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Inhibition of melanin content by *Punicalagins* in the super fruit pomegranate (*Punica granatum*)

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Synopsis

Current efforts to develop effective skin lightening products through the inhibition of melanin production have focused on compounds that inhibit the function and activity of tyrosinase, the rate-limiting enzyme in the melanin biosynthesis pathway. Synthetic tyrosinase inhibitors, such as hydroquinone, kojic acid, and arbutin, have been reported to cause skin irritation or acute dermatitis, raising concerns about the safety of these compounds. As a result, there is a need for safe natural ingredients that show effective skin lightening. In this report, we have identified a natural ingredient, pomegranate fruit extract, that inhibits melanin production in melanocytes and shows potential for use as a cosmetic skin lightening agent. In addition, we have identified a polyphenolic compound, punicalagins, as the active melanin inhibitor in pomegranate fruit extract based on its capacity to directly inhibit melanin production.

INTRODUCTION

Skin pigmentation is primarily determined by the amount of melanin produced by melanocytes in the skin epidermal–dermal junction. Melanin biosynthesis is catalyzed by tyrosinase, the rate-limiting enzyme in the melanin biosynthetic pathway in melanocytes. Tyrosinase catalyzes two steps in melanin synthesis, the oxidation of L-tyrosine to L-dopa (L-3, 4-dihydroxyphenylalanine), and L-dopa to dopaquinone and its subsequent conversion to dopachrome, which autocatalyzes to a series of intermediate products to form the brown-black pigment, melanin (1). Skin pigmentation due to synthesis and dispersion of melanin in the epidermis is of great cosmetic significance. Lower amounts of melanin in the skin epidermis signify lighter skin, whereas higher amounts of melanin are found in darker skin.

Current efforts to develop effective skin lightening products have focused on agents that inhibit the function and activity of tyrosinase. Synthetic tyrosinase inhibitors, such as hydroquinone, kojic acid, and arbutin, have been shown to cause skin irritation or acute dermatitis raising concerns about the safety of these compounds (2,3). As a result, there

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is a need for safe natural ingredients that show effective skin lightening. Recently, there have been extensive reports in the literature that plant-derived polyphenols and tannins inhibit the catalytic activity of tyrosinase, thus providing a natural alternative to synthetic tyrosinase inhibitors (4).

Pomegranate (*Punica granatum*) extracts have been used in ancient folk medicines by numerous cultures (5,6). Extracts from different portions of the plant such as seeds, peels, and leaves have been reported to exhibit strong antimicrobial (7) and antioxidant activity (8). It has also been shown that ingestion of pomegranate juice significantly reduces the progression of atherosclerosis in hypercholesterolemic mice (9). Several of the health-promoting properties of pomegranate fruit have been attributed to its polyphenolic compounds and tannins. Prominent among them are the hydrolyzable ellagitannins that, upon hydrolysis, produce ellagic acid (10) a compound known to possess anti-inflammatory, antitumorigenic, antiproliferative, and antiapoptotic activities (11).

In recent years, ellagic acid has been shown to inhibit tyrosinase activity and melanin production (12). Topical application of ellagic acid to UV-induced hyperpigmented skin of guinea pigs and human subjects significantly reduced skin pigmentation (12–14) and oral dosing of ellagic acid to guinea pigs significantly lowered UV-induced skin hyperpigmentation (15). In addition, a study showing antioxidant, antiglycation, and tyrosinase inhibiting activities of a polysaccharide fraction from pomegranate has been reported (16). On the basis of this evidence, we examined the effects of pomegranate fruit extract and its predominant polyphenol, punicalagins (Fig. 1), on the inhibition of melanin production in melanocytes.

MATERIALS AND METHODS

REAGENTS

Gallic acid [97.5–102.5% (titration); Sigma-Aldrich, St. Louis, MO] and punicalagins (PhytoLab GmbH & Co. KG, Germany) were used as standards for phytochemical analysis.

