

Evaluation of Punicalagin Niosomes for Skin Aging

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Abstract

Skin aging is one of the most common problems facing humanity. It occurs because of altering the balance between free radicals and antioxidants and increasing the amount of the reactive oxygen species (ROS) in skin cells, which leads to oxidative stress especially when exposed to UV radiation. Antioxidants can neutralize the harmful effects of ROS, and secondary plant metabolites can help protect against UV radiation. In this study, punicalagin was extracted from pomegranate and concentrations of total polyphenolics and flavonoids were determined and antioxidant activities measured. Punicalagin was loaded onto niosomes and its morphology and release were studied. An in vitro study was performed on human fibroblast cell line HFB4 cells with aging induced by H₂O₂ and UV radiation. Cell cycle arrest was studied and different genes (*MMP3*, *CollA1*, *TimP3*, and *TERT*) involved in the skin aging process were selected to measure punicalagin's effect. Punicalagin succeeded in reducing the growth arrest of HFB4 cells, activated production of the *CollA1* and *TimP3* genes, maintained collagen level, and lowered of *MMP3*. Punicalagin increased human *TERT* concentration in skin cells. Punicalagin is promising as a natural antioxidant to protect human skin from aging.

Keywords: skin aging, punicalagin, niosomes, UV radiation, collagen

Introduction

During the human life cycle, the skin is exposed to various harmful factors (Zouboulis et al. 2019). Our skin is the largest organ, accounting for one-sixth of total body weight, and it acts as a barrier to protect the body from water loss and environmental factors such as chemicals, pathogens, and ultraviolet (UV) rays (Lephart 2016).

Human skin is divided into three layers: epidermis, dermis, and hypodermis. In the dermis, collagen fibers and elastin fibers are found. Aging skin shows thinning of the skin, loss of elasticity, dryness, wrinkles, and delayed wound healing. In general, with aging skin becomes pale in appearance (Lephart 2016).

Intrinsic aging is defined as the aging process that occurs naturally and begins when a person is in their mid-twenties. In the skin, the collagen production slows down, and elastin has a little less spring (Sjerobabski-Masnec & Šitum 2010). In contrast, extrinsic aging is a phenomenon that can be avoided to some extent. Many extrinsic or external factors often work in conjunction with the natural aging process to prematurely age our skin. Extrinsic factors are frequent facial expressions, gravity, sun, and smoking. The most harmful environmental factor that leads to extrinsic aging is repeated UV exposure, which is referred to as photo-aging (Sjerobabski-Masnec & Šitum 2010). UV A radiation (UVA) (400-320 nm) accounts for about 95-98% of the total UV rays that reach the surface of the earth. Besides damaging the epidermis, UVA rays penetrate deep into the dermis to break down collagen and elastin (Lephart 2016). Exposure to a high amount of UV radiation makes young people's skin prematurely aged due to the occurrence of mutations unless damaged skin cells are repaired (Sjerobabski-Masnec & Šitum 2010; Manosroi et al. 2012; Lephart 2016). UV radiation exposure leads to the generation of ROS and its accumulation in the skin cells, which results in skin aging.

Many genes were proved to have influence on the aging process. The group of genes known as the Collagens (COL) Gene group (Gagliano et al. 2000) are responsible for collagen synthesis, in addition to the matrix metalloproteinases (MMPs) that play an important role in inflammation, tumor invasion, and skin aging. MMPs degrade dermal collagen and elastin during the aging process (Kang et al. 2018; Lago & Puzzi 2019). Previous research has also demonstrated the importance of tissue inhibitors of metalloproteinase (TIMPs), which play a considerable role in the inhibition of protein degradation of the extracellular matrix (Lago & Puzzi 2019).

Skin as a self-renewing tissue goes through massive proliferation during life and telomere shortening acts to control mitotic division and prevent abnormal proliferation such as cancer. And as a result, cellular ageing occurs. The telomerase enzyme complex (Telomerase Reverse Transcriptase *TERT*) ensures telomere length maintenance in germ and cancer cells. Telomerase is also active in some somatic cells as in the epidermis but is hardly detectable in the dermis. Shortening of telomeres in skin cells may be accelerated due to proliferation and DNA-damaging agents as reactive oxygen species (ROS) (Erin & Aloysius 2011).

