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Anti-photoaging potential of propolis extract in UVB-irradiated human dermal fibroblasts through increasing the expression of FOXO3A and NGF genes

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ABSTRACT

Propolis is a resinous compound that has been widely used in folk medicine. Different biological activities and therapeutic applications of propolis have been studied before. However, the effects of propolis on longevity-associated genes expression in the prevention of skin photoaging still remained unclear. Therefore in this study the protective effects of propolis on the expressions of two longevity-associated genes, FOXO3A and NGF genes, against UVB-induced photoaging in human dermal fibroblasts (HDF) were investigated. Propolis extract demonstrated a concentration-dependent free radical scavenging activity that was determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. Also, Folin-Ciocalteu method was used to measure the total phenolic content of the extract. The viability of HDF cells was decreased gradually with increasing UVB radiation doses and 248 mJ/cm^2 was selected as the sub-cytotoxic dose. Pre-treatment with propolis extract increased the viability of UVB-irradiated human dermal fibroblasts and decreased the number of β-galactosidase positive cells as senescent cells among them. It also increased the expression of FOXO3A and NGF genes in irradiated and non-irradiated cells. Consequently, these findings suggest that propolis extract has anti-photoaging potential and this property, in addition to its strong antioxidant activity, may be due to its effects on upregulation of longevity-associated genes.

1. Introduction

Skin is the largest organ with various physiological functions and has a great impact on social relations among individuals. One of the noteworthy factors in dermatological researches and industry is aging that make diverse changes in the skin [1]. Injury intolerance and normal function decline occur during aging. Two mechanisms that lead to aging are telomere shortening during cell cycle and cumulative external damages like UV radiation and free radicals. Both mechanisms are involved in skin aging and have the same final pathway [2]. In the extrinsic origin mechanism, UV radiation has significant role in skin aging especially in facial skin. Frequent exposures to UV radiation from sun or tanning beds cause specific pathophysiological alternations in the skin namely photoaging. These alternations may occur before or along with genetically based aging and can intensify skin senescence signs [3]. Deep wrinkle, roughness, irregular pigmentations and shallowness are some visual markers in sun-exposed areas of skin due to photoaging [4]. UV radiation has destructive influences on cellular membrane, DNA and protein especially extracellular matrix proteins in dermal connective tissues [5,6]. UVB (280–320 nm) affects fibroblasts and extracellular matrix located mainly in upper dermis through interaction with chromophores and photosensitizers. It also activates cytoplasmic signaling pathways related to growth, differentiation and connective tissue degradation. Previous experiments have shown that subcytotoxic doses of UVB may change gene expression profiles and lead to premature senescence in human dermal fibroblasts (HDFs) [7,8].

Forkhead box O (FOXO) proteins are conserved transcription factors that have four members: FOXO1, FOXO3A, FOXO4 and FOXO6. Extracellular stimuli result in post-translational modifications and translocation of these transcription factors between cytoplasm and nucleus. Hence, extracellular stimuli regulate FOXO-dependent genes expressions [9]. FOXO-dependent downstream genes contribute to proliferation, apoptosis, metabolism, immunity, inflammation and stress resistance. FOXO3A, in

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particular, plays an important role in cellular longevity and prevention of age related diseases via reduction of oxidative stress and upregulation of genes involved in cellular metabolism, cell cycle arrest and apoptosis [10,11]. In human dermal fibroblasts, FOXO3A expression is inversely related to cellular senescence in a conserved mechanism [12].

Nerve growth factor (NGF) as a member of neurotrophin (NT) family, has biological effects on neuronal and non-neuronal cells including endocrine, immune and skin cells [13,14]. Skin cells that express NGF include keratinocytes, mast cells, dermal fibroblasts and myofibroblasts [15]. NGF participates in skin inflammation and tissue repair [16]. It stimulates fibroblast migration [14] and thereby accelerates skin wound healing [17]. A previous study has reported that NGF expression in the skin decreases in association with aging [18].

Recent laboratory data have indicated that phenolic acids, flavonoids and polyphenols of plant origin can protect skin against harmful damages of UV radiation and have efficient antiaging power. Accordingly, particular attention has been paid to botanical compounds because of their photoprotective features and little side effects [19–21]. Propolis as a resinous material is collected by *Apis mellifera*. The word "propolis" is originally a Greek word that is made up of pro meaning "in front of" or "at the entrance to" and polis meaning "community" or "city". This word refers to a compound that is used to defend the beehive. The origin of this material is from secretions and wound exudates of plant buds and trees such as birch, poplar, pine, alder, *etc*. The primary purpose of the bees to produce propolis is to maintain the structure of their hive and eliminate its defects like cracks and crevices. Other applications of propolis include using as thermal insulation, embalming the carcass of killed invaders and preventing biological contamination inside the hive. It mainly consists of flavonoids, phenols and aromatic compounds; however, its chemical composition may vary according to the diversity of plants, season and collector bees. Propolis has different pharmacological activities such as antibacterial, antiviral, antifungal, antioxidative and anti-inflammatory properties [22,23]. It has been shown that propolis has UV-protection impact via its antioxidant effect [24] and prevents GSH depletion in the skin [25]. In addition, it reduces apoptosis and DNA damage of UVB-exposed skin cells [26].