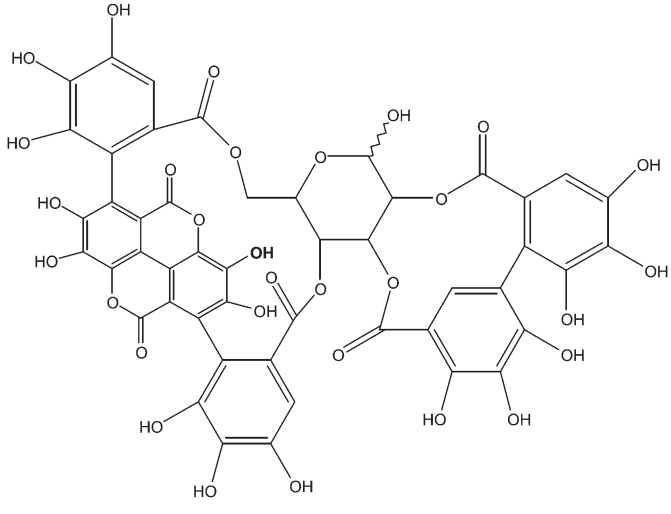

Figure 1. Chemical structure of punicalagins.

All solvents were HPLC grade from Fisher Scientific (Pittsburgh, PA). Cell culture media and reagents were from Life Technologies (Grand Island, NY), and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

PREPARATION OF POMEGRANATE EXTRACT

Fresh pomegranate fruit (*Punica granatum*) was harvested from our Nutrilite Farm (Lakeview, CA) in July 2008. The fruit (including peels) were macerated and extracted for 2 h at 60°C in a 75–80% (w/w) ethanol solution using a ratio of 1:4 (pomegranate : solvent). The extract was pressed, filtered, and the ethanol removed under vacuum. The aqueous extract was spray dried without carriers and stored under ambient conditions until used. The overall yield of extract was approximately 10%. The total punicalagins content and structure was confirmed by HPLC–MS, as reported earlier by our laboratory (17).

CELL CULTURE

Melan-a cells were purchased from Welcome Trust Functional Genomic Cell Bank, St. George Medical School, University of London, London (United Kingdom). Cells were grown in a 37°C, 10% CO_2 incubator in RPMI-1640 media supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% amphotericin B, 2 mM L-glutamine, and 200 nM tetradecanoyl phorbol acetate (TPA). Melan-a cells at passage 30 or less were used for the experiments (18).

MELANIN INHIBITION ASSAY

Melan-a cells were seeded at a density of 5×10^4 cells per well in 24-well tissue culture plates and grown overnight. The cells were treated, in triplicate, with the indicated concentrations of pomegranate extract. Phenylthiourea (60 µg/ml), a known tyrosinase inhibitor, was used as a positive control. All compounds were prepared in 70% dimethyl sulfoxide (DMSO) and diluted to respective concentrations with cell culture medium.

Melan-a cells were treated with compound for 4 days, refreshing the compound and supplemented media at the end of day 2. Following treatment, melanin was extracted from the cells as described by Ni-Komatsu. Briefly, the cell media were removed and the cells were lysed. The lysed cells were centrifuged, and the resulting pellet was washed with ethanol : ether (1:1) solution and then solubilized in 100 µl of 20% DMSO in 2 N NaOH. The melanin extract was transferred to a 96-well, clear-bottom plate, and total melanin content determined by reading absorbance at 490 nm on a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). All the samples were normalized to total protein content and reported as percent of melanin in untreated controls (19,21). The protein content in each sample was determined using the BCA protein assay kit (Pierce, Rockford, IL) as outlined by the manufacturer. An identical set of treated Melan-a cells were used to determine cell viability.

CELL VIABILITY ASSAY

Cell viability was determined, following treatment of Melan-a cells with pomegranate fruit extracts, using WST-1 assay reagent (Pierce, Rockford, IL) as outlined by the manufacturer.

