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Tyrosinase inhibitory constituents from a polyphenol enriched fraction of rose oil distillation wastewater

Jessica Solimine [a], Eliane Garo [b], Jonas Wedler [a], Krasimir Rusanov [c], Orlando Fertig [b], Matthias Hamburger [b], Ivan Atanassov [c], Veronika Butterweck [a],*

[a] Institute for Pharma Technology, School of Life Sciences, University of Applied Sciences Northwestern Switzerland, Gründenstrasse 40, CH-4132 Muttenz, Switzerland
[b] Institute of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Klingelbergstrasse 50, CH-4056, Basel, Switzerland
[c] AgroBioInstitute, Agriculture Academy, Dragan Tzankov 8, Sofia 1164, Bulgaria

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ABSTRACT

During the water steam distillation process of rose flowers, the non-volatile phenolic compounds remain in the waste. We recently developed a strategy to separate rose oil distillation water (RODW) into a polyphenol depleted water fraction and a polyphenol enriched fraction (RF20-SP207). Bioassay-guided investigation of RF20-SP207 led to the isolation of quercetin, kaempferol and ellagic acid. Their structures were elucidated by spectroscopic analysis as well as by comparison with literature data. Tyrosinase inhibition studies were performed with RF20-SP207, fractions I–IV, and the isolated compounds of the most active fraction. RF20-SP207 strongly inhibited the enzyme with an IC_{50} of 0.41 μg/mL. From the tested fractions only fraction IV (IC_{50} = 5.81 μg/mL) exhibited strong anti-tyrosinase activities. Quercetin, kaempferol and ellagic acid were identified in fraction IV and inhibited mushroom tyrosinase with IC_{50} values of 4.2 μM, 5.5 μM and 5.2 μM, respectively, which is approximately 10 times more potent than that of the positive control kojic acid (56.1 μM). The inhibition kinetics, analyzed by Lineweaver–Burk plots, indicated that RF20-SP207 and fraction IV are uncompetitive inhibitors of tyrosinase when L-tyrosine is used as a substrate. A mixed inhibition was determined for ellagic acid, and a competitive inhibition for quercetin and kaempferol. In conclusion, the recovered polyphenol fraction RF20-SP207 from RODW was found to be a potent tyrosinase inhibitor. This value-added product could be used as an active ingredient in cosmetic products related to hyperpigmentation.

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1. Introduction

The production of rose oil by steam distillation of rose flowers (*Rosa damascena* Mill. f. trigintipetala Dieck, Rosaceae) leaves a water fraction of the distillate as main part of the waste. Therefore, rose oil distillation wastewater (RODW) represents a serious environmental problem due to the high content of polyphenols which are difficult to decompose. In addition, they are biopollutants when discarded into the rivers. On the other hand, natural polyphenols are valuable compounds with useful properties as bioactive substances for the pharmaceutical and cosmetic industries. Until now there is no established practice for processing of RODW, and utilization of contained substances. Distillation of each kilogram of raw flower material results in approximately 4 L of liquid waste, and less than 1 kg of flower residue on a wet weight basis. During a single cycle of industrial distillation 500 to 1000 kg of rose petals is used [1], and the waste problem in the area of production is thus substantial. Therefore, we recently developed a strategy to separate RODW into a polyphenol depleted water fraction and a polyphenol enriched fraction (RF20-SP207) [2].

While the rose oil contains mainly the essential oils which are known for their antimicrobial activities, the medicinal properties of rose flowers are partly attributed to the abundance of polyphenols, particularly flavonoids and anthocyanidins [3]. Current evidence strongly supports a contribution of polyphenols to the prevention of cardiovascular diseases, cancers, and osteoporosis, and suggests a role in the prevention of neurodegenerative diseases and diabetes mellitus [4]. However, one of the significant challenges of elucidating the health effects of polyphenols is linked to major differences in their bioavailability. It has been known meanwhile for quite some time that polyphenols, and especially flavonoids, are heavily metabolized by colonic microorganisms [5–8]. Considering the unfavorable pharmacokinetic profile and the intensive metabolism of flavonoids after oral intake, the development of a polyphenol based dietary supplement for oral application is therefore difficult. Thus, indications involving topical application seem to be more suitable for plant derived polyphenols, since the application to the skin should circumvent issues associated with the relatively low oral bioavailability of flavonoids. However, the industrial development and utilization of flavonoids for medicinal uses are limited, because

* Corresponding author.
E-mail address: veronika.butterweck@fhnw.ch (V. Butterweck).

