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# *Betula pendula* leaves: Polyphenolic characterization and potential innovative use in skin whitening products

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ABSTRACT

The research of new tyrosinase inhibitors is currently important for the development of skin whitening agents; particularly, birch leaves extracts are included in many skin cosmetic products. In this study, the potential ability of *Betula pendula* leaves ethanolic extract (BE) was evaluated on mushroom tyrosinase activity. Results showed that BE was capable to inhibit dose-dependently L-DOPA oxidation catalyzed by tyrosinase. The inhibition kinetics, analyzed by Lineweaver–Burk plots, showed a noncompetitive inhibition of BE towards the enzyme, using L-DOPA as substrate. The inhibitory mechanism of BE as studied by spectrophotometric analysis, demonstrated its ability to chelate copper ion in the active site of tyrosinase. In addition, BE exhibited $Fe^{2+}$-chelating ability ($IC_{50} = 614.12 \pm 2.14$ μg/mL), reducing power and radical-scavenging properties ($IC_{50} = 137.22 \pm 1.98$ μg/mL). These results suggest the usefulness of birch leaves extracts in cosmetic and pharmaceutical industries for their skin-whitening and antioxidant effects. Determination of the polyphenolic compounds in BE extracts was afterward achieved by means of high-performance liquid chromatography (HPLC) with photodiode array (PDA) and mass spectrometry (MS) detection. A total of 25 compounds were positively identified, through the complementary analytical information, and are reported in such a matrix for the first time. Knowledge on the qualitative composition and contents of these natural sources in fact represents mandatory information, for rational consumption and correlation of the beneficial effects to the specific amounts.



## 1. Introduction

Tyrosinase is a copper-containing enzyme catalyzing the oxidation of monophenols and *o*-diphenols in the first stages of melanin biosynthesis. This enzyme is also responsible for the undesired browning reactions in damaged fruits during post-harvest handling and processing [1]. Many tyrosinase inhibitors are topically used for treating such localized hyperpigmentation in humans such as lentigo, nevus, ephelides, melasma, and post-inflammatory state [2,3].

Therefore, the research for new tyrosinase inhibitors is valuable both in the development of skin whitening agents and in the control of enzymatic browning during manufacturing process in food industry.

The genus *Betula* (Betulaceae) is represented by common trees and shrubs of boreal and north temperate zones [4]. Several organs and products of birch such as leaves, bark, bugs, juice and distilled oil have been used to increase diuresis and against rheumatic pain and inflammation [5]. Birch leaves are also employed for skin diseases, against hair loss and dandruff in cosmetic products [6,7].

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Regarding the chemical composition, triterpene alcohols and malonyl esters of the dammarane type have been reported in *Betula pendula* [8]; flavonol glycosides (basically hyperoside and other quercetin glycosides together with glycosides of kaempferol and myricetin) [9,10] and phenolic compounds (caffeic and chlorogenic acid) in *B. pendula* and *B. pubescens* [11]. Lignans and diarylheptanoids have been reported in *B. platyphylla* [12–14]. *B. pendula* leaves also contain remarkable amounts of polymeric proanthocyanins, *viz.* 39 mg/g (expressed as dry weight) [15]. The monograph of Betulae folium has been enclosed in European Pharmacopoeia. A large number of phenolic compounds seem to be strong tyrosinase inhibitors for their structural resemblance to tyrosine and L-DOPA, which represent natural substrates of tyrosinase [3]. Moreover, phenolic compounds are known to have multifunctional properties, acting as reducing agents (free radical terminator/scavenger), metal chelators, singlet oxygen and free-radical quenchers [16].

In the present study, we evaluated the polyphenolic content along with the tyrosinase inhibitory activity, $Fe^{2+}$-chelating properties, reducing power and radical-scavenging effects of an ethanolic extract from *B. pendula* leaves. Polyphenolic fingerprint of ethanolic extracts from the leaves obtained by high-performance liquid chromatography (HPLC) with photodiode array (PDA) and mass spectrometry (MS) detection allowed correlation of the observed activities with the presence and respective amounts of particular polyphenolic compounds.

## 2. Experimental

### 2.1. Reagents

All the chemicals and reagents were purchased from Carlo Erba, Milan, Italy and Sigma Chem. Co., MO, USA. HPLC–MS grade acetonitrile (ACN), water, and formic acid (FA) were obtained from Riedel-de Haën (Germany).