Briefly, the cell media were replaced with phenol red-free RPMI-1640 media, WST-1 reagent was added, and the cells incubated for 4 h in a 37°C, 10% CO_2 incubator. The plate was read at 480 nm using a SpectraMax M5 microplate reader and cell viability was determined by measuring production of formazan dye by metabolically active cells. The results were analyzed and reported as percent of untreated controls.

HPLC ANALYSIS OF ELLAGIC ACID IN CELL MEDIA AND LYSATES

Melan-a cells treated with punicalagins as described earlier for the melanin inhibition assay and Melan-a cells treated for 48 h with 50 µg/ml ellagic acid were prepared for HPLC analysis of ellagic acid content. Cell media were subsampled and the cells lysed as described earlier. HPLC fractionation was achieved using an Agilent Technologies, Santa Clara, CA HP1100 System equipped with photodiode-array detection and a Waters 4 µm NovaPak column (250 × 4.6 mm). Samples were separated with a 0.2% phosphoric acid [v/v with deionized (DI) water] and acetonitrile (ACN) elution gradient as outlined in Table I. The column elution rate was held at 1 ml/min at ambient temperature with an injection volume of 10 µl for both samples and standards. The chromatogram was taken at 252 nm, integrated, and analyzed with Agilent Chemstation, a chromatography data system. An ellagic acid standard curve from 4 to 400 µg/ml ($r^2 > 0.99$) was used for this analysis. Ellagic acid standards were used as positive controls with a lower detectable limit of 11 µg/ml.

RESULTS AND DISCUSSION

To investigate the effect of pomegranate fruit extract on melanin production, we used an *in vitro* Melan-a melanocyte cell culture model. Melan-a melanocytes are derived from normal mouse embryonic skin; they are pigmented and dendritic, and retain almost all the characteristics of primary cells other than phenotypic variability and senescence. The cells have been shown to be an excellent model for screening antimelanogenic inhibitors for basal skin lightening products (19). Treatment of Melan-a cells with pomegranate fruit extract, standardized to 20% punicalagins, resulted in inhibition of total melanin production. Melanin content was reduced by approximately 40% and 60% at extract

Table I
HPLC Column Elution Gradient for Ellagic Acid

	Gradient Mobile Phase	
Time (Minutes)	0.2% Phosphoric Acid (v/v DI Water) (%)	Acetonitrile (%)
0.0	92	8
12.0	90	10
14.0	88	12
26.0	78	22
29.0	78	22
29.5	92	8
32.0	92	8

concentrations of 50 µg/ml and 100 µg/ml, respectively (Fig. 2). Pomegranate fruit extract had no effect on Melan-a cell viability at any of the concentrations tested (Fig. 3).

Because punicalagins are the major fraction of the polyphenols and tannins present in pomegranate fruit extract, we next looked at the effects of punicalagins on melanin production in Melan-a cells. We tested purified punicalagins (Fig. 1) isolated from pomegranate fruit in the melanin inhibition assay. Punicalagins treatment of Melan-a cells reduced melanin production by approximately 60%, 70%, and 75% of control levels at 20 µg/ml, 60 µg/ml, and 100 µg/ml, respectively (Fig. 4). The reduction of melanin with 20 µg/ml punicalagins is comparable to the inhibition of melanin content by 100 µg/ml pomegranate fruit extract, standardized to 20% punicalagins, indicating that punicalagins are the active melanin biosynthesis inhibitor in pomegranate fruit extract.

Currently, the mechanism of inhibition of melanin production by punicalagins is unclear. Studies of pomegranate punicalagins in oral, colon, and prostate cancer cell lines indicate that punicalagins are not directly absorbed by humans and are instead hydrolyzed to ellagic

