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Anti-tyrosinase and antioxidant activity of meroterpene bakuchiol from Psoralea corylifolia (L.)

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1	Anti-Tyrosinase and Antioxidant Activity of Meroterpene Bakuchiol from Psoralea
2	corylifolia (L.)
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27 Abstract

28 Bakuchiol is gaining major interest for treatments against skin photoaging. The kinetics of mushroom tyrosinase inhibition by bakuchiol, by real-time oxygen sensing and UV-Vis monitoring (475nm), 29 30 showed competitive inhibition with average K_i constant (μ M, 30°C, pH 6.8) of 6.71±1.23 and 1.15 ± 0.34 for monophenolase and diphenolase reactions respectively, with respective IC₅₀ 31 37.22 ± 5.18 and $6.91\pm0.96 \sim$ at 1 mM substrate, compared to kojic acid IC₅₀ 34.02 ± 5.51 and 32 16.86 \pm 3.28 μ M. Fluorescence quenching showed a single binding mode with formation constant K_a 33 1.02×10^{6} M⁻¹. The antioxidant activity was studied by inhibited autoxidation of styrene and cumene 34 (PhCl, 30°C) affording inhibition constant $k_{inh}=18.1\pm6.6$ (10⁴M⁻¹s⁻¹, 30°C) and of MeLin in TritonTM 35 X-100 micelles giving $k_{inh}=0.16\pm0.03$ (10⁴M⁻¹s⁻¹, 37°C). Stoichiometric factor was 1.9±0.1. ReqEPR 36 spectroscopy afforded the BDE(OH) as 81.7±0.1 kcal/mol. Bakuchiol is a potent tyrosinase inhibitor 37 38 with good antioxidant activity having major potential as natural food preservative against oxidation 39 and food-browning. 40 41 42 Keywords: bakuchiol; skin-whitening; melanin; food browning; antioxidant; peroxyl radicals. 43 44 45 46 **Compounds investigated in this study:** 47 (S)-Bakuchiol, CAS 10309-37-2 (PubChem CID: 5468522) 48 49 O-methylbakuchiol, CAS 10309-44-1 (PubChem CID: 14610678) Kojic acid, CAS: 501-30-4 (PubChem CID: 3840) 50

51 Mushroom Tyrosinase (EC 1.14.18.1), CAS: 9002-10-2

53 1. Introduction

Psoralea corylifolia (L.) is an annual erect herb (30-180 cm in height) native of India and the subtropical regions, which has wide and long-standing use in Traditional Chinese Medicine and in Indian Ayurvedic medicine (Chopra, Dhingra & Dhar, 2013; Alam, Khan & Asad, 2018). The edible seeds (legumes) have the highest medicinal value for the content in bioactive phytochemicals including sterols, flavonoids, chalcones, psoralenes, terpenes and, most notably, the meroterpene bakuchiol (Figure 1) (Chopra et al., 2013), named after the Indian traditional name of the plant (bakuchi, or baguchi, or babchi).

61 Several studies have outlined important and diverse bioactivities of bakuchiol, such as 62 antinflammatory, antimicrobial, anticancer, estrogenic, protection from organ damage, from diabetes 63 and from anxiety and neurological disorders (Chopra et al., 2013; Alam et al., 2018; Oh et al., 2010). 64 However, the main interest in recent research has been polarized by the structural similarity with 65 retinol (Vitamin A), which enables mimic bioactivity, particularly in dermatological applications (Krishna, Edachery & Athalathil, 2022), such as in anti-acne, anti-psoriasis and anti-age treatments 66 67 (Chaudhuri & Bojanowski, 2014). Clinical investigations proved similar efficacy to retinol in topical 68 skincare treatments against photoaging, accompanied by much lower side effects (Dhaliwal et al., 69 2019).

Such efficacy is in part attributed to a reported antioxidant activity (Dhaliwal et al., 2019; Haraguchi, Inoue, Tamura & Mizutani, 2000). The reactivity of bakuchiol with selected radicals was investigated and its ability to protect rat brain homogenates from autoxidation was shown using the TBARS assay (Adhikari et al., 2003); however, the absolute antioxidant activity and the trapping of most relevant alkylperoxyl radicals, which represent the main mechanism sustaining direct antioxidant activity was not determined, allowing no quantitative comparison with other antioxidants and no rational exploitation of its properties.

