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Populus nigra (Salicaceae) absolute rich in phenolic acids, phenylpropanoïds and flavonoids as a new potent tyrosinase inhibitor

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Chemical compounds:
L-DOPA
L-Tyrosine
Kojic acid
1,1-Diphenyl-2-picrylhydrazyl radical
1-(2,6-Dihydroxy-4-methoxyphenyl)-3-phenyl-2-propenone
Pinocembrin

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B16F10 murine melanoma cells culture
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ABSTRACT

The purpose of this study was to evaluate the tyrosinase inhibitory capacity of *Populus nigra* buds absolute (PBA) and compare it to kojic acid (KA), controversial reference tyrosinase inhibitor. *Populus nigra* buds were extracted with hexane and ethanol to obtain PBA. The inhibitory effect of this absolute was first tested on the mushroom *Agaricus bisporus* tyrosinase. Then the depigmenting potential of PBA was tested on B16F10 murine melanocytes by assaying the activity of tyrosinase and melanin content. Consecutively, a microscopic analysis of intracellular melanin granules was performed. Finally, melanised reconstructed human epidermis (RHE) were used to assess the lightening potential activity of this PBA on human skin. Results show that PBA inhibits *A. bisporus* tyrosinase (IC_{50} = 77 ± 8 ppm) and inhibits melanocytes B16F10 tyrosinase (IC_{50} = 27 ± 1 ppm). PBA decreases intracellular melanin levels, with 50% loss at 39 ± 9 ppm. Finally, PBA at 1000 ppm lightens RHE and decreases their melanin content of 20%. PBA is a strong inhibitor of tyrosinase and reduces melanogenesis in melanocytes B16F10. Thus, PBA has potential applications in skin-lightening cosmetics.

1. Introduction

Skin pigmentation results from the quantity and distribution of melanin pigment. In melanocytes, melanin synthesis is caused by exposure to ultraviolet (UV) radiation, or by α-melanocyte stimulating hormone (αMSH) [1]. Melanin is a major defense mechanism against ultraviolet light and plays an important role in the prevention of sun-induced skin damages. Tyrosinase (L-tyrosine, L-DOPA, oxygen oxidoreductase, EC 1.14.18.1) is the key enzyme in the melanogenesis pathway, also involved in the browning of fruits and vegetables [2]. This copper-dependent protein catalyzes two different reactions, both utilizing molecular oxygen: the oxidation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) by monophenolase activity and the oxidation of L-DOPA to DOPAquinone by catecholase activity [3].

A sharp increase of melanin in the epidermis leads to numerous hyperpigmentary disorders such as lentigo, chloasma, and postinflammatory pigmentation [4–6]. These hyperpigmentations can be anaesthetic and motivate some people to use whitening products. For instance, Caucasian women looking to eradicate or delay the development of aging spots (solar lentigo). Moreover in Africa and Asia, the use of depigmenting products is common for "aesthetic" reasons supported by fashion and social phenomena. This booming market leads to numerous studies to find skin-whitening agents in order to prevent or cure these hyperpigmentary disorders.

Many compounds have been demonstrated to show inhibitory effects on melanogenesis through inhibition of the enzymatic activity of tyrosinase: hydroquinone, ascorbic acid derivatives, kojic acid (KA), azelaic acid, corticosteroids, retinoids, arbutin [2,4,7–10]. However, most of these compounds are prohibited in oil based cosmetics and skin-care products [8]. In addition, long term effects of these chemicals on skin are not optimal and may cause serious skin damages. Thus, people seek natural and harmless compounds. Since plants are a major source of bioactive molecules, we are looking for essential oils or other plant extracts which inhibit melanogenesis.

In this study, we showed that PBA is a good tyrosinase inhibitor and we compared its activity with the reference inhibitor, KA. The inhibitory action of PBA on mushroom tyrosinase was determined first. Then, the

Abbreviations: KA, kojic acid; PBA, poplar (*Populus nigra*) buds absolute; RHE, reconstructed human epidermidis; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical.

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established murine B16F10 melanoma cell line which provides a model system with easily quantifiable markers of melanogenesis was used. We also examined the inhibitory effect of PBA on melanin biosynthesis in B16 cells. We checked PBA cytotoxicity. Then, we confirmed the melanogenesis inhibition by PBA on reconstructed human epidermis (RHE).

2. Materials and methods

2.1. Populus nigra buds absolute

In accordance with ISO 9001 norms, heating 150 kg of *Poplus nigra* buds with volatile and non-aqueous solvent hexane gives 16.5 kg of concrete. The concrete that is extracted with ethanol, cooled and filtered to remove waxes, gives the *Poplus nigra* buds absolute with a yield of 85%, after removal of ethanol by distillation.

2.2. Gas chromatography–mass spectrometry (GC–MS) analysis

The chemical composition of the volatile part of PBA, from Robertet SA (Grasse, France), was investigated by gas chromatography–mass spectrometry (GC–MS).

GC–MS analysis was carried out on a 100% polydimethyl-siloxane column, 30 m × 0.25 mm external diameter × 0.25 μm. Injection conditions were: injection mode: split/split ratio: 1/100; injection volume: 1 μl; temperature of injector: 240 °C. Carrier gas used was helium with initial flow rate 1.2 ml min^{-1} in constant linear velocity mode. Temperature program used was: from 50 °C (2 min) to 300 °C (20 min) at 4 °C min^{-1}. We used a quadrupole mass analyzer (QMS) with a mass range of 35–350 *m/z* (mass-to-charge ratio) suitable to cover the full fragmentation pattern of all analytes investigated in full scan mode.