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their chemical synthesis is complex and expensive. Therefore, our approach represents a promising biotechnology paradigm to meet the dual ends of wastewater treatment and recovery, and biological activity of high value added 'bioproducts' for use in the cosmetic industry.

In the current study we initially focussed on tyrosinase inhibition for bioactivity screening, since there is a high need for potential tyrosinase inhibitors in the cosmetic industry as skin whitening agents that prevent hyperpigmentation. Tyrosinase is a key enzyme in the biosynthetic pathway of melanin production and catalyzes the hydroxylation of L-tyrosine to L-DOPA and the oxidation of L-DOPA to dopaquinone, a reactive intermediate which spontaneously cyclizes to dopachrome [9]. In humans, dopaquinone is converted by a cascade of cyclization and polymerization steps into melanin [10]. In many cultural settings irregular melanin pigmentation is considered as an esthetic disadvantage [11,12]. As a result, several depigmenting modalities are being investigated for their efficacy in treating skin hyperpigmentary lesions [13]. The best known tyrosinase inhibitors are hydroquinone, kojic acid and arbutin, but they all have been associated with serious side effects, such as permanent depigmentation following chronic application (hydroquinone), paradoxical hyperpigmentation in higher but more efficacious concentrations (arbutin), or erythema and contact dermatitis (kojic acid) (for reviews see [11,13]). For these reasons a broad spectrum of naturally-derived skin whitening agents have been investigated that efficiently inhibit tyrosinase without causing harmful side effects (for reviews see [14,15]). Many flavonoid derivatives have been identified to be strong inhibitors of tyrosinase [16]. The objective of the present study was to investigate RF20-SP207, a polyphenol enriched fraction of RODW, for possible tyrosinase inhibitory effects. In a bioassay guided approach fractions and isolated compounds from RF20-SP207 were further screened for their inhibitory activity against mushroom tyrosinase.

2. Experimental

2.1. General

Technical grade solvents were used for extraction and CC. HPLC grade solvents were purchased from Scharlau (Spain) and Lab-Scan (Poland). HPLC grade water was obtained using EASY-pure II (Barnstead, USA) water purification system. DMSO, phosphate buffer, hydrochloric acid, sodium hydroxide and mushroom tyrosinase (EC 1.14.18.1) were purchased from Sigma (Switzerland), ellagic acid (purity >95%), kaempferol (purity >99%), quercetin (purity >99%), L-tyrosine (purity >98%) and kojic acid (purity >98%) were from Sigma (Switzerland). For the preparation of TLC spray solutions, Naturstoffreagenz A (diphenylboryloxyethylamine) was purchased from Roth (Karlsruhe, Germany) and polyethylene glycol-4000 (PEG-4000) from Sigma (Switzerland).

2.2. Rose oil distillation wastewater (RODW) and resin fractions

Wastewater obtained after distillation of full-blown *R. damascena* flowers was obtained from the distillery of the Institute of Roses, Essential and Medicinal Crops (IREMC) in Kazanlak, Bulgaria, in June 2013. Wastewater was filtered through a cheese-cloth to afford RODW which was stored at +4 °C in 10 L plastic bottles. RF20-SP207 was prepared from RODW as described previously [2].

