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Full Paper

Effects of Fumarprotocetraric Acid, a Depsidone from the Lichen *Cladonia verticillaris*, on Tyrosinase Activity

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Abstract: Lichens are widely distributed around the world. Their phenolic compounds, consisting mainly of depsides and depsidones, have been extensively studied for important biological activities. More recently, these compounds have been evaluated for their inhibitory activity against enzymes such as tyrosinase, a key agent in melanin biosynthesis. In the present investigation, the depsidone fumarprotocetraric acid isolated from the lichen *Cladonia verticillaris* (Raddi) Fr. was evaluated for its inhibitory activity against this critical enzyme. Kinetic study showed that depsidone at 0.6 mM inhibited tyrosinase activity by 39.8%. Lineweaver–Burk plots revealed that fumarprotocetraric acid can act as an uncompetitive or mixed-type inhibitor, depending on concentration.

Keywords: depsidone; phenolic compounds; fumarprotocetraric acid; lichens; tyrosinase

1. INTRODUCTION

Lichens are widely distributed around the world, from tropical to polar regions [1]. Lichenization—an association between an alga and/or cyanobacterium and a fungus—is not an uncommon phenomenon, as it occurs with 20% of all members of the Fungi Kingdom [2]. Lichens are sources of phenolic compounds synthesized mainly via the acetate–polymalonate pathway, which yields dibenzofurans, chromones, naphthoquinones, anthrones, anthraquinones, depsones, depsides, and depsidones [3].

Depsides and depsidones, metabolites commonly isolated from lichens, are biosynthesized through esterification of two or more units of orsellinic or β-methyl orsellinic acid [4]. Depsidones are more stable than depsides, owing to an additional ether linkage between both aromatic rings, making up a rigid third ring [5]. Over the past few decades, depsidones have been tested in biologic assays for potential anti-tumor, anti-HIV, anti-microbial, and other activities [6-12]. Studies have explored the potential of depsides, depsidones, and other phenolic compounds to inhibit enzymes such as histidine decarboxylase, 5-lipoxygenase, prostaglandin synthase, and monoamine oxidase [13-28] (Table 1S).

Tyrosinase (EC 1.14.18.1), a copper-containing enzyme that is crucial for melanin production [29], oxidizes monophenol (tyrosine) to *o*-diphenol L-DOPA and subsequently to *o*-quinone dopachrome, which then undergoes chemical and enzymatic reactions to yield melanin [30]. The enzyme's structure and catalytic oxidation mechanism have been elucidated [31, 32].

The importance of tyrosinase in pigmentation and exoskeleton formation has been demonstrated,

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suggesting that compounds capable of inhibiting this enzyme could find utility in skin whitening, pest control, and food conservation applications [33-34]. Considerable tyrosinase inhibitory activity has been observed in phenolic compounds isolated from natural products [29, 35, 36]. Although tyrosinase inhibition by lichen extracts has been reported [37-43], this type of activity has not, to the best of our knowledge, been investigated in compounds isolated from lichens. This article reports an investigation of fumarprotocetraric acid isolated from the lichen *Cladonia verticillaris* (Raddi) Fr. as a potential tyrosinase inhibitor.

2. MATERIAL AND METHODS

General Experimental Procedures

NMR spectra (^{1}H, ^{13}C, and DEPT-135) were obtained in DMSO-d_6. Solvent resonances were used as the internal references. The experiments were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C NMR on a Bruker DPX300 spectrometer. IR spectra were recorded using an FT/IR Perkin Elmer Frontier spectrometer over the wavenumber range of 4000-400 cm^{-1} using a potassium bromide disk. Melting points were determined on a Uniscience Melting Point apparatus without corrections. Sample purity was checked by TLC on pre-coated silica gel GF_{254} plates (0.25 mm thickness, Merck) under UV radiation (254 nm), while methanol : sulfuric acid (10%) and *p*-anisaldehyde : sulfuric acid were used as spraying reagents.

Mushroom (*Agaricus bisporus*) tyrosinase (3393 units/mg) and L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Sigma-Aldrich and maintained at temperatures below –10 ºC. NIH 3T3 cells (mouse embryonic fibroblast ATCC CRL-1658) were purchased from the Rio de Janeiro Cell Bank. All aqueous solutions were prepared with deionized water on the first day of the experiment, then frozen and used within three days. Phosphate buffer (Na_2HPO_4–NaH_2PO_4) at 66.7 mM, pH 6.8, was used in all reactions. Analytical-grade organic reagents were acquired from Synth and Aldrich.