Oxidation processes in cell metabolism produce ROS normally to produce the energy required to fuel other biological processes, and this explains the inevitable production of free radicals through aerobic chemical reactions of cells in chronological aging that lead to mitochondrial damage, altering cell function, and if the damage is excessive, infected cells may die (Lephart 2016; Zouboulis et al. 2019). Although a strong antioxidant defense system exists in skin cells, excessive production of ROS will alter the balance between free radicals and

antioxidants, a process known as oxidative stress that may damage cells because ROS affects cellular constituents such as membranes, enzymes, proteins, lipids, RNA, and DNA in addition to reducing levels of antioxidants in the skin, which leads to many diseases and disorders as well as aging (Gilchrest 1989; Lephart 2016; Stress 2017; Zouboulis et al. 2019).

It has been shown that hydrogen peroxide (H_2O_2) enhances the development of skin injury and aging like other free radicals because it increases the expression of inflammation-related proteins and downregulates collagen I/III formation and expression of antioxidative proteins (Shen et al. 2017; Wen et al. 2017). Previous studies showed that treating human skin fibroblasts with 0–500 μM H_2O_2 decreased the viability of cells in a concentration-dependent manner.

Antioxidants are any substances that can directly scavenge ROS or indirectly act to enhance antioxidant defenses or inhibit ROS output (Petruk et al. 2018; Abd-Elghany and Mohamad 2020; Mohamad and Fahmy 2020). They are produced to counteract oxidative stress generated by the presence of excess ROS (Stress 2017). Also, antioxidants are distinguished by their ability through intra-molecular hydrogen bonding to further oxidize to form a more stable radical (Petruk et al. 2018).

Plants' secondary metabolites can be used as natural antioxidants to protect human skin from photo-aging (Petruk et al. 2018). The natural antioxidants from plant secondary metabolites can protect human skin from photo-aging (Petruk et al. 2018). They mainly contain polyphenols (phenolic acids, anthocyanins, flavonoids), carotenoids, and vitamins (vitamin E and C) (Xu et al. 2017). Pomegranate (*Punica granatum L.*) is a fruit that belongs to the family Punicaceae and consists of several substances such as fiber, vitamins, minerals, and bioactive plant compounds and sugar that are useful in reducing the risk of disease (Viuda-Martos et al, 2010; Zarfeshany et al, 2014).

Pomegranate fruits are divided into anatomical origins: peel (husk), arils, and seeds, and each has valuable compounds. The juice, which can be obtained from arils or from whole fruit, is also an important product. The edible part as well as seeds contain biologically active compounds like flavonoids and phenolics, principally anthocyanins capable of suppressing the effect of ROS on the body (Aloqbi et al, 2016). The peel weighs 50% of the total fruit weight and it is considered an important source of bioactive compounds such as flavonoids, phenolics, highly polymerized compounds as lignins, melanins, tannins, and proanthocyanidin compounds, complex polysaccharides, and minerals (Viuda-Martos et al, 2010). It was shown in previous studies that the peel extracts are more effective than juice and seed extracts as a potential source of natural antioxidants (Khan et al, 2017; Orak et al, 2012). The peel extract has antioxidant ability significantly higher than other known natural antioxidant plants such as green tea, and this is the main reason peel extract was used in this study.

To improve the therapeutic performance of the peel extract, niosomes can be used to protect them from the biological environment. Niosomes became popular in the field of topical drug delivery because of its unique characteristics (Mishra et al, 2016; Kazi et al, 2010; Rahimpour & Hamishehkar 2012). Its bilayer is formed by mixing non-ionic surfactants at variable combinations and molar ratios and may have cholesterol added (Rane et al, 2018; Yeo et al, 2018; Osanloo et al, 2018). Niosome is spherical in shape and consists of microscopic lamellar structures. Niosomes can deliver insoluble agents into vesicular bilayer membranes and hydrophilic agents in aqueous compartments, to the target region with lower doses of the drug to reduce side effects, increase the stability of the drug, enhance the therapeutic effects, prolong the blood circulation time in the

body, and enhance the absorption of the drugs into the target area. The toxicity of the drug is reduced due to the decreased absorption of drug by nonspecific tissues (Fang et al, 2001; Maibach 2005; Karim and Sattwa 2010; Sathali et al, 2010; Varun et al, 2012; Gupta et al, 2014; Khoei & Yaghoobian 2017).