2.3. Column chromatography and TLC analysis

Column chromatography was performed on a Sephadex® LH-20 column (100 × 5.5 cm I.D.) using 90% methanol as mobile phase, at a flow rate of 2 mL/min. A portion of the RF20-SP207 resin extract (6.8 g) was dissolved in 60 mL 80% methanol and loaded into the column. Fractions were collected every 10 min using a SuperFrac autosampler (GE Healthcare). A total of 300 fractions were collected in glass tubes and combined to 12 main fractions based on TLC patterns. Four additional late eluting fractions were collected in flasks giving rise to a total of 16 main fractions: F1 (1–74, 1.32 g), F2 (75–88, 0.22 g), F3 (89–104, 0.40 g), F4 (105–110, 0.17 g), F5 (111–122, 0.64 g), F6 (123–130, 0.31 g), F7 (131–136, 0.28 g), F8 (137–152, 0.42 g), F9 (153–174, 0.26 g), F10 (175–194, 0.23 g), F11 (195–226, 0.45 g), F12 (227–300, 0.56 g), F13 (0.19 g), F14 (0.47 g), F15 (0.07 g) and F16 (0.07 g). TLC analysis of fractions was performed on precoated silica gel 60 F_{254} plates (Merck) with a mobile phase composed of ethyl acetate/formic acid/glacial acetic acid/water (100/11/11/26). Detection was at UV 254 and 366 nm. Spots were visualized at UV-366 nm after spraying 1% methanolic Naturstoffreagenz A followed by 5% ethanolic PEG-4000. Fractions F1 to F16 were combined based on HPLC and TLC data to give four pool fractions I to IV in order to simplify pharmacological testing. Fractions F1 and F2 were combined in pool "I", fractions F3, F4 and F5 in pool "II", fractions F6, F7, and F8 in pool "III", fractions F9 to F16 in pool "IV" (Fig. 1S).

2.4. Compound purification

Compounds **1** to **14** (Table 1) were purified from Sephadex® LH-20 fractions F1 to F16 by preparative HPLC. The prep HPLC system consisted of a SCL-10VP controller, LC-8A binary pumps, a UV–Vis SPD-M10A VP detector and Class-VP 6.12 software (all Shimadzu). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) with the exception of fraction 12 where 0.1% formic acid was added to both solvents. The flow was set to 20 mL/min. Equilibration time between the injections was 10 min. UV spectra were recorded from 200 to 400 nm. Separations were performed on a SunFire Prep C_{18} OBD (30 × 150 mm, 5 μm, Waters) column equipped with a SunFire Prep guard column (19 × 10 mm, 5 μm, Waters). Preparative HPLC was performed under following isocratic conditions: 20% ACN for F1, 22% ACN for F3, 25% ACN for F6 and F8, 30% ACN for F4 and 22% + 0.1% formic acid for F12 and compounds were isolated as previously described [2]. Quercetin (**16**) and Kaempferol (**15**) (Table 1) were major peaks of F13 and F12 respectively and their identity was confirmed by co-chromatography of reference compounds as previously described [2].

2.5. HPLC–ELSD-DAD-MS analysis

Analytical HPLC analyses of fractions I–IV were performed on a LC-20 AD instrument system (Shimadzu) equipped with a SPD-M20A PDA detector, an evaporative light scattering detector (ELSD) serie 3300 (Alltech) and a LCMS-8030 detector (Shimadzu). For the ELSD, N_2 flow was 2.5 L/min, and evaporation temperature was 60 °C. The mobile phase consisted of 0.1% formic acid (solvent A) and ACN + 0.1% formic acid (solvent B) and the flow was set to 0.4 mL/min. Separations were performed on a C_{18} SunFire™ column (3.0 × 150 mm, 3.5 μm, Waters) equipped with a guard column (3.0 × 20 mm, 3.5 μm, Waters) which were thermostated at 40 °C. Each sample was prepared at a concentration of 3.5 mg/mL and 10 μL was injected. The following gradient was used; 10%B isocratic for 4 min, gradient 4–5 min to 17%B, 5–26 min to 27%B, 26–27 min to 50%B, 27–35 min 50%B isocratic, 35–36 min to 100%B, 36–41 min 100%B isocratic, and 41–42 min to 10%B. All HPLC-ELSD chromatograms are shown in Fig. 1.

2.6. Tyrosinase inhibition (EC 1.14.18.1)

Tyrosinase inhibitory activity was assayed as described previously with slight modifications [24–26], using L-tyrosine as substrate. For determination of the absorption maximum of dopachrome, 130 μL of a 10 mmol/L phosphate buffer (pH 6.8) (PBS) and 70 μL L-tyrosine solution at a concentration of 0.4 mg/mL were pipetted into a 96 well plate. Afterwards, 10 μL of mushroom tyrosinase (250 U/mL in PBS) was added and the absorption spectrum recorded between 350 nm

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