Extraction and Isolation of Fumarprotocetraric Acid

The lichen *Cladonia verticillaris* (Raddi) Fr. (Cladoniaceae) was collected by Prof. N.P.S. Santos in Saloá (24L0742998, UTM 9001885), Recife, PE, Brazil. Identification was conducted by Prof. E.C. Pereira. After drying at room temperature, the lichen was pulverized and exhaustively extracted with hexane at room temperature, followed by acetone. The concentrated acetone extracts were centrifuged with cold acetone at 3000 rpm until a white precipitate (fumarprotocetraric acid) was obtained [m.p. 287 °C (dec.)] The structure of fumarprotocetraric acid was confirmed by ^{1}H and ^{13}C NMR and IR spectra and were in accordance with the literature [44 -47]. Supplementary Material available: Fumarprotocetraric acid – NMR (^{1}H, ^{13}C, and Dept-135) and IR data.

Enzyme Assay

The effect of fumarprotocetraric acid on L-DOPA oxidation mediated by tyrosinase was evaluated spectrophotometrically (Bioespectro SP220) at 475 nm. Absorbance was recorded every 30 s for 5 min in order to calculate the rate of dopachrome formation (ε = 3700 M^{-1} cm^{-1}) [48]. In a glass cuvette, 0.1 mL of tyrosinase (final concentration, 2.0 μg mL^{-1} in phosphate buffer) was added to 1.7 mL of fumarprotocetraric acid solution (at a final concentration of 0.2-0.6 mM in phosphate buffer) containing DMSO. After 10 min, 0.2 mL of aqueous L-DOPA solution (final concentration, 0.2-0.6 mM) was added to start the reaction and absorbance was recorded as described above. All experiments were performed in triplicate with 10% DMSO at 28-30 °C. A blank experiment was also carried out without the acid. To evaluate the type of inhibition, Lineweaver–Burk plots were constructed and statistical analysis performed using Origin 6.0 software. Percent inhibition (%*I*) was calculated based on the absorbances of the blank system (B) and the fumarprotocetraric acid solution (A) system at 5 min of reaction, applying the formula $[(B - A) / B] \times 100$.

Toxicity to *Artemia salina* [49]

100 mg of dried brine shrimp (*A. salina*) eggs were bred in saline solution (38 g L^{-1} in distilled water). The container was illuminated with a 40 W lamp for 48 h. The larvae were then transferred to the solutions (10 larvae per vial). Fumarprotocetraric acid was solubilized in saline solution containing 3% DMSO to give final concentrations of 0.54, 0.18, and 0.10 mM. Quinine sulfate was used as the positive control; the saline solution was the negative control. Bioassays were conducted in triplicate and survivors were counted after 24 h. LC_{50} values (95% confidence interval) were calculated using Probitos software [50].

Toxicity on NIH/3T3 fibroblast cells

The toxicity of fumarprotocetraric acid against NIH/3T3 fibroblasts was evaluated as per Brandão et al. [51]. Acid concentrations from 5.2×10^{-4} to 5.2 x 10^{-1} mM were employed.

3. RESULTS AND DISCUSSION

Figure 1 depicts the structure of fumarprotocetraric acid.

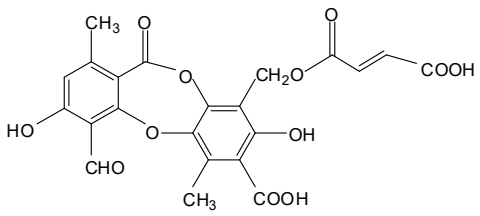

Figure 1. Structure of fumarprotocetraric acid.

The kinetic parameters of tyrosinase employed in the assays and the rates of tyrosinase inhibition by fumarprotocetraric acid are shown in Tables 1 and 2, respectively.

Table 1. Tyrosinase kinetic parameters employed in the assays.*

Parameters	Values**
Enzyme unit [product (mmol min^{-1})]	1.3×10^{-4} mmol min^{-1}
Specific enzyme activity [unit/enzyme (mg)]	3.3×10^{-2} mmol min^{-1} mg^{-1}
Catalytic constant [unit/enzyme (mol***)]	3.9×10^{3} mol min^{-1} mol^{-1}
Turnover (catalytic constant/active site)	3.9×10^{3}

* L-DOPA: 0.6 mM; tyrosinase: 2.0 μg mL^{-1}. ** Calculation based on *y*-intercepts (V_{max} values) of controls with Lineweaver–Burk plots. *** Tyrosinase MW = 120 kD [29].

Table 2. Tyrosinase inhibition (%*I*) by fumarprotocetraric acid at 5 min of reaction.*

Fumarprotocetraric acid (mM)	%*I*
0.2	12.5
0.3	23.4
0.5	32.2
0.6	39.8

* L-DOPA = 0.6 mM; tyrosinase = 2.0 μg mL^{-1}.

At 0.6 mM, the acid inhibited tyrosinase activity by 39.8%. Inhibition rose with increasing acid concentration. Tyrosinase parameters and inhibition rates were calculated for 0.6 mM L-DOPA, the concentration at which the enzyme exhibited maximum velocity in the experiments. No significant difference in reaction velocity (curve slope) was observed between experiments conducted with 0.6 and 0.9 mM L-DOPA (Figure 2).

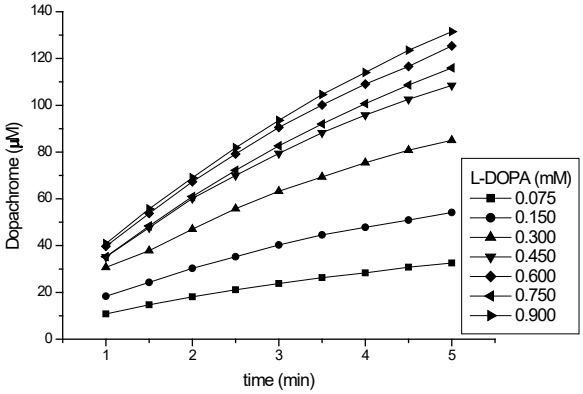

Figure 2. Dopachrome formation (μM) *vs*. time (min) during catalytic oxidation of L-DOPA by tyrosinase (2.0 μg/mL).

Lineweaver–Burk plots ($1/V_0$ *vs*. $1/[S]$) for acid concentrations are depicted in Figure 3. The curves for acid concentrations in the 0.2-0.5 mM range are parallel to the control curve. An explanation for this phenomenon is that fumarprotocetraric acid decreases K_m (1.78 mM) and V_{max} (64.5 μM min^{-1}) as its concentration is increased—a characteristic behavior of uncompetitive inhibition [52]. K_m and V_{max} also decreased at an acid concentration of 0.6 mM, but not proportionally, causing the intersection with the control curve to shift to quadrant III (Figure 3, arrow). This is characteristic of mixed-type, cooperative inhibition, since the substrate increases inhibitor (fumarprotocetraric acid) affinity ($\alpha < 1$) for the enzyme, shifting the intersection to quadrant III [52]. Inhibition type is dependent on fumarprotocetraric acid concentration. These findings corroborate the relevance of elucidating tyrosinase inhibition mechanisms, as well as further investigating dosages in formulations containing tyrosinase inhibitors [53].

Fumarprotocetraric acid showed low toxicity against *Artemia salina* larvae (LC_{50} > 0.54 mM) and murine fibroblasts (IC_{50} 0.4 mM) [54].

Phenolic compounds isolated from lichens may

represent a promising target for research on tyrosinase modulation (inhibition or activation). This appears to be the first study of tyrosinase inhibition kinetics conducted with a compound isolated from a lichen.

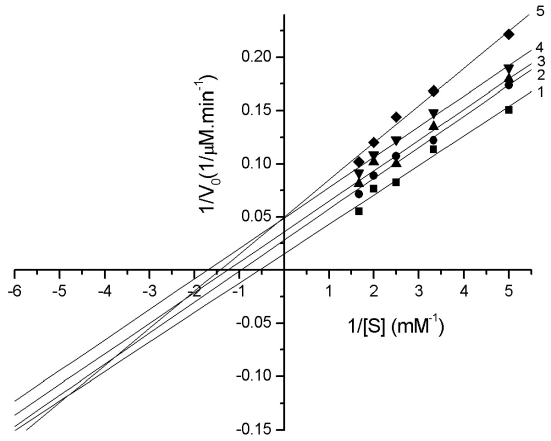

Figure 3. Lineweaver–Burk plots of L-DOPA ([*S*]) oxidation catalyzed by tyrosinase (2.0 μg/mL) in the presence of varying concentrations of fumarprotocetraric acid: **1** (blank); **2** (0.2 mM); **3** (0.3 mM); **4** (0.5 mM); **5** (0.6 mM). $R^2 > 0.99$; $SD < 0.01$; $P < 0.0001$.

4. CONCLUSION

Fumarprotocetraric acid isolated from *Cladonia verticillaris* (Raddi) Fr. showed uncompetitive, mixed-type inhibition of tyrosinase. This inhibitory activity was concentration-dependent, suggesting that the use of this or other tyrosinase modulators in pharmaceutical formulations should be guided by extensive investigation of the mechanisms of inhibition of this enzyme.

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