In this study, we extracted punicalagin from pomegranate peel and then encapsulated it in niosomes to treat the HFB4 cell line as an anti-aging agent. We evaluated the antioxidant and anti-aging effects of punicalagin coated in niosomes by studying cell cycle arrest and different skin aging genes (*MMP3*, *Col1A1*, *Timp3*, and *TERT*).

Materials and Methods

Materials

The fruits of pomegranate were purchased from a local market, Giza. Methanol, ethanol and Tween 80 were supplied from Sigma-Aldrich Co. (Schnelldorf, Germany). Cell line HFB4 (Human fibroblast cell lines) was provided by the Tissue Culture Department, Vaccines & Sera (VACSERA), Egypt, and fetal bovine serum (FBS) was purchased from Invitrogen Corp. (Carlsbad, CA, USA). Penicillin-streptomycin, trypsin, EDTA, and Hank's buffer were purchased from Gibco, New York, NY, USA. Dimethyl sulfoxide (DMSO) was obtained from Merck (Darmstadt, Germany). Propidium Iodide Flow Cytometry Kit for cell cycle analysis was from abcam, Ciro, Egypt. Qiagen® RNA extraction kit, BioRad SYBR® green PCR MMX kit, and *TERT* (Human) ELISA kit were from BioVision, Inc., Milpitas CA, USA. A punicalagin reference standard was obtained from Aktin Chemicals Inc., Chengdu, China.

Methods

Preparation of methanol extract from peels of pomegranate

The fruits were washed and peeled, then the peels were dried in a solar oven with temperature ranging from 27°C to 30°C (according to climate temperature) for nearly 10 days. The dried peels were then collected and ground in a grinding mixer until the powder form was obtained.

Powdered peels were macerated with 20% methanol for 7 days in a dark place to avoid degradation by the effect of light. After the soaking process was completed, the thick mixture was squeezed by hand and filtered through a muslin cloth to obtain a dark brown aqueous extract. The extract was then centrifuged, and the supernatant contained the extract (Hanu & Harmanpreet 2012).

Isolation of punicalagin from the extract with HPLC

An HPLC system was used to isolate punicalagin and consisted of an HPLC pump, an automatic sampler cooled to 4°C (Thermo Finnigan, San Jose, CA, USA), and a PDA detector (scanning 200 to 700 nm). The HPLC column was C18, 5µm, 4.6 x 250 mm (Thermo Finnigan). For the chromatographic conditions, ChemStation software (Agilent Technologies, Inc., Sant Clara, CA, USA) was used. The mobile phase was solvent A (2% acetic acid in distilled water) and solvent B (0.5% acetic acid in 50% acetonitrile in water). The flow rate was 1 ml/min. Samples

(50 μ L) were injected onto the HPLC column (25°C) (González-Barrio et al, 2010). The chromatogram peak regions were quantified at 260 nm using a calibration curve obtained with a punicalagin reference standard.

Determination of total polyphenolics

The phenolic compounds were complexed with Folin-Ciocalteau reagent and the intensity of the blue color formed was measured using gallic acid as a standard (Aliyu et al, 2009). Total phenolics concentration was calculated. The absorbance of the resultant blue color was read at 765 nm using a UV-visible spectrophotometer (UVD-2960 LABOMED Inc., Los Anglos, CA, USA). For each concentration, three replicates were carried out and the average was calculated.

Determination of total flavonoids

The flavonoids were combined with aluminum chloride and the produced color intensity was measured (Makky et al, 2012). The concentration of flavonoids was calculated as quercetin equivalent (QE) with reference to a pre-established standard calibration curve. The content was incubated for 30 min at room temperature and the absorbance was measured at 415 nm. Total content of flavonoids in the extracts was calculated as QE.