77 Interestingly, clinical investigation also outlined a skin depigmenting activity, which was judged not
78 due to the most common mechanism, *i.e.* the inhibition of tyrosinase enzyme activity (West, Alabi &

79 Deng, 2021). Other studies attributed the skin depigmenting activity to reduction of pre-formed 80 melanin (owing to the antioxidant activity), or to the blocking of α -melanocyte-stimulating hormone 81 activation, as well as to (not investigated) inhibition of tyrosinase (Dhaliwal et al., 2019). Inhibition 82 of tyrosinase was shown using immobilized mushroom tyrosinase (mTYR) with an electrophoresis 83 assay and it was also supported by molecular docking (Cheng & Chen, 2017); however, the reported 84 IC_{50} value of 100.30 μ M for inhibition of diphenolase activity, much higher than for reference 85 inhibitor kojic acid (5.55 µM) (Cheng & Chen, 2017), appears too high to justify the depigmenting 86 efficacy outlined in clinical studies, leaving the matter essentially unsettled. Moreover, the inhibition mechanism and the Michaelis-Menten related constants, which are most relevant in quantifying 87 88 enzyme inhibition, have never been determined.

89 Owing to the major importance of bakuchiol as emerging bioactive food component (Chopra et al., 90 2013; Alam et al. 2018; Chaudhuri & Bojanowski, 2014; Dhaliwal et al., 2019; West et al., 2021), 91 and to the current high interest in plant-derived depigmenting compounds (Mahdavi, 92 Mohammadsadeghi, Mohammadi, Saadati & Nikfard, 2022; Zhu et al., 2022; He, Fan, Liu, Li & Wang, 2021; Yang et al., 2021; Song, et al., 2021; Panzella & Napolitano, 2019), particularly for their 93 94 efficacy in post-harvest food protection (Zhou et al., 2022), we set up to fill in such gap of knowledge. 95 The kinetics of mTYR inhibition was investigated in depth, by exploiting our newly developed and 96 validated method based on real-time oxygen sensing during tyrosinase reaction (Guo, Cariola, Matera, 97 Gabbanini & Valgimigli, 2022), which we matched to the conventional spectrophotometric and 98 spectrofluorometric approaches for enhanced reliability. Additionally, we investigated the antioxidant activity of bakuchiol by state-of-the-art inhibited autoxidation studies both in 99 100 homogenous solution and in heterogeneous system (Amorati & Valgimigli, 2018; Guo, Baschieri, Amorati & Valgimigli, 2021; Guo et al., 2021b), and by electron paramagnetic resonance (EPR) 101 102 (Amorati, Pedulli, Valgimigli, Johansson & Engman, 2010), so to complete the picture, affording the 103 mechanism and absolute kinetics and thermodynamics of peroxyl radical trapping (Valgimigli & Pratt,

104 2015).

Our hypothesis was that bakuchiol's anti-tyrosinase potency is substantially higher that previously
 suggested and that the mechanism of its antioxidant activity would require significant revision.

Our hypothesis was particularly that bakuchiol has a previously unrecognized great potential as
 natural food preservative, which stems from the combined abilities to protect from air oxidation and
 from enzymatic food browning.