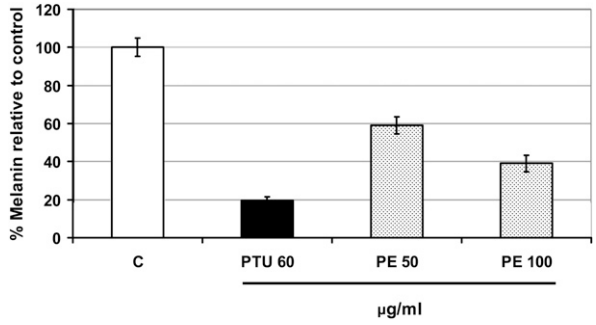

Figure 2. Effect of pomegranate fruit extract (PE), standardized to 20% punicalagins, on melanin formation in Melan-a cells treated with 50 and 100 µg/ml pomegranate fruit extract. Melan-a cells were treated with 60 µg/ml phenylthiourea (PTU), a known tyrosinase inhibitor, as a positive control. Results are reported relative to untreated control cells (C). The data shown is representation of two different experiments conducted under the same conditions. Each column represents the mean ± SD, with n = 3.

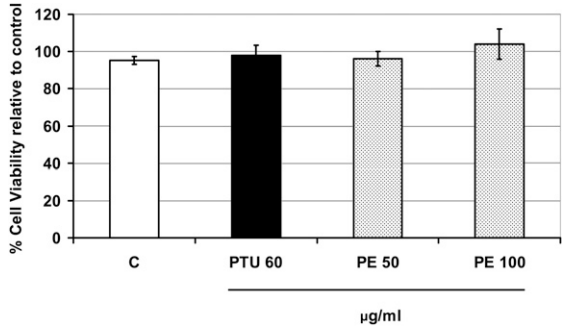

Figure 3. Effect of pomegranate fruit extract, standardized to 20% punicalagins, on cell viability of Melan-a cells treated with 50 and 100 µg/ml pomegranate fruit extract (PE), and 60 µg/ml phenylthiourea (PTU), a tyrosinase inhibitor. Results are reported relative to untreated control cells (C). The data shown are representations of two different experiments conducted under the exact same conditions. Each column represents the mean ± SD, with n = 3.

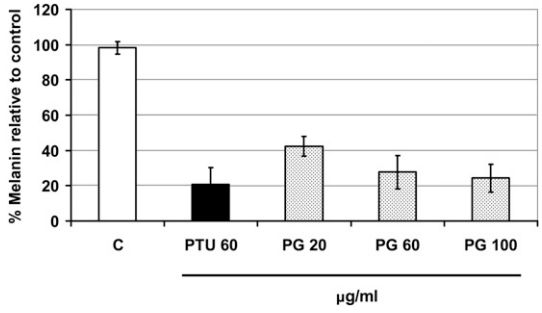

Figure 4. Effect of punicalagins on melanin formation in Melan-a cells treated with 20, 60 and 100 µg/ml punicalagins (PG), and 60 µg/ml phenylthiourea (PTU), a known tyrosinase inhibitor, as a positive control. Results are reported relative to untreated control cells (C). The data shown are representations of two different experiments conducted under the same conditions. Each column represents the mean ± SD, with n = 3.

acid in the gastrointestinal tract and then converted to 3,8-dihydroxy-6H-dibenzo(b, d) pyran-6-one (Urolithin A) in the colon to become bioavailable (20). In colon cancer cells, punicalagins are hydrolyzed to ellagic acid at physiological pH (10), which is consistent with reports that polyphenols are unstable and tend to be metabolized at physiological pH (16).

Because ellagic acid has been established as a potent inhibitor of tyrosinase, the rate-limiting enzyme in the melanin biosynthesis pathway, and therefore, melanin synthesis (12,15), we attempted to measure ellagic acid production in punicalagins treated Melan-a cell cell lysates. HPLC analysis of Melan-a cell lysates at 48 h after punicalagins treatment found no detectable levels of ellagic acid (Fig. 5). HPLC analysis of ellagic acid treated Melan-a cell lysates showed that all of the ellagic acid was detected in both the cell culture medium (Fig. 6B) and cell lysates (Fig. 6C) after 48 h of treatment. Because the ellagic acid levels were unchanged and punicalagins were not hydrolyzed to ellagic