Determination of antioxidant activity (DPPH method)

The extract stock solution was diluted by methanol to concentrations of 250, 125, 50, 25, 10, and 5 μ g/ml. One ml of 0.3 mM DPPH was added to 2.5 ml of the extracts or standard, and the mixture was incubated at room temperature in the dark for 30 min. Absorbance was measured at 518 nm and converted to antioxidant activity percentage (AA %) (Besada 1987; Menezes et al, 2001).

Preparation of niosomes

Using a thin film hydration method, the mixture of 10 μ l Tween 80 and 3 mg cholesterol was dissolved in ethanol in a round flask. Ethanol was evaporated using a rotary evaporator at 50°C, and a thin solid mixture appeared on the flask wall (Ravalika & Sailaja 2017). To form multilamellar niosomes, that layer was rehydrated with 10 ml phosphate buffered saline (PBS, pH 7.4) with bath sonication (Akbarzadeh et al, 2013).

Study of niosome morphology

The morphology of free and punicalagin loaded niosomes was examined using a transmission electron microscope (TEM) (JEOL JEM.1230, Shizuoka, Japan.)

Release test for free punicalagin and punicalagin entrapped into niosomes:

Two dialysis sacs were washed and soaked in distilled water. Each sample (free punicalagin and punicalagin entrapped in niosomes) was pipetted into a sac and the sac was

sealed. Then the sacs were placed each in a 250 ml beaker containing 200 ml PBS with constant shaking at 37°C. At different time intervals, the buffer was analysed for punicalagin content using a UV spectrophotometer.

In vitro cell culture study

Preparation of cell culture: HFB4 cell lines were preserved as a culture of monolayer in Dulbecco's Modified Eagle's Medium (DMEM) containing 10FBS, L-glutamate, sodium pyruvate, and 1% penicillin-streptomycin and then incubated at 37°C. DMEM medium was added to cells in a 15 ml tube and then centrifuged to get rid of any freezing medium. The pellet was resuspended in DMEM, then incubated at 37°C in a T-75 flask for 2 days. When the cells reached at least 80% confluence, they were washed twice with PBS and were detached, then the HFB4 cells were collected and counted.

MTT (cytotoxicity) assay

The MTT test was used to assess the cell viability. The test depends on the reduction of MTT. The cells (cell count was 1×10^6) were suspended in 10 ml DMEM + 10% FBS and seeded in 96-well plates at $8-10 \times 10^3$ /well for 24 hrs. Then treatment with two-fold dilution was applied on punicalagin and H₂O₂ to determine the safe concentration for each. The plates were incubated for 24 hrs at 37°C and then filtered from the medium and 10 μ l MTT was added to each well. After incubation for 4 hrs, the supernatants were removed, and the absorbance was analyzed with an ELISA reader (Exert 96, Asys Hitch, Ec, Neudorf, Austria) at 570 nm.

Effect of H₂O₂ and punicalagin on HFB4

Subculture of HFB4 cells was placed into 3 T-75 flasks and in a 6-well plate, and all were incubated at 37°C until the cells attained at least 80% confluence. The cells then were distributed into three groups in T-75 flasks: untreated cells (CC), cells treated with safe concentration (0.11 mM) of H₂O₂ (H₂O₂/HFB4), and cells treated with punicalagin (0.27 Mm) in addition to hydrogen peroxide (0.11 mM) (s + H₂O₂). Another two groups of cells were placed in 6-well plates: untreated cells (cR), and cells treated with safe concentration (0.27Mm) of punicalagin (s.R), then the two 6-well plates were exposed to UV (365 nm for 15 min at height 12.5 cm²). After 24 hrs incubation at 37°C, cells were taken from the 6-well plates and put into 5 tubes (15 ml), then centrifuged to get rid of any remaining medium. The pellet was resuspended and washed twice with PBS then dispersed in 0.25% trypsin/EDTA in Hank's buffer. Then the HFB4 cell lines were collected in five tubes to be ready for the next tests.

Cell cycle analysis (DNA content)

Cell cycle analysis was preformed using a Propidium Iodide Flow Cytometry Kit from abcam®. The cells were prepared and stored at 4°C until use, then they were transferred to the bench-top to equilibrate to room temperature, and then resuspended by gentle inversion. The cell pellet was resuspended in 200 μ l 1X propidium iodide + RNase staining solution, then incubated for 20 min in the dark at 37°C. The tube was placed in ice (in the dark) and the cells resuspended by gentle inversion. Then the samples were analyzed using flow cytometry. To exclude debris and

cell aggregates, FSC vs. SSC gates were set, and the propidium iodide fluorescence was collected in FL2.