110

111 2. Materials and Methods

112 *2.1 Materials*

(S)-(+)-Bakuchiol (4-[(3S,1E)-3-ethenyl-3,7-dimethylocta-1,6-dien-1-yl]phenol; 99%) was from 113 114 Cymit Quimica (Barcellona, Spain). L-Tyrosine (\geq 98%), L-DOPA (3,4-dihydroxy-L-phenylalanine; \geq 98%), kojic acid (5-hydroxy-2-hydroxymethyl-4H-4-pyranone; \geq 98.5%) and mushroom 115 tyrosinase (mTYR; EC 1.14.18.1, activity = 3130 units/mg) were purchased from Sigma-Aldrich and 116 117 used without further purification. Fresh mTYR solutions were prepared every day and tyrosinase 118 activity was analysed spectrophotometrically to adjust solutions to fixed tyrosinase Sigma units for 119 consistent results. Briefly, one Sigma unit corresponds to the amount that will cause an increase in 120 absorbance at 280 nm of 0.001 per minute at pH 6.8 in a 3 mL reaction mixture containing L-tyrosine. Sigma units were used throughout this study. One Sigma unit corresponds to 1.65×10^{-4} international 121 units (I.U.) for monophenolase activity and to 2.24×10^{-2} I.U. for diphenolase activity, as defined by 122 123 Fenoll et al. (2002). AAPH (2,2'-azobis(2-methylpropionamidine) dihydrochloride), methyl linoleate (≥ 98%) and TritonTM X-100, were used as received. AIBN (2,2'-Azobis(isobutyronitrile); 98%) 124 125 was recrystallized from methanol, while 2,4,6-tri-tert-butylphenol (TBP, 98%) was recrystallized 126 from hexane. Stock solutions of AAPH or AIBN in the desired solvents were prepared immediately prior to use and/or maintained for maximum 2h or 4h, respectively, at 4°C between subsequent uses, 127 128 to avoid significant decomposition. Cumene (98%) and styrene (\geq 99%) were purified by double 129 percolation through silica and activated basic alumina columns. Solvents and other chemicals were

- 130 of the highest grade commercially available (Sigma-Aldrich, Merck, VWR; Milan, Italy) and were 131 used as received.
- 132 2.2 Synthesis of (S)-(+)-Bakuchiol methyl ether
- 133 MeOBak ((3S,1E)-1-(3,7-dimethyl-3-vinylocta-1,6-dien-1-yl)-4-methoxybenzene) was prepared by cautious addition of 0.2 mmol of bakuchiol (in dry DMF) to a suspension of 1.4 eq. NaH in dry DMF 134 135 at 0°C under N₂. The mixture was stirred for 30 min while allowing to reach r.t., then MeI (1.4 eq)
- was added dropwise and the mixture was stirred for 4h at r.t. then quenched with brine and extracted 137 with hexane. The dried (Na_2SO_4) extract was evaporated under vacuum do afford a yellowish oil
- 138 which was purified by column chromatography on silica gel, eluting with hexane/EtOAc 95:5 (yield
- 139 85%; purity 99% by GC-MS, see Appendix).

- 140 ¹H NMR (400 MHz; CDCl₃) δ 7.30 (2H, d, J = 9 Hz), 6.80 (2H, d, J = 9 Hz,), 6.30 (1H, d, J = 16
- 141 Hz), 6.10 (1H, d, *J* = 16 Hz), 5.88 (1H, dd, *J* = 18, 11 Hz), 5.11 (1H, t, *J* = 7 Hz), 5.02 (2H, m), 3.80
- 142 (3H, s, OC<u>H</u>₃), 1.95 (2H, dt, *J* = 9, 8 Hz), 1.70 (3H, s), 1.57 (3H, s), 1.50 (2H, m), 1.20 (3H, s) ppm;
- in agreement with literature (Hu & Brenner-Moyer, 2022). 143
- 144 MS (EI⁺, 70 eV) *m/z*: 271 (2), 270 (M⁺, 10), 255 (3), 227 (10), 188 (17), 187 (100), 173 (10), 172
- 145 (23), 159 (29), 158 (18), 144 (19), 135 (19), 121 (66), 93 (24), 91 (18), 83 (17), 79 (25), 69 (29), 55 146 (39), 41 (69).
- 147 2.3 mTYR kinetic studies by UV-vis spectrophotometry