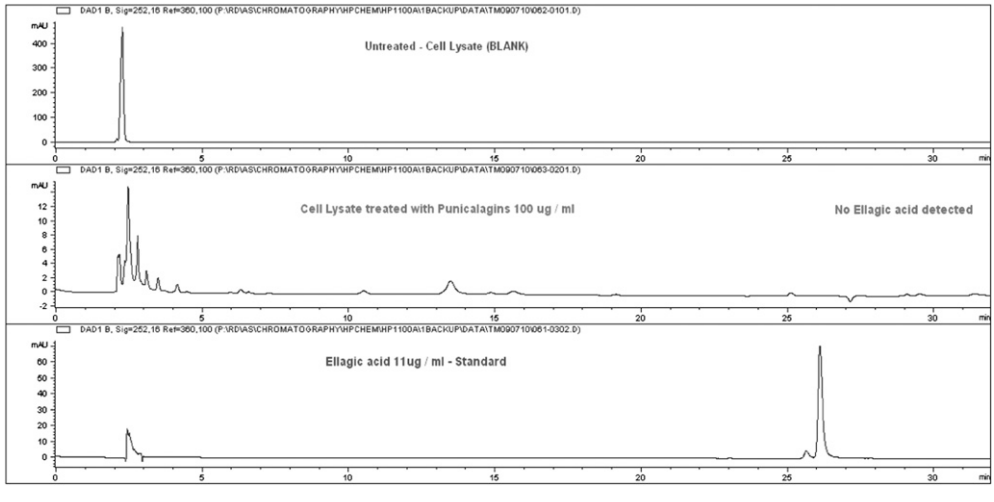

Figure 5. Analysis of ellagic acid levels in Melan-a cell cultures treated with punicalagins. Melan-a cells were treated with punicalagins, and cell lysates were prepared and analyzed by HPLC for ellagic acid. Ellagic acid was undetectable in the untreated controls and punicalagins treated (100 µg/ml) Melan-a cells. Pure ellagic acid (11 µg/ml) was used as a positive control.