So far among broad therapeutic applications of propolis that have been studied [27], photoprotection properties are taken into consideration. Recent studies have reported that propolis UV-protection is due to its antioxidant effect. However, the effects of propolis extract on UVB-induced photoaging through the evaluation of the longevity associated genes expressions in human dermal fibroblast have not been investigated yet. Therefore in this study the ani-photoaging effects of propolis extract in human dermal fibroblast were studied by evaluating FOXO3A and NGF genes expressions.

2. Material and methods

2.1. Preparation of ethanol extract of propolis (EEP)

Pure raw propolis was obtained from the beehives of Maragheh, East Azarbaijan, Iran. The color of propolis was dark brown and its consistency was hard. To prevent unwanted oxidation, it was kept in a dark closed container at −20 °C. Freezed propolis was cut into small pieces and powdered in a grinder. The powder and 70% (v/v) ethanol (Merck, Germany) was mixed with a ratio of 1:10 (w/v) in an ultrasonic bath for 1 h at room temperature. After overnight incubation in the dark, the suspension was filtered through a Whatman filter paper No. 4 (Millipore, USA) and concentrated in a rotary evaporator (Laboratory 4000, Heidolph, Kelheirn, Germany) under reduced pressure at 40 °C. Then, the filtrate was lyophilized and preserved in a dark vessel at −20 °C until used.

2.2. Measuring 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

The principle of DPPH free radical scavenging method as an antioxidant assay is based on reduction of deep violet color DPPH solution to a pale yellow one by antioxidant molecules. Free radical scavenging activity of EEP was measured based on the protocol of Sulaiman et al. [28] with slight modifications. Hence, serial dilutions of EEP in absolute ethanol (ranging from 10 to 200 μg/ml) were mixed 1:1 (v/v) with DPPH solution (60 μM) and placed in a 96-well microplate (Jet Biofil, Guangzhou, China) in a final volume of 200 μl in each well. The plate was incubated for 30 min at room temperature in the dark. Then, the absorbance was read at 517 nm using ELISA reader (BioTek, Powerwave XS2, Vermont, USA). Ascorbic acid was used as antioxidant standard. The control reaction contained equal volumes of ethanol and DPPH solution and 200 μl of ethanol was used as blank. Each test was performed in triplicate. The scavenging activity of the samples was determined according to the following equation:

$$\%/\text{DPPH radical scavenging activity} = ((OD\ of\ control - OD\ of\ blank) - (OD\ of\ sample - OD\ of\ blank))/(OD\ of\ control - OD\ of\ blank) \times 100 \quad (1)$$

2.3. Determination of total phenolic content of ethanol extract of propolis (EEP)

Total phenolic content of EEP was measured according to the Folin-Ciocalteu method described by Wang et al. [29] with minor modifications. The 1 mg/ml concentration of EEP in absolute ethanol (Merck, Germany) was prepared. Then, 20 μl of this concentration was mixed with 100 μl of 0.2 N Folin-Ciocalteu's reagent (Merck, Germany). After 5 min incubation at room temperature, 80 μl of sodium carbonate (75 mg/ml) was added to the mixture and incubated an additional 1 h at room temperature. The absorbance was measured at 735 nm using a microplate spectrophotometer (BioTek, Powerwave XS2, Vermont, USA) against ethanol as a blank. Gallic acid was used as a standard (150–400 μg/ml) to generate a calibration curve. The phenolic content was calibrated using the linear equation based on the calibration curve. Each experiment was performed in triplicate and the total phenolic content was expressed as mg of gallic acid equivalent (GAE)/g EEP.

2.4. Cell culture

Human dermal fibroblasts (NCBI C646, National cell bank of Iran, Pasteur Institute of Iran) were grown as monolayer cultures in DMEM medium (Caisson, North Logan, UT, USA), supplemented with 10% (v/v) FBS (Gibco, Grand Island, NY) plus 1% (v/v) PenStrep (Gibco, Grand Island, NY) at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. The medium was exchanged every 2–3 days until cells were confluent. The cells were used between passages 3 and 6 in all experiments.

2.5. Ethanol extract of propolis (EEP) treatment and UVB irradiation

Ethanol extract of propolis (EEP) was dissolved in dimethyl sulfoxide (DMSO) (Merck, Germany) and diluted in culture medium followed by filtration through 0.22-μm filters (30 mm, MCE filter; Jet Biofil, Guangzhou, China). Afterwards, serial dilutions of stock were prepared so that the final concentration