Kinetic evaluation of tyrosinase reaction with or without inhibitor was carried out using UV-Vis 148 spectrophotometry similarly to previous methods (Copeland, 2000; Song et al., 2021; Yu & Fan, 149 150 2021), following our recent protocol (Guo et al., 2022). Measurements were performed at 30°C in 151 phosphate buffer (50 mM, pH 6.8) in polystyrene low-volume cuvettes (1.5 mL, l = 1 cm) with a double-beam spectrophotometer. L-Tyrosine and L-Dopa (5 levels, 0.125-2 mM) were used as the 152 substrate of mushroom tyrosinase (mTYR, 0.5-5.0 U/ml) for monophenolase and diphenolase 153 154 reactions, respectively. Bakuchiol (0 to 12 μ M) and kojic acid (0 to 50 μ M) were comparatively tested 155 as inhibitors. Absorbance of dopachrome was measured at 475 nm for a time-period of 20 to 60 min

reading the solution against a reference cuvette containing all reaction components except the 156 157 substrate. Absorbance variation vs time at different substrate concentration allowed to obtain initial velocity (V = $\Delta A/\Delta min$) which was converted in $\mu M/min$ according to Lambert-Beer law as follows: 158 V (μ M/min) = V (Δ A/ Δ min) × 10⁶/ $\epsilon_{\lambda max}$ × *l*. The molar extinction coefficient (ϵ) for dopachrome at 159 λ_{max} = 475 nm is 3700 M⁻¹ cm⁻¹. Michaelis-Menten parameters (K_m e V_{max}) were obtained by 160 161 processing initial velocity vs substrate concentration data by nonlinear fitting to M-M equation (1), using Sigmaplot 11.0 (Systat Software Inc., San Jose, California) (Guo et al., 2022). Linearized 162 163 Lineweaver-Burk equation (2) was used to identify the inhibition mode (Copeland, 2000). In both 164 equations, V indicates the measured initial rate of reaction, [S] is the initial substrate concentration, 165 while V_{max} and K_{m} are respectively the maximum reaction rate (at saturating substrate concentration) and the M-M constant. (the substrate concentration yielding half-maximum reaction rate).[†] 166

$$V = \frac{V_{max}\left[S\right]}{K_m + \left[S\right]} \tag{1}$$

$$\frac{1}{V} = \frac{K_m}{V_{max}\left[S\right]} + \frac{1}{V_{max}} \tag{2}$$

169 2.4 mTYR kinetic studies by oximetry

170 Evaluation of tyrosinase monophenolase and diphenolase kinetics and inhibition by bakuchiol or kojic acid was carried out by monitoring the oxygen consumption, at 30°C, by miniaturized oxygen 171 172 sensing apparatus, based on NIR (760-790 nm) fluorescence quenching, which has previously been 173 described in detail (Guo et al., 2022). The samples were contained in stirred 2.4 mL glass flasks, 174 immersed in a water bath. The oxygen consumption was recorded every second, and all reaction 175 components were maintained as those set for UV-vis spectrophotometry. The raw data collected 176 directly from the oxygen sensor is a percentage (P) of the saturating oxygen concentration in the sample, corresponding to 0.236 mM at 30°C which reflects in the sensor reading as 20% (P₀). Thus, 177 178 the oxygen concentration during the enzymatic oxidation of L-DOPA or L-tyrosine were converted 179 into mM by the equation: $[O_2] (mM) = P \times 0.236 (mM) / 20\%$. The initial rate of oxygen consumption was obtained as (ΔP) vs time (in seconds) by regression of the initial data range of oxygen 180

181 consumption, and it was converted by equation: $V (\mu M/min) = V (\Delta P/\Delta t) \times 0.236 \times 10^3 \times 60 / P_0$ 182 which was used for analysis by non-linear fitting to Michaelis-Menten kinetics (eq. 1). Previous 183 studies indicate that the stoichiometry of O₂ uptake / dopachrome formation is 1.5:1 and 1:1 for 184 substrate L-tyrosine and L-DOPA, respectively (Guo et al. 2022), therefore the rate of O₂ uptake 185 recorded for monophenolase reaction was divided by 1.5.[†]

186 2.5 Fluorescence quenching studies

187 Fluorescence spectra arising from interactions between bakuchiol and tyrosinase were recorded by a 188 fluorescence spectrometer (Horiba FluroMax-4) at r.t. similarly to literature (Yu & Fan, 2021). A 2.5 189 mL solution of tyrosinase (20 U/mL) in 50 mM sodium phosphate buffer (pH 6.8) was placed in 190 guartz cuvettes and a bakuchiol stock solution in methanol was added portionwise, for a total of 10 191 additions, to obtain various concentration of bakuchiol in the solution, from 0 to 1.320 µM. Each 192 resultant solution was incubated for 1 min to equilibrate, before measurement. The excitation 193 wavelength was set at 280 nm and the emission spectra were collected from 290 to 500 nm with 194 excitation and emission slits kept at 5 nm.