RT-PCR assay

RT-PCR was performed to detect the fold change in the expression of the *MMP3*, *Col1A1*, and *Timp3* genes with the aid of beta actin as housekeeping gene. mRNA isolation was performed using RNeasy® extraction kit. Cells were torn and homogenized in Buffer RLT. Then ethanol was added to the mixture, creating conditions that enhance selective binding of RNA to the RNeasy membrane. The sample was then added to the RNeasy Mini spin column. Total RNA was bound to the membrane, contaminants were efficiently isolated, and high-quality RNA was filtered in RNase-free water. Ligation, washing, and rinsing steps were carried out by centrifugation in a micro-centrifuge. Then the Master Mix was prepared, and the amplification was performed by incubating the reaction mix in the RT-PCR device.

Telomerase reverse transcriptase (*TERT*) enzyme assay

The reagents, samples, and standards were prepared from *TERT* (Human) ELISA kit from BioVision. The clean plate was washed 2 times using 1X wash solution, 100 μ l of each sample and standard were added, and the wells were covered for 1.5 hrs incubation at 37°C. The plate content was discarded without washing or letting the wells completely dry. Biotin-detection antibody work solution (0.1 ml) was added into the wells, then the plate was sealed and left for 60 min for incubation. The solution was discarded, and the plate washed 3 times by filling the wells with the buffer solution and soaked for 1-2 min, decanted, and clapped by absorbent filter papers. SABC working solution (0.1 ml) was added to the wells and then the plate was incubated at 37°C for 30 min, then the solution discarded and washed 5 times. TMB substrate (90 μ l) was added, and the plate was incubated in a dark place for 15-30 min at 37°C. At the end, 50 μ l of stop solution was added and the results were read at 450 nm within 20 min.

Statistical Analysis

Data were examined using SPSS v. 15.0. Significant variations between groups were estimated using one-way ANOVA analysis. $p \leq 0.05$ was considered significant.

Results

Isolation of punicalagin with HPLC

Punicalagin was isolated with HPLC by comparing the pomegranate methanol extract with a punicalagin reference standard (Figure 1 a and b). Two peaks appeared, representing α -punicalagin and β -punicalagin, and they were both isolated. A calibration curve was made and the concentration of the isolated punicalagin was determined as 7.34 mg/g (Figure 2).

Total polyphenolics

The total polyphenolics were determined by using a standard curve between different standard concentrations of gallic acid and the absorbance at 765 nm. Total polyphenolics were 138 mg/g.

Total flavonoids

The total flavonoids were determined by using a standard curve between different concentrations of quercetin. Total flavonoids were 58 mg/g.

Antioxidant activity

The scavenging value of DPPH was 73.33% and was closely related to the total polyphenol contents. It can be assumed that these compounds have significant oxidation capacity. This result is compatible with the previous studies (Jacob et al. 2018; Zeghad et al. 2019) as the total phenolic content in punicalagin reflects high antioxidant activity.

Study of the morphology using TEM:

TEM examination displayed the morphology of the niosomes and the difference in size between the free ones (135-200 nm) and those loaded with punicalagin (185-335 nm) (Figure 3). Also, it showed that the sizes are in the nano-range.

Release test for free punicalagin and niosomes loaded with punicalagin:

The entrapment of punicalagin in the niosomes caused a decrease in the release rate of the punicalagin from the sac to the medium (Fig. 4).

MTT (cytotoxicity) assay

Cytotoxicity assay showed the safe doses to be used on the HFB4 cells. For H₂O₂ the safe concentration was determined between dilution factor 4096 and 8192, while the punicalagin was at dilution factor 128 (Figure 5).

Cell growth rate

The cell percentage arrested in the G2/M phase increased in the case of cR and H₂O₂/HFB4 samples, however the percentage of cells arrested in the G0/G1 phase diminished in the case of H₂O₂/HFB4 sample. The samples (s + H₂O₂ and s.R) that were pretreated with punicalagin showed an effect that opposed the effect resulting from H₂O₂ and UV radiation (Figure 6).