### 2.2. Plant material

Fresh leaves of *B. pendula* L. (Betulaceae) were collected during April 2009 from the Natural Park of Nebrodi Mountains (Messina, Italy). The samples were botanically identified using available literature [17]. Voucher specimens are deposited in the Herbarium of the Pharmaco-Biological Department, School of Pharmacy, University of Messina (B-09#31-5).

### 2.3. Extraction

*B. pendula* leaves were air dried and milled. The powdered material (150 g) was extracted twice with 80% ethanol using an Ultra-Turrax homogenizer for 3 min [10]. The obtained extract (BE) was filtered and evaporated to dryness (yield: 32.73 g).

### 2.4. Phytochemical screening

The total flavonoid content of BE was determined according to the method reported in the European Pharmacopoeia, 6th edition [18]. The calculated percentage content was expressed as hyperoside.

### 2.5. Determination of polyphenolic compounds by HPLC–PDA–ESI–MS analysis

A complete fingerprint was also carried out to identify and quantify the phenolic-type compounds and flavonoids contained in BE. The latter (10 mg) was dissolved in ethanol (1 mL) and filtered through 0.45 μm membrane filters (Whatman, Clifton, USA).

The analyses were carried out using a Shimadzu HPLC system (Milan, Italy) equipped with a CBM-20A controller, two LC-20AD pumps, a DGU-$20A_3$ degasser, a SIL-20AC autosampler, a SPD-M20A photo diode array detector (PDA) and a quadrupolar mass analyzer (LCMS-2020, Shimadzu), equipped with an electrospray ionization (ESI) interface, operated in the negative mode. Data acquisition was performed by Shimadzu LabSolution software ver. 5.10.153.

Analyses were carried out on an Ascentis Express C18, 15 cm × 4.6 mm i.d. packed with 2.7 μm partially porous particles (Supelco, Bellefonte, PA). The injection volume was 2 μL, and the mobile phase consisted of water/formic acid (99.9:0.1) (solvent A) and ACN/formic acid (99.9:0.1) (solvent B), the linear gradient profile was as follows: 0 min, 0% B, 5 min, 5% B, 15 min, 10% B, 30 min, 20% B, 60 min, 50% B, 70 min, 100%B, 71 min, 0%B. The mobile phase flow rate was 1 mL/min, and it was splitted to 0.2 mL/min prior to MS detection. PDA wavelength range was 190–400 nm and the chromatograms were extracted at 280 nm. Time constant was 0.16 s and sample frequency 6.25 Hz. MS acquisition was performed using ESI, in the negative mode, under the following conditions: mass spectral range 100–800 *m/z*; interval: 0.5 s; scan speed: 1500 amu/s; nebulizing gas ($N_2$) flow: 1.5 L/min; Heat block: 300 °C, DL temperature: 300 °C; DL voltage: −34 V; probe voltage: 4.5 kV; Qarray voltage: 1.0 V; RF voltage: 90 V; detection gain: 1.0 kV.

Quantitative analysis was carried using calibration curves of four standards, namely catechin, chlorogenic acid, isoquercitrin and kaempferol. Standard calibration curves were obtained in a concentration range of 1–50 mg/L at three concentration levels (1, 10, and 100 mg/L). Triplicate injections were made for each level, followed by linear regression analysis of the chromatographic peak areas plotted *vs.* the injected concentrations. Integration of the PDA peak areas was performed at a wavelength of 280 nm for Flavan-3-ol and Flavanone-like compounds, 325 nm for Cinnamic acid and Flavone-like compounds, 354 nm for Flavonol-glycoside-like compounds and 369 nm for Flavonol-like compounds. Excellent linearity was attained for all the standard compounds (catechin: $y = 1425.4x - 422.48$, $R^2 = 0.999$; chlorogenic acid: $y = 6021.6x - 6409.1$, $R^2 = 0.998$; isoquercitrin: $y = 2428.66x - 1897.1$, $R^2 = 0.999$; quercetin: $y = 803.66x + 818.28$, $R^2 = 1$). The amount of each compound was finally expressed in mg/g of extract.

### 2.6. Anti-tyrosinase activity

#### 2.6.1. Tyrosinase inhibition assay

Tyrosinase inhibition was assayed according to the method of Masamoto [19]. Briefly, aliquots (0.05 mL) of BE (80–200 μg/mL) were mixed with 0.5 mL of L-DOPA solution (1.25 mM), 0.9 mL of sodium acetate buffer solution (0.05 M, pH 6.8) and preincubated at 25 °C for 10 min. Then, 0.05 mL of an aqueous solution of mushroom tyrosinase (333 U/mL) was added last to