195 2.6 Autoxidation studies in homogenous solution

196 Autoxidation experiments were performed in a two-channel oxygen uptake apparatus, based on a 197 Validyne DP 15 differential pressure transducer built in our laboratory (Amorati, Pedulli & 198 Valgimigli, 2011; Baschieri, et al., 2019; Amorati et al., 2016). In a typical experiment, an airsaturated solution of the oxidizable substrate containing AIBN (0.01-0.1M) was equilibrated at 30 °C 199 200 with an identical reference solution containing excess 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMHC, 25 mM). After equilibration, and when a constant O₂ consumption was reached, a stock 201 202 solution of the antioxidant (typically 1.0 mM in PhCl) was injected in the sample flask, so to reach 203 the desired concentration in the range 1-20 μ M in the sample flask. The oxygen consumption in the 204 sample was measured after calibration of the apparatus from the differential pressure recorded with 205 time between the two channels. Initiation rates, R_i , were determined for each condition in preliminary

experiments by the inhibitor method (eq. 3), where τ is the length of the inhibition period, using TOH as a reference antioxidant (Guo et al., 2021a; Guo et al., 2021b).

The inhibition rate constant for peroxyl radical trapping k_{inh} was obtained from oxygen consumption plots by eqs. 4 or 5, where k_p , and $2k_t$, are the rate constants for chain propagation and termination of the substrate, R_0 and R_{inh} are respectively the rate of oxygen consumption $(-d[O_2]/dt)$ in the absence and presence of the antioxidant, while *n* is the stoichiometric factor, *i.e.* the number of peroxyl radicals trapped by one molecule of antioxidant (Amorati & Valgimigli, 2018; Amorati et al., 2016). Styrene ($k_p = 41 \text{ M}^{-1}\text{s}^{-1}$; $2k_t = 4.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, at 30°C) and cumene ($k_p = 0.34 \text{ M}^{-1}\text{s}^{-1}$; $2k_t = 4.5 \times 10^4 \text{ M}^{-1}$

215
$$\tau = \frac{n[Antiox.]}{R_i}$$
(3)

216
$$-\frac{d[O_2]}{dt} = \frac{k_p[Substrate]R_i}{nk_{inh}[Antiox]}$$
(4)

$$\frac{R_0}{R_{inh}} - \frac{R_{inh}}{R_0} = \frac{nk_{inh}[Antiox]}{\sqrt{2k_t R_i}}$$
(5)

218 2.7 Autoxidation studies in aqueous micelles

217

219 Measurement were performed as previously described (Konopko & Litwinienko, 2022), with 220 modifications. In a typical experiment, 2.5 mL of air-saturated buffered (PBS, 50 mM, pH 7.4) 221 aqueous dispersion of MeLin (final concentration 2.74 mM) in TritonTM X-100 (final concentration 8 mM) micelles were prepared by vortex mixing, then adding a freshly prepared stock solution of 222 223 AAPH (final concentration 5 mM), immediately followed by addition 4-16 µL of a (0.5-1 mM) stock 224 solution of the antioxidant in acetonitrile (final concentration of 2-8 µM) at room temperature. After 225 brief additional vortex stirring (5-10 sec.) the mixture used to fill in a sealed 2.4 mL glass vial 226 provided with a PTFE-coated stirring bar. The sample was equilibrated at 37 °C in a thermostatted 227 bath equipped with a sealed magnetic stirrer and O₂ consumption was recorded. Oxygen concentration was monitored with the same equipment described for mTYR kinetics and data were 228 similarly treated by the equation: V (M/s) = V ($\Delta P/\Delta t$) × 0.210 × 10⁻³ / P₀, where P₀ is the initial 20% 229

O₂ saturation reading that corresponds to 0.210 mM at 37°C. Oxygen consumption in the presence of the antioxidant was compared with that recorded with identical reference mixtures lacking the antioxidant. The inhibition rate constant was obtained from oxygen consumption plots by eqs. 3-5, as described for autoxidation studies in homogenous solution, using $k_p = 36 \text{ M}^{-1}\text{s}^{-1}$ for MeLin in micelles (Konopko & Litwinienko, 2022).

