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Photoprotection by *Punica granatum* seed oil nanoemulsion entrapping polyphenol-rich ethyl acetate fraction against UVB-induced DNA damage in human keratinocyte (HaCaT) cell line

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ABSTRACT

There has been an increase in the use of botanicals as skin photoprotective agents. Pomegranate (*Punica granatum* L.) is well known for its high concentration of polyphenolic compounds and for its antioxidant and anti-inflammatory properties. The aim of this study was to analyze the photoprotection provided by *P. granatum* seed oil nanoemulsion entrapping the polyphenol-rich ethyl acetate fraction against UVB-induced DNA damage in the keratinocyte HaCaT cell line. For this purpose, HaCaT cells were pretreated for 1 h with nanoemulsions in a serum-free medium and then irradiated with UVB (90–200 mJ/cm^2) rays. Fluorescence microscopy analysis provided information about the cellular internalization of the nanodroplets. We also determined the *in vitro* SPF of the nanoemulsions and evaluated their phototoxicity using the 3T3 Neutral Red Uptake Phototoxicity Test. The nanoemulsions were able to protect the cells' DNA against UVB-induced damage in a concentration dependent manner. Nanodroplets were internalized by the cells but a higher proportion was detected along the cell membrane. The SPF obtained (~25) depended on the concentration of the ethyl acetate fraction and pomegranate seed oil in the nanoemulsion. The photoprotective formulations were classified as non-phototoxic. In conclusion, nanoemulsions entrapping the polyphenol-rich ethyl acetate fraction show potential for use as a sunscreen product.

1. Introduction

Ultraviolet radiation (UVR) is an important exogenous factor in skin pathogenesis and can lead to the development of a number of skin disorders including sunburn, immunosuppression, carcinogenesis, and photoaging. UVR can be divided into three types: ultraviolet C (UVC — from 200 to 290 nm); ultraviolet B (UVB — from 290 to 320 nm) and ultraviolet A (UVA — from 320 to 400 nm) [1]. UVC radiation is filtered by the ozone layer before reaching the Earth. UVA is the radiation most responsible for photoaging; it penetrates deep into the epidermis and dermis of the skin and as it is barely able to excite the DNA molecule directly, it is assumed that much of its mutagenic and carcinogenic action is mediated through oxidative stress [1]. UVB radiation (290–320 nm) is responsible for damage due to sunburn (erythema and edema) and, induction of oxidative stress, and is highly genotoxic agent. Direct absorption of UVB photons leads to disruption of DNA, with cyclobutane-pyrimidine dimers (CPD) and pyrimidine-pyrimidone (6-4) photoproducts as a result, which, if remains unrepaired, can initiate photocarcinogenesis. It is less penetrating than UVA, mostly only reaches the epidermal basal cell layer of the skin and thus affects mainly epidermal cells, possibly altering the proliferation, differentiation and metabolism of these cells [2–4].

Thus, protection of the skin against excessive sunlight exposure is essential to forestall damage. Exogenous application of protective dermatological preparations containing sunscreens (organic and/or inorganic filters) is commonly recommended. In this regard, naturally occurring plant products have also been investigated and play a role in a broad range of physiological processes including protection against harmful UVR. Due to their sunscreen effect and, potent antioxidant, anti-inflammatory and immunomodulatory properties, polyphenols are among the most promising group of compounds that can be exploited as chemopreventive agents for a variety of skin disorders [1,3].

Punica granatum (pomegranate) is an ancient fruit, considered as "a pharmacy unto itself" with enormous health benefits [5–7]. The main compounds responsible for most of its functional properties are phenolic compounds. They can be found in substantial quantities and in different parts of the fruit (bark, flower, leaves, and arils) but are much more concentrated in the peel and juice. The peel is

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rich in hydrolysable tannins, mainly punicalin, peduncalagin and punicalagin; hydroxybenzoic acids such as gallic acid and ellagic acid; anthocyanidins and flavonoids. They account for 92% of the antioxidant activity associated with the fruit [8]. The pomegranate seed oil contains phytosterols, tocopherols and a unique fatty acid mixture, mainly consisting of punicic acid (50–70%), which is considered to be one of the strongest natural antioxidants [9].

We recently developed pomegranate seed oil nanoemulsions (PSO-NE) and medium chain triglyceride nanoemulsions (MCT-NE), both of them entrapping a pomegranate peel polyphenol-rich ethyl acetate fraction (EAF), for topical administration, and evaluated their antioxidant activity using *in vitro* methods [10]. Nanoemulsions (NE) present a large surface area and with low surface tension of the oil droplets, which could help increase the permeation of the incorporated polyphenol compounds through the skin, enhancing their topical effect [11–13]. In another study erythrocyte-based assays were employed to determine whether if EAF-loaded NE could protect the membrane lipid bilayer against the oxidative stress induced by oxidant agents (erythrocytes are well known as a biomembrane model that mimics a cellular environment), and to determine whether nanoemulsion component (mainly surfactants) could damage the cell membrane and lead to hemolysis [14].

The main purpose of this study was to investigate whether the free EAF and EAF-loaded nanoemulsions have photoprotective effect against DNA damage induced by UVB irradiation, of monolayer cultures of human keratinocyte HaCaT, and to determine the cytotoxicity and phototoxicity of the formulations. Finally, cell internalization studies were conducted to predict the possible localization of the nanoemulsion when in contact with cells.