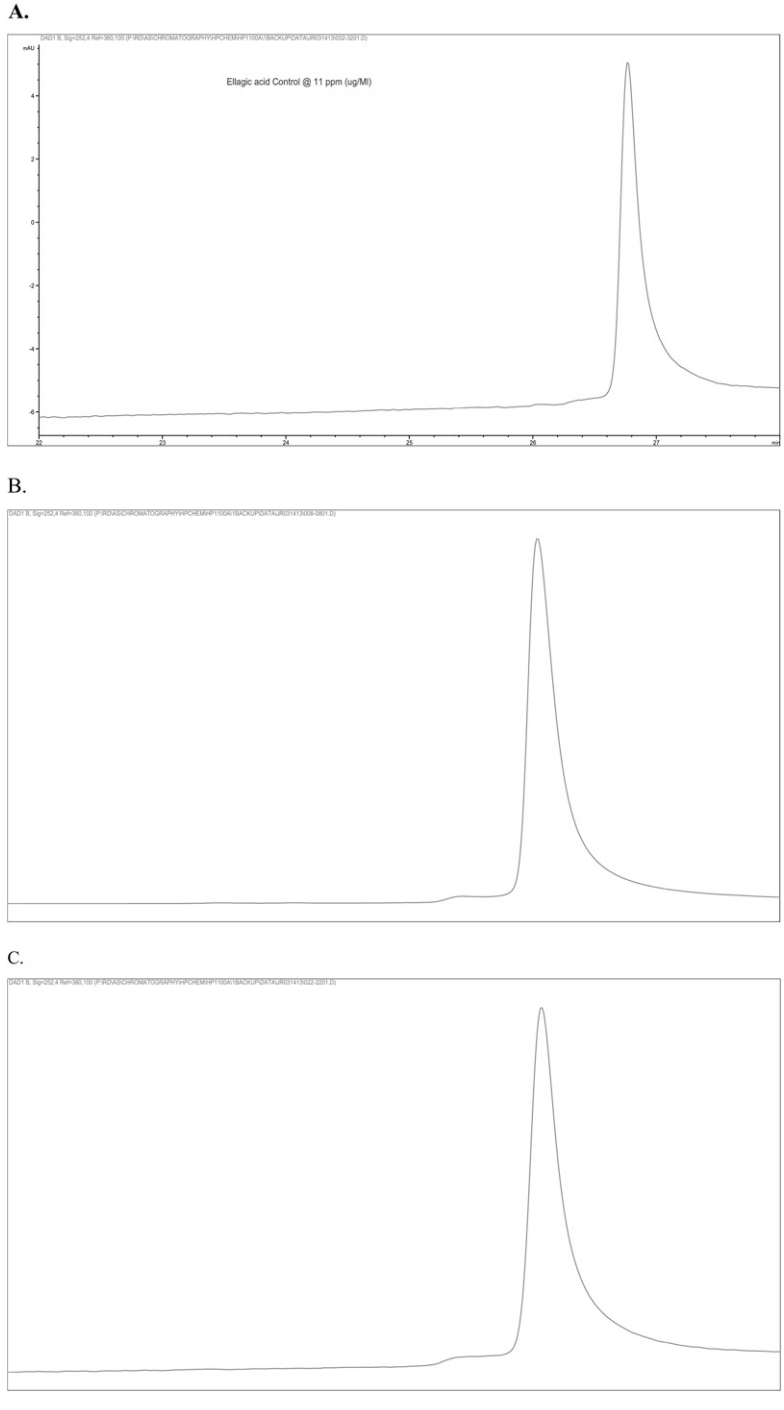

Figure 6. Analysis of ellagic acid levels in Melan-a cell cultures treated with ellagic acid. The HPLC was calibrated for ellagic acid with a detection limit set at 11 μg/ml (A). Melan-a cells were treated with 50 μg/ml ellagic acid for 48 h and the culture medium (B) and cell lysates (C) were analyzed for ellagic acid content by HPLC.

acid, our data indicate that punicalagins act directly on melanocytes to inhibit melanin content. These results are further supported by reports from our laboratory that punicalagins, unlike ellagic acid, do not inhibit tyrosinase activity in Melan-a cell melanocytes (17). In addition, we have also found that a combination of pomegranate extract standardized to 20% punicalagins and Siberian larch standardized to 80% taxifolin synergistically reduced melanin content more than pomegranate extract or Siberian larch alone (25,26).

Most inhibitors of melanogenesis (e.g., arbutin, kojic acid, licorice extract, and ascorbic acid) are potent antioxidants and show anti-inflammatory activity (22). Consistent with the antioxidant and anti-inflammatory activity of most skin lightening ingredients, punicalagins have been reported to inhibit oxidative stress and activity of the proinflammatory transcription factor NFκb (23). Furthermore, because of its solubility and stability in aqueous medium and its high bioavailability characteristics, punicalagins are well suited for cosmetic formulation (24).

In recent years, many publications have reported *in vitro* and *in vivo* skin lightening efficacy of ellagic acid rich pomegranate extracts. In a double-blind placebo-controlled study with formulations containing synthetic ellagic acid and another with pomegranate extract containing natural ellagic acid, the authors found that topical application of synthetic or natural ellagic acid were comparable in skin lightening efficacy (13). In another study, topical application of pomegranate juice extract mitigated melanin levels and erythema index in female subjects (14). Furthermore, it has been shown that oral intake of pomegranate extract rich in ellagic acid ameliorates skin pigmentation induced by UV irradiation (15). On the basis of these reports of *in vivo* and *in vitro* skin lightening efficacy of pomegranate extracts containing ellagic acid, our findings suggest that the melanin-inhibiting function of punicalagins and pomegranate extract (standardized to 20% punicalagins) could provide skin lightening benefits.

CONCLUSIONS

These results show that pomegranate fruit extract with 20% punicalagins directly reduce melanin content in Melan-a cells and is noncytotoxic at the effective concentrations. Our data combined with the recent reports on the *in vivo* effects of pomegranate extract suggest that pomegranate punicalagins are an attractive candidate for topical skin lightening application.

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