235 2.8 EPR measurements

236 Deoxygenated tert-butylbenzene solutions containing the phenols (0.01-0.001 M) and di-tert-butyl peroxide (10% v/v) were sealed under nitrogen in a suprasil quartz EPR tube. The sample was inserted 237 in the thermostatted (30°C) cavity of an X-band EPR spectrometer and photolyzed with a mercury-238 xenon lamp (240-400 nm, max 4500 mW/cm²). Spectra were recorded with the following settings: 239 240 modulation amplitude 0.1-1 Gauss, sweep width 60 Gauss, modulation frequency 100 kHz, frequency 241 9.76 GHz, sweep time 60s, microwave power 0.1-1 mW. Measured g-factors, were corrected with 242 respect to that of the perylene radical cation in concentrated H_2SO_4 (g = 2.00258) (Amorati et al., 2011; Valgimigli et al., 2000). When using mixtures of TBP and bakuchiol in ReqEPR experiments, 243 244 the molar ratio of the two equilibrating radicals was obtained from the EPR spectra and used to 245 determine the equilibrium constant, K_{eq} (Johansson et al. 2010; McGrath, Garrett, Valgimigli & Pratt, 246 2010). Different irradiation power levels (20% to 100%) and different ratios of the two phenols were 247 tested to guarantee that the two species where at the equilibrium (Amorati et al., 2010). Spectral 248 parameters and relative radical concentrations were determined by comparison of the digitized 249 experimental spectra with computer simulated ones, as previously described (Amorati et al., 2010; 250 Amorati et al., 2011; Brigati, Lucarini, Mugnaini, & Pedulli, 2002).

251 2.9 Statistical analysis

Each measurement was performed at least in triplicate. Values of V_{max} and V_{max}^{app} , and of K_m and K_m^{app} in the absence and presence of inhibitors were determined from non-linear regression of M-M plots based on 5-6 concentrations of the substrate, which were analysed by Shapiro-Wilk Test with significance set at $P \le 0.05$. Results are expressed as regression value \pm standard error.

257 3. Results

258 3.1 Kinetics of mTYR inhibition

н

$$HO \xrightarrow{\text{COOH}}_{\text{L-Tyrosine}} \xrightarrow{\text{TYR}}_{\text{HO}} \xrightarrow{\text{HO}}_{\text{HO}} \xrightarrow{\text{COOH}}_{\text{NH}_2} (6)$$

260

259

$$HO \qquad COOH \qquad TYR \qquad O \qquad COOH \qquad HO \qquad NH_2 \qquad 1/2 O_2 \qquad H_2O \qquad O \qquad NH_2 \qquad (7)$$

$$O + COOH + COH$$

261

The kinetics of monophenolase and diphenolase reactions of mTYR were studied at 30°C (pH 6.8) 262 by monitoring the oxygen consumption, respectively during the oxidation of L-tyrosine to L-Dopa 263 264 (6) or of L-Dopa to dopaquinone (DQ) (7), and recording the initial rate as a function of the 265 concentration of the substrate, to afford the typical Michaelis-Menten (M-M) hyperbolic curve (eq.1) 266 (Guo et al., 2022). At each concentration of substrate, the reaction was performed under identical 267 settings either in the absence of inhibitors or in the presence of different concentrations of bakuchiol, 268 clearly showing significant inhibition already in the low micromolar range (Figure 2). Kinetic analysis was performed by non-linear fitting to M-M equation (eq. 1, Figure 2A,C) while the 269 linearized double reciprocal Lineweaver-Burk (L-B) plot (eq. 2, Figure 2B,D) was used to identify 270 271 the inhibition mode. This approach was found to offer higher accuracy than obtaining the relevant 272 kinetic parameters (V_{max} and K_{m}) from the intercepts using Lineweaver-Burk plots (Guo et al., 2022; 273 Copeland, 2000).