2.3. Cell culture

2.3.1. B16F10 melanocytes

B16F10, from Naval Biosciences Laboratory of University of California (Berkeley, USA), is an immortalised cell line derived from mouse skin melanosomes. These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) Glutamax (Gibco) with 10% fetal bovine serum, at 37 °C in a humidified atmosphere containing 5% CO_2. Trypsin/EDTA 0.04% in Phosphate Buffer Saline (PBS) was used to re-suspend adherent cells. B16 cells were cultured in 12-well plates at a density of 25.000 cells cm^{-2}. Two days after plating, B16 cells were incubated for 24 h with αMSH 100 nM and various concentrations (0–200 ppm) of the PBA.

2.3.2. Reconstructed human epidermis (RHE)

RHE, from Sterlab SA (Vallauris, France), are pigmented epidermis cultivated for 10 days from human keratinocytes and melanocytes of phototype VI derived from prepuce. They are reconstituted by airlifted culture on insert polycarbonate filter. RHE were cultured on Sterlab proprietary growth medium.

Immediately upon receipt (day 10), RHE were transferred aseptically into the 6-well plates containing 1 ml of culture medium and are incubated at 37 °C in a humidified atmosphere with 5% CO_2, overnight (at least 16 h), to acclimate the tissues. The medium was changed every day. Systemic treatments were performed daily, twice a day during 15 min, from day 11 to 14. RHE were placed, in triplicate, in medium with KA 1%, or medium containing Tween 20 0.2% and PBA at 0.1% or 1%. RHE controls were placed in medium only, or in medium with Tween 20 0.2%. After 15 min of treatment, RHE were amply rinsed with sterile PBS and incubated at 37 °C. At day 17, determination of color, viability and melanin content was realised.

2.4. Assay on tyrosinase activity

2.4.1. Mushroom Agaricus bisporus tyrosinase

Tyrosinase activity was realised as reported by Kim et al. [12] with minor modifications. Briefly, we used a 96-well plate (Nunc, Denmark), with 120 μl assay mixture containing 1 mM L-tyrosine solution, and 50 mM potassium phosphate buffer (pH 6.5). 40 μl of Dimethyl sulfoxide (DMSO) with various concentrations of PBA (0 to 200 ppm, with dilutions made weight by weight) was added. At the last moment, 40 μl of mushroom tyrosinase (14 units.$well^{-1}$) was added and the absorbance at 490 nm was read every 10 min for 1 h at 25 °C in a microplate reader (Tecan Infinite M200 pro). A control reaction was conducted with DMSO alone. KA was used as reference inhibitor. Each condition was realised in triplicate. The percentage of tyrosinase activity was calculated for each condition as follows: (A / B) × 100, where A represents the difference of absorbances of test samples with and without the enzyme at 1 h; and B represents the difference of control sample absorbances with and without the enzyme at the same moment. IC_{50} refers to the concentration of a substance that inhibits standard response by 50%. In the present experiments, IC_{50} values were derived from the X-axis of the plot (sample concentrations), when Y-axis (inhibition rate of tyrosinase activity) revealed 50%.

2.4.2. Kinetic analysis on tyrosinase inhibition

Various concentrations of L-tyrosine (0.2 to 3.2 mM) as substrate, 50 mM potassium phosphate buffer (pH 6.5), 40 μl of DMSO with or without test sample (0 to 80 ppm of poplar bud absolute), and 40 μl of mushroom tyrosinase (14 units/well), were added to a 96-well plate in a total volume of 200 μl. The initial rate of dopachrome formation from the reaction mixture was determined as the increase of absorbance at wavelength 490 nm per minute by using a microplate reader. The Michaelis constant (Km) and maximal velocity (Vmax) of the tyrosinase activity were determined by Lineweaver-Burk's plot using various concentrations of L-tyrosine as substrate [2].

2.4.3. Tyrosinase activity in B16 cells

Tyrosinase activity in B16 cells was estimated by measuring the rate of oxidation of L-DOPA. After 24 h of treatment with PBA, and washing with PBS, cells were lysed in 100 μl of 50 mM sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 (Sigma) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and then frozen at −80 °C for 30 min. After thawing at room temperature, cellular extracts were clarified by centrifugation at 15.000 rpm for 5 min. Pellets were used to assay the intracellular melanin, whereas supernatants were used to assay the enzyme activity. 80 μl of the supernatant and 20 μl of L-DOPA (2 mg ml^{-1}) were placed in 96-well plate (Nunc, Denmark), and the absorbance at 490 nm was read every 10 min for 1 h at 37 °C [8,10,12].

2.5. Determination of cell viability with PBA

2.5.1. B16 cells

After treatment and removal of PBA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) solution at 1 mg ml^{-1} was added. After 3 h of incubation, the formazan derivative formed by cellular dehydrogenase was solubilized with DMSO 100%. The absorbance was read at 590 nm. The optical density of formazan formed by control cells was taken as 100% cell viability [8,10,12].

2.5.2. RHE

After 1 week of treatment, RHE were placed in an incubator for 3 h in a 24-well plate containing culture medium with 1 mg ml^{-1} of MTT. RHE were placed in a new plate with 500 μl/well of isopropanol and shaken gently at room temperature for 2 h. The absorbance was read at 590 nm. The optical density of formazan formed by control RHE was taken as a 100% cell viability [13].