2. Materials and Methods

2.1. Materials

Polysorbate (Tween 80®), triethanolamine, dimethyl sulfoxide (DMSO), 2,5 diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT), neutral red dye, Nile red (NR), calcein and 4,6 diamino-2-phelylindole dihydrochloride hydrate (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). NaCl, Na_2HPO_4 and KH_2PO_4 were purchased from Merck (Darmstadt, Germany). Sodium acetate, ethyl acetate, dichloromethane, chloride acid, and ethanol were obtained from Vetec® (Rio de Janeiro, Brazil). Pomegranate seed oil and pomegranate fruit peel dry extract were purchased from Via Farma (São Paulo, Brazil). Soy lecithin (Lipoid® S100) was from Lipoid AG (Steinhausen, Switzerland). Medium chain triglyceride was from Brasquim (Porto Alegre, Brazil) and water was purified in a Milli-Q system (Millipore, Bedford, MA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine solution (200 mM), trypsin–EDTA solution (170,000 U/L trypsin and 0.2 g/L EDTA) and penicillin–streptomycin solution (10,000 U/mL penicillin and 10 mg/mL streptomycin) were obtained from Lonza (Verviers, Belgium). The 75 cm^2 flasks, 96-well and 24-well plates were obtained from TPP (Trasadingen, Switzerland).

2.2. Methods

2.2.1. Ethyl Acetate Fraction (EAF)

The polyphenol-rich ethyl acetate fraction (EAF) from *P. granatum* peel extract was obtained following the method described by [15] with some modifications. Briefly, the *P. granatum* fruit peel dry extract was commercially bought and then extracted for 24 h by dynamic maceration with methanol containing 10% (v/v) water. The obtained extract was dried *in vacuo* and then suspended in 2% aqueous acetic acid. The suspended extract was partitioned with dichloromethane and ethyl acetate. After that, the pooled ethyl acetate fractions were evaporated to dryness *in vacuo*.

2.2.2. Preparation of Nanoemulsions

EAF-loaded pomegranate seed oil nanoemulsions (EAF-PSO-NE) were prepared using an ultrasonic emulsification method followed by solvent evaporation [16]. Briefly, the ethyl acetate fraction (EAF) (0.5%; w/v), soy lecithin (0.4%; w/v) and PSO (2%; w/v) were dissolved in 10 mL of ethyl acetate. This ethyl acetate solution was slowly poured into 40 mL of a polysorbate 80 (2.1%; w/v) aqueous solution, which was then adjusted to pH 5.0–6.5 with triethanolamine. The oil in water dispersion was sonicated for 3 min using an Ultrasonic Processor UP200S (Hielscher, Germany), and kept under magnetic stirring for 24 h. The resulting nanoemulsion was evaporated under reduced pressure to a volume of 15 mL.

EAF-loaded medium chain triglyceride nanoemulsions (EAF-MCT-NE) were prepared using the spontaneous emulsification method [17]. For this, 10 mL of an ethanolic solution containing EAF (0.5%; w/v), soy lecithin (0.4%; w/V), and MCT (1.8%, w/v) was poured into a 2.1% (w/v) polysorbate 80 aqueous solution and adjusted to pH 5.0–6.5 with triethanolamine, under magnetic stirring. The NE were then evaporated under reduced pressure to eliminate the organic solvent and concentrated to a volume of 15 mL. All formulations were filtered through 8 μm quantitative filter paper. Unloaded PSO-NE and MCT-NE were prepared in the same manner.

2.2.3. Droplet Size and Zeta Potential

Droplet size and zeta potential were analyzed by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, UK) at 25 °C and a detection angle of 173°. Before measurement unloaded and EAF-loaded NE were appropriately diluted in ultrapurified water, or cell culture medium with 5% (v/v) FBS. Readings were taken immediately after preparation ($t = 0$ h) and after a 24 h incubation at 37 °C ($t = 24$ h). Each measurement was performed using at least three sets of a minimum of 10 runs.

2.2.4. Culture of HaCaT and 3T3 Cell Line

The spontaneously immortalized human keratinocyte cell line HaCaT and the murine Swiss albino 3T3 fibroblast cell line were grown in DMEM medium (4.5 g L^{-1} glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}) at 37 °C, 5% CO_2. Both cell lines were routinely cultured in 75 cm^2 culture flasks and trypsinized using trypsin/EDTA when the cells reached approximately 80% confluence. All cell lines were obtained from Eucellbank (Universitat de Barcelona, Spain).

2.2.5. Cytotoxicity Assays

The cytotoxic effect of the free EAF, unloaded and EAF-loaded NE was measured by tetrazolium salt MTT assay [18] and neutral red uptake (NRU) assay [19]. 3T3 and HaCaT cells were seeded into the central 60 wells of a 96-well plate at a density of 8.5×10^4 cells mL^{-1} and 1×10^5 cells mL^{-1}, respectively. After incubation for 24 h under 5% CO_2 at 37 °C, the spent medium was replaced with 100 μL of fresh medium supplemented with 5% FBS containing free EAF, unloaded or EAF-loaded NE at the required concentration (7.8–500 μg mL^{-1}). After 24 h, the surfactant-containing medium was removed, and 100 μL of MTT in PBS (5 mg mL^{-1}) diluted 1:10 in medium without FBS and phenol red was then added to the cells. Similarly, 100 μL of 50 μg mL^{-1} NR solution in DMEM without FBS and phenol red was added to each well for the NRU assay. The plates were further incubated for 3 h, after which the medium was removed, and the cells were washed once in PBS. Thereafter, 100 μL of DMSO was added to each well to dissolve the purple formazan product (MTT assay) and for the NRU assay, 100 μL of a solution containing 50% absolute ethanol and 1% acetic acid in distilled water was added to extract the dye. After 10 min on a microtiter plate shaker at room temperature, the absorbance of the