274 Concerning the inhibition mode, both for monophenolase and diphenolase reactions, regression lines 275 in L-B plots obtained at different concentration of inhibitor crossed at (or in close proximity to) the Y axis Figure 2B,D), implying a constant value of V_{max} – the maximum rate of enzyme reaction at 276 277 saturating substrate concentration – on increasing the concentration of the inhibitor, which is indicative of a reversible competitive inhibition (Copeland, 2000). Competitive inhibition is confirmed by analysis of V_{max} and K_{m} values obtained by fitting M-M curves (Figure 2A, C), collected in Table 1. While V_{max} remains constant within experimental error, the M-M constant K_{m} – the concentration of substrate required to produce half-maximum reaction rate – increases linearly with the concentration of the inhibitors (Guo et al., 2022; Copeland, 2000). Confirmation of this behavior, typical of competitive inhibitors, is given by the secondary M-M plots of $K_{\text{m}} vs$ [bakuchiol] (Figures S4, S5 in Appendix).

To further confirm the above findings, the reactions were also investigated in parallel by the 285 286 conventional spectrophotometric approach, monitoring the formation of dopachrome at 475 nm (6-287 8), under otherways identical settings. Results (Figure S2, S3 in Appendix) were in excellent agreement and the corresponding V_{max} and K_{m} are compared to those obtained by oxygen sensing in 288 289 Table 1. Average inhibition constant K_i – the dissociation constant of the enzyme-inhibitor complex 290 – was 6.71 \pm 1.23 μ M for monophenolase and 1.15 \pm 0.34 μ M for diphenolase reactions. Such low values indicate high inhibition potency and should be compared with the higher K_i values of 10.91 291 292 µM and 9.91 µM previously measured under similar settings for reference inhibitor kojic acid (Guo 293 et al., 2022).

294

295 *3.2 IC*₅₀ values for mTYR inhibition by bakuchiol

Although K_i is the most reliable parameter to quantify and compare the potency of enzyme inhibitors, as it does not depend on enzyme and substrate concentrations (Copeland, 2000), it is most common in the literature to find activity quantified by the IC₅₀ values: the concentration of the inhibitor affording 50% reduction of the enzyme reaction rate. Hence IC₅₀ values for bakuchiol were measured by comparing the rate of reaction at fixed enzyme activity in the absence (V_0) and presence (V_1) of increasing inhibitor concentration [I], according to Langmuir equation (9) (Guo et al., 2022; Copeland, 2000).

$$\frac{V_I}{V_0} = \frac{1}{1 + \frac{[1]}{1 + \frac{[1]}{1 + \frac{[1]}{C_{50}}}}} \tag{9}$$

The resulting values obtained both by O_2 uptake and by spectrophotometry are given in the Appendix (Tables S1, S2) and, as expected (Copeland, 2000), they grow linearly with the concentration of substrate. While this somewhat limits their usefulness, comparison among inhibitors is possible by referring to similar settings, *e.g.* to a standard 1 mM substrate concentration.

308 Our values for monophenolase and diphenolase reaction, averaged between O_2 uptake and 309 spectrophotometry, were $37.22 \pm 5.18 \ \mu\text{M}$ and 6.76 ± 0.73 , respectively (Table 1). Reference kojic 310 acid was investigated for comparison, affording $34.02 \pm 5.51 \ \mu\text{M}$ and $16.86 \pm 3.28 \ \mu\text{M}$, at 1 mM L-311 tyrosine and L-DOPA, respectively, in excellent agreement with previous studies by our group (Guo 312 et al., 2022),²² and by others (He at al., 2021).

