Industrial Crops & Products 122 (2018) 498–505

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Screening of a hundred plant extracts as tyrosinase and elastase inhibitors, two enzymatic targets of cosmetic interest

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ARTICLE INFO

Keywords:
Skin ageing
Tyrosinase
Elastase
Phytocosmetics
Polyphenols
Hypericum scruglii

ABSTRACT

In search for natural products of cosmetic interest, a hundred plant extracts were *in vitro* tested against elastase and tyrosinase. The inhibitors of these enzymes find application as skin whitening, anti-ageing, anti-wrinkle agents as well as in the treatment of dermatological disorders.

Among the tested samples, seventeen extracts resulted strongly active. In particular, eleven out of them were capable to inhibit both enzymes, five showed a strong activity only against tyrosinase and one only against elastase. The IC_{50} values of the selected samples ranged from 7 to 100 μg/mL and from 20 to 100 μg/mL against elastase and tyrosinase, respectively. Leaves extract of *Pistacia lentiscus* emerged as the most potent elastase inhibitor and, together with *Cytinus hypocistis* (aerial parts) and *Limonium morisianum* (aerial parts), it showed also the lowest IC_{50} of tyrosinase inhibition.

The tested plants were collected in India, Africa and Mediterranean area. Interestingly, among the most active ones, two are endemic and exclusive of Sardinia Island (Italy), namely: *Limonium morisianum* and *Hypericum scruglii*, moreover, the latter resulted the only plant which hydroalcoholic extract was capable to inhibit elastase selectively.

Moreover, a positive correlation was established among the potency of enzymatic inhibitions and the total phenolic and flavonoid content of the samples. The presence of these aromatic compounds in the most active plants confers them a potential additional value as skin protectors from oxidative damage.

1. Introduction

Skin ageing processes are generally divided into intrinsic and extrinsic, both responsible for drastic changes in skin structure and elasticity. The intrinsic or chronological skin ageing is irremediably related to the passage of time, although it is also influenced by the inherited genes. Conversely, the extrinsic skin ageing is caused by environmental factors, such as chronic exposure to sunlight (photoageing) or pollutants, and it is influenced by miscellaneous lifestyle components (*i.e.* smoking and diet) (Farage et al., 2008). In particular, photoageing is caused by overexposure to UV radiations, which increases the production of reactive oxygen species (ROS) (Rittié and Fisher, 2002), causing lipid peroxidation, DNA damage, and proteins alterations. Moreover, ROS can also contribute to skin ageing by direct activation of enzymes responsible for the cleavage of extracellular matrix (ECM) components (Mukherjee et al., 2011; Rittié and Fisher, 2002).

Natural products from plants are widely used as cosmetic or cosmeceutical ingredients because of their capability to slow down the intrinsic skin ageing processes and to contrast the extrinsic ones. Plants anti-ageing properties are generally attributed to their antioxidant metabolites, which minimize free radical activity and protect skin against solar radiations (Sahu et al., 2013). Additionally, several plant metabolites are also reported to modulate the activity of enzymes involved in the ageing processes (Cefali et al., 2016; Mukherjee et al., 2011). Among these enzymatic targets of cosmetic interest, elastase and tyrosinase are of remarkable importance.

Elastase belongs to chymotrypsin family of proteases and it is responsible for the breakdown of elastin and other proteins, such as collagen and fibronectin, which are fundamental for the ECM elastic properties (Imokawa and Ishida, 2015). Misregulations of this enzyme are involved in skin ageing processes (Korkmaz et al., 2010). In fact, the excessive hydrolysis of the dermal elastin fiber network leads to the loss of skin elasticity and consequent skin sagging (Thring et al., 2009). On this basis, elastase inhibitors are endowed with anti-wrinkles activity promoting the preservation of skin elasticity.

Tyrosinase is a copper-containing enzyme, also known as

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https://doi.org/10.1016/j.indcrop.2018.06.029
Received 12 February 2018; Received in revised form 20 April 2018; Accepted 8 June 2018

polyphenol oxidase (PPO). It catalyzes two distinct reactions, namely: the hydroxylation of a monophenol and the conversion of an *o*-diphenol to the corresponding *o*-quinone. This enzyme is responsible for the rate-limiting first two steps of melanin biosynthetic pathway, and thus, for skin, hair, and eyes color in humans (Pillaiyar et al., 2017). Tyrosinase misregulated expression and/or activity causes skin pigmentation disorders such as: lentigo senilis, urticaria pigmentosa, and age-related skin hyperpigmentation (Slominski et al., 2004). Therefore, tyrosinase inhibitors are candidate skin-whitening agents.

In this work, aimed at identifying natural products endowed with anti-ageing potential, the *in vitro* tyrosinase and elastase inhibitory activity of a hundred hydroalcoholic plant extracts was evaluated. Moreover, the total phenolic and flavonoid content of the tested extracts was also determined, considering the importance of these compounds as antioxidants. In order to investigate on the involvement of these classes of phytochemicals in the tested bioactivities, total phenolic and flavonoid content was also statistically correlated to the percentages of enzymatic inhibitions.

2. Methods and materials

2.1. Plant material

The Indian plants (used in Ayurveda tradition), dried and powdered, were kindly supplied by Maharishi Ayurveda Product Italy (Verona, Italy). They were collected in Ram Bagh (Rajasthan, India) and authenticated by Dr. MR Uniyal, Maharishi Ayurveda Product Ltd., Noida, India.

The samples of African plants were collected in six villages of Baskoure and Songretenga communes (Burkina Faso) and identified by Prof. Joseph Issaka Boussim. Among the Mediterranean plants, the ones collected in Sardinia Island (Italy) were identified by Dr. Cinzia Sanna and Prof. Andrea Maxia, while the two *Sedum* species were collected in Emilia Romagna (Italy) and identified by Prof. Ferruccio Poli. The other Mediterranean plants samples were kindly supplied by Biokyma S.r.l, Anghiari (AR) Italy, and identified by Dr. Franco Maria Bini. Vouchers of crude drugs of the Indian plants and Mediterranean plants were deposited in Department of Pharmacy and Biotechnology, University of Bologna (*via* Irnerio 42, Bologna, Italy). Vouchers of the African plants were deposited in Herbarium of the Botanical Laboratory of the University of Ouagadougou (Burkina Faso). Vouchers of the Sardinian plants were deposited at the General Herbarium of the Department of Life and Environmental Sciences, University of Cagliari and vouchers of the two *Sedum* species were deposited in the Herbarium of the Department of Pharmacy and Biotechnology, University of Bologna. All the information (including vouchers) of the considered plants are reported in Table 1.

2.2. Preparation of the extracts

Thirty mg of dried and powdered plant material were extracted by sonication for 30 min using 1.5 mL of MeOH/H_2O (1:1). Subsequently, the samples were centrifuged for 20 min, the supernatant was separated from the pellet and dried to yield the crude extracts.

2.3. Tyrosinase inhibitory assay

The enzymatic inhibitory assay was performed according to Venditti et al. (2013) with slight modifications. Mushroom tyrosinase (2 mU) and sample (50 µg/mL) were incubated for 5 min in 0.1 M sodium phosphate buffer pH 6.8, in 0.1 mL of final volume. L-DOPA (final concentration 2 mM) was added up to a final reaction volume of 0.2 mL. The formation of dopachrome was immediately monitored for 5 min at 490 nm in a microplate reader (Victor™ *X*3 PerkinElmer, Waltham, Massachusetts, United States) under constant temperature of 30 °C. The IC_{50} (concentration necessary for 50% inhibition of enzyme activity) was calculated by constructing a linear regression curve showing extracts concentrations (from 1 to 250 µg/mL) on the *x*-axis and percentage inhibition on the *y*-axis. A negative control was obtained by adding water instead of extracts, while kojic acid (solubilized in water) was used as positive control, finding an IC_{50} of 3 ± 0.37 µg/mL (21 µM).

The percentage of enzyme inhibition was calculated using the following formula:

$$\%\text{Inhibition} = [1 - (\Delta\text{Abs/min}_{\text{sample}} / \Delta\text{Abs/min}_{\text{negative control}}) \times 100]$$

In order to determine the kinetic parameters for the enzymatic reaction the Lineweaver-Burk plot was built, using substrate concentration in the range from 0.5 to 4 mM. In the assay conditions, the obtained K_M value was of 0.2 mM and V_{max} of 10 µmol/min (ΔAbs/min = 0.03), considering dopachrome ε at 490 nm = 3.6201 mM^{-1} cm^{-1} and a light path length of 0.8 cm.

2.4. Elastase inhibitory assay

The assay was performed according to the method of Liyanaarachchi et al. (2018) whit some modifications. Porcine pancreatic elastase (1.5 mU) and extract sample (50 µg/mL) were incubated for 5 min in 0.1 M TRIS buffer pH 8.1, in 0.1 mL final volume. Substrate N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide (2 mM) was added to start the reaction in a final volume of 0.2 mL. The variation of absorbance was monitored for 5 min at 420 nm in the microplate reader under constant temperature of 30 °C. For the IC_{50} calculations, samples and quercetin (positive control) were tested at different concentrations ranging from 1 to 250 µg/mL. In the case of quercetin the assay was performed in 2% DMSO, thus a proper negative control in the same conditions was used for the IC_{50} calculation.

Lineweaver-Burk plot was built, using substrate concentration in the range of 0.25–2 mM. In the assay conditions, the obtained K_M value was of 0.2 mM and V_{max} of 6 µmol/min (ΔAbs/min = 0.04), considering ε of p-nitroanilide at 420 nm = 8.8 mM^{-1} cm^{-1} and a light path length of 0.8 cm.

2.5. Total phenolic and flavonoid content

The assays were performed in Spectrophotometer Jasco V-530 as described by Di Pompo et al. (2014) with slight modifications. Briefly, for total phenolic content analysis a calibration curve was constructed using 50 µL of different gallic acid stock solutions prepared in MeOH 80% (from 10 to 200 µg/mL) mixed with 250 µL of Folin-Ciocalteu reagent (diluted 1:10) and 500 µL of H_2O. Different stock solutions of extracts were prepared in water (from 0.05 to 0.2 mg/mL) and 50 µL of each stock were mixed with the same reagents as described above. Both calibration curve and samples were incubated at room temperature for 5 min before adding 800 µL of sodium carbonate solution (Na_2CO_3 20%). After 30 min of incubation at 40 °C, absorption was recorded at 760 nm. Total phenolic content was calculated by interpolation in the calibration curve and expressed as: mg GAE (gallic acid equivalent)/g of extract (dried weight).

Total flavonoid content was determined using rutin to perform the calibration curve. Different stock solutions of extracts were prepared in water (from 0.05 to 0.2 mg/mL) and 50 µL of each one were mixed with 450 µL of methanol and 500 µL of $AlCl_3$ (2% w/volume of methanol). The absorption at 430 nm was recorded after incubation (15 min) at room temperature. The calibration curve was obtained using 50 µL of different rutin stock solutions prepared in DMSO (from 1 to 100 µg/mL). Total flavonoid content of the extracts was calculated by interpolation in the calibration curve and expressed in terms of mg RE (rutin equivalent)/g of extract (dried weight).