RT-PCR assay

MMP3 expression increased in cR and H₂O₂/ HFB4 samples, and the expression of *CollA1* and *Timp3* decreased in those two samples. The s + H₂O₂ and s.R samples showed an effect that opposed that resulted from H₂O₂ and UV radiation (Figure 7).

TERT assay

Telomere shortening occurred significantly in both H₂O₂/ HFB4 and cR samples by 64.20% and 14.22 %, respectively, while punicalagin treated samples showed telomere shortening in s.R and s + H₂O₂ samples by only 31.19 % and 6.5%, respectively, and cells were protected from telomere shortening (Figure 8).

Discussion

This work was done to show the effect of punicalagin as a strong antioxidant against skin aging. The final concentration of punicalagin in the extract was 7.34 mg/g (73.4%), which is a relatively high concentration compared to that found in other natural sources of antioxidants. Total polyphenolics and flavonoids were 138 mg/g and 58 mg/g, respectively. Furthermore, the scavenging activity percentage showed high activity (73.33%), proving the high antioxidant activity of pomegranate husk extract (Aloqbi et al 2016).

Those active compounds work to protect the skin absorbing UV radiation, inhibiting UV-induced free radical reactions, or modifying endogenous antioxidant and inflammatory systems (Petruck et al, 2018).

TEM analysis showed that the ultra structure of the arranged niosomes and the niosomes loaded with punicalagin was non-aggregated circular shaped particles. The morphology demonstrated the homogeneity of the molecule, and the niosomes appeared spherical. The sizes ranged from 180 nm to 220 nm for the free niosomes, which is smaller than those loaded with niosomes (218-437 nm). This shows that the sizes were within the reasonable large unilamellar vesicle size range (Yeo et al, 2018). The electrostatic charge between the comparable charged particles prevents accumulation and so guarantees a scattered state of the nanosuspension in tests.

Release tests showed a decrease in the absorbance of punicalagin because the niosomes dragged the release of punicalagin slightly in the medium compared to its free state. The target of a long-time sustained drug release inside the body was achieved here.

The cytotoxicity (MTT) assay was done to ensure a safe dose was used and that the percentage of cell viability was more than 90%.

The cR sample showed higher number of cells in phase of G2/M, while the s.R sample showed a low number of cells in phase of G2/M. As for the H₂O₂/ HFB4 sample showed a higher value of cells in the phase of G2/M and a low number of cells in the G0/G1 phase, while the s + H₂O₂ sample showed a lower number of HFB4 cells in phase of G2/M and a high number of cells in G0/G1 phase. This proved the protection ability of punicalagin to cells versus the effects of UV radiation and H₂O₂ (Lee et al, 2017).

RT-PCR assay was carried out to determine the level of expression of 3 genes that are responsible for skin aging process and considered as markers for aging. The MMPs degrade collagen in the dermal layer and proteins in the extracellular matrix, so the upregulation of *MMP3* accompanied by the decrease in *CollA1* and *TimP3* compared to the control was shown in the cR and H₂O₂/ HFB4 samples. In s + H₂O₂ and s.R samples, there was an increase in both *CollA1* and *TimP3* and a decrease in *MMP3* compared to the other samples.

In the *TERT* assay it was observed that H₂O₂ and UV-irradiation affected telomerase activity and promoted telomerase shortening, which leads to cell senescence and skin aging, but the samples pretreated with punicalagin showed an increase in the concentration of human *TERT*.

Conclusion

There are many factors that increase the rate of skin aging by changing the balance between free radicals and antioxidants in the body. The solution to counteracting negative changes is to increase antioxidants. This study demonstrated the high antioxidant effect of punicalagin extracted from pomegranate and its loading into niosomes as a carrier.

Punicalagin protects HFB4 cells from cell growth arrest caused by UV radiation and H₂O₂, activates production of the *CollA1* and *TimP3* genes, maintains collagen level, and reduces *MMP3*. Punicalagin increases human *TERT* concentration. We conclude that punicalagin treatment efficiently protects cells from aging caused by oxidative stress.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

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