314 *3.3 Fluorescence quenching study*

315 The quenching of intrinsic tryptophan fluorescence of mTYR by inhibitors is often investigated to confirm the nature of enzyme-inhibitor interaction (Yu & Fan, 2021). To this end, the fluorescence 316 of mTYR in 50 mM phosphate buffer (pH 6.8) in the range 290-500 nm (λ_{max} 338 nm) upon excitation 317 318 at 280 nm was recorded either in the absence of inhibitor (F₀) and in the presence of growing 319 concentration of bakuchiol (Figure 2E), in the absence of substrate. On increasing the concentration 320 of bakuchiol fluorescence intensity (F) progressively decreased, without significant shifting of the 321 band maxima, indicating no major change in the conformation of the protein (Yu & Fan, 2021). Stern-Volmer plot (eq.10), relating the relative fluorescence intensity to the concentration of a quencher 322 323 [Q], was used to analyze the quenching type, either static or dynamic.

$$F_0/F = 1 + k_q \tau_0[Q] = 1 + K_{SV}[Q]$$
(10)

In eq. 10 and k_q and K_{SV} are respectively the quenching rate constant and the Stern-Volmer constant, while τ_0 (=10⁻⁸ s) is the typical lifetime of the fluorophore in the absence of the quencher (Mátyus, Szöllósi & Jenei, 2006). Figure 2F shows that the plot was linear only at low quencher concentration, 328 while it shows upward curvature for higher Bak levels. This behavior is often encountered in case of 329 static quenching, *i.e.* when the protein and the quencher form a non-emitting stable complex (Mátyus et al., 2006). The K_{SV} and k_q constants determined by fitting the plot in the linear region afforded K_{SV} 330 $=4.32 \times 10^5 \text{ M}^{-1}$ and $k_p = 4.32 \times 10^{13} \text{ M}^{-1}\text{s}^{-1}$. The bimolecular quenching rate constant k_p for bakuchiol 331 332 is much greater than the maximum scatter collision quenching constant of dynamic quenchers with proteins, $(2.0 \times 10^{10} \text{ M}^{-1} \text{s}^{-1})$, which indicates that the static quenching mechanism is dominating, 333 implying the formation of a stable complex with mTYR (Yu & Fan, 2021). Dominance of the static 334 quenching mechanism is also confirmed by fitting the data to the modified Stern-Volmer plot for 335 static quenching (see Figure S6 in Appendix) (Yu & Fan, 2021; Mátyus et al., 2006). The apparent 336 337 binding constant (K_a) and the number of binding sites (n) for complex formation between bakuchiol and mTYR were obtained by processing fluorescence data with eq. 11 (Figure 2G) (Yu & Fan, 2021). 338 Results were n = 1.06 and $K_a = 1.02 \times 10^6 \text{ M}^{-1}$ indicating a single binding site and high affinity of 339 340 complex formation, fully consistent with the results of enzyme inhibition.

$$Log [(F_0 - F)/F] = Log K_a + nLog[Q]$$
(11)

342

343 3.4 EPR and ReqEPR spectroscopy of bakuchiol phenoxyl radical

Reaction of the O-H group with oxidizing or chain-carrying radicals to afford the corresponding stabilized phenoxyl radical is the key process sustaining the activity of phenolic antioxidants (Valgimigli & Pratt, 2015), hence we investigated the phenoxyl radical stability to shed more light on the antioxidant mechanism of bakuchiol.

348

$$^{t}BOO^{t}B \xrightarrow{\text{fiv}} 2^{t}BO \bullet$$
 (12)

 $^{t}BO \bullet + BakOH \longrightarrow ^{t}BOH + BakO \bullet$ (13)

Photolysis of di-*tert*-butylperoxide in the presence of bakuchiol in the cavity of the EPR spectrometer generated alkoxyl radicals that were trapped forming bakuchiol phenoxyl radical (eqs. 12,13), which was identified by its spectrum, reported here for the first time (Figure 3A). It showed large hyperfine splitting constants (hfsc/Gauss, Table 2) due to coupling with the two *ortho* hydrogens (6.67 G) and with the *para* -CH= (7.05 G), and lower values for coupling with the hydrogen in vinylic position
(3.27 G) and with the hydrogens in *meta*-position (1.94 G) in line with structurally related radicals
(Amorati et al., 2010; Brigati et al., 2002; