causing pre-mature aging (photo-aging). Moon et al. (2008) have suggested the development of MMP inhibitors to be a promising strategy for photo-aging therapy. Fujii et al. (2008) and Chanvorachote et al. (2009) have reported that *Emblca officinalis* stimulates fibroblast proliferation and extracellular matrix deposition in human and mouse skin fibroblast via increased collagen and tissue inhibitors of metalloproteinase-1 (TIMP-1) production with enhanced fibroblast proliferation and inhibition of MMP-1. However, no, investigation of *Emblca officinalis* on protection against UVB-induced photo-aging in human skin fibroblasts have been reported to date. Hence, we investigated the protective effect of *Emblca officinalis* against UVB-induced photo-aging in human skin fibroblast and tried to elucidate the mechanism underlying.

We have demonstrated that irradiation of human skin fibroblasts with UVB causes decrease in pro-collagen-1 content, cell viability loss and an increased MMP-1 production. There was an

increase in intracellular ROS levels (data not shown) associated with disturbed cell cycle. Treatment with *Emblca officinalis* augmented these manifestations in a dose dependent manner.

Fujii et al. (2008) have reported that *Emblca officinalis* contains 2% ascorbic acid, which is known to enhance proliferation and collagen production in fibroblasts and concluded that ascorbic acid contributes to the overall activity of EO. However, Majeed et al. (2009) have concluded that the presence of ascorbic acid in *Emblca officinalis* fruit is questionable. For several decades, *Emblca officinalis* has been claimed to be a very rich source of ascorbic acid, however, during their attempt to quantify the ascorbic acid with newly developed, modern scientific techniques, they have found that ascorbic acid was present below the quantifiable limits of HPLC–DAD (LOD = 1 ppm and LOQ = 3 ppm). This study is suggestive of the fact that *Emblca officinalis* contains ascorbic acid only in trace quantities and thus the photo-protective activity of *Emblca officinalis* lies in the molecules other than ascorbic acid, which are yet to be identified and isolated. Besides, the mechanism of photo-protection offered by *Emblca officinalis* seems to be entirely different from that of ascorbic acid. In this respect, the photo-protective effect of *Emblca officinalis* is of great importance compared to other fruits which contain high quantities of ascorbic acid and their cosmetic use is attributed to the presence of ascorbic acid.

Results of the present study showed that EO not only, enhanced the fibroblast proliferation in a concentration dependent manner but also exhibited a highly significant photo-protective effect against UVB-induced cytotoxicity (Fig. 1). These results are suggestive of strong skin protective ability of *Emblca officinalis*.

Present results shows that EO pretreatment (pre-UVB) for 24 h, followed by another 24 h incubation period (post-UVB), inhibited UVB-induced MMP-1 levels and increased the otherwise UVB depleted pro-collagen 1 content as a function of concentration of *Emblca officinalis* and ascorbic acid compared to UVB only irradiated cells (Fig. 2). This is in agreement to earlier reports where, UVB irradiation has been shown to induce MMP-1 expression as a function of time and dose in cultured cells (Di Girolamo et al., 2003; Kim et al., 2005).

EO contains large amounts of functional tannins, and therefore may exert its activities through varied mechanisms as shown by green tea tannins through the involvement of MAPKs, modulation of MMP expression and production through AP-1 and NF- κ B activation, suggesting a strong anti-photo-aging effect of tannins (Trautinger, 2001; Fisher et al., 2002; Chaudhuri, 2002; Bae et al., 2008, 2009).

We further evaluated the protective effect of EO against UVB radiation-induced cell death. Our results showed that fibroblast cell viability was reduced due to UVB irradiation, however, EO pretreatment significantly protected against this loss in cell viability in a concentration dependent manner (Fig. 1). Poltanov et al. (2009) have recognized the strong protective functions of EO against oxidative stress. These results demonstrated that EO possess the potential inhibitory effects on intracellular oxidative damage induced by UVB irradiation, and the antioxidant activities were associated with the improved cell viability. The results are in agreement with earlier reports which clearly mention the role of intracellular generation of ROS leading to a state of cellular oxidative stress as key mediators in photo-aging process as described by Wang et al. (2006). Increased cellular levels of ROS lead to cellular damage and scavenging of ROS through the use of antioxidants like EO, protecting the cells from such cellular damage has been a good strategy for development of photo-protective agents of cosmetic interests (Finkel and Holbrook, 2000; Martindale and Holbrook, 2002; Kim et al., 2008).

Cell cycle analysis (Fig. 4) also clearly demonstrated that *Emblca officinalis* reduced DNA damage induced by UVB radiation. Numerous natural compounds from plant sources have been shown to have strong skin photo-protective effects through their ability to quench ROS generated by UVB irradiation and thus preventing DNA damage (Booij-James et al., 2000; Sudheer et al., 2007; Skandrani et al., 2009; Hsu and Chiang, 2009). EO is such a natural plant product which has an antioxidant activity related to UV protection (anti-photo-aging). Such plant products are intensively focused due to the currently growing demand for beauty care products from natural sources world wide.

Our results also demonstrate a strong anti-hyaluronidase activity of EO (Fig. 3), suggesting that EO treatment can increased hyaluronic acid, and is highly beneficial for prevention of premature skin aging (wrinkle formation).

5. Conclusion

In conclusion, present results are strongly suggestive of the photo-protective effect of EO against UVB irradiation in human skin fibroblast via its strong ROS scavenging and antioxidant potential, MMP-1 inhibitory effect, protection and enhancement of pro-collagen-1 and Hyaluronic acid contents and inhibition of UVB-induced cell death. We conclude that *Emblca officinalis* extract protects the UVB irradiated human skin fibroblast against photo-aging and has a potential to be used as therapeutic and cosmetic product for anti-aging problems.

Conflict of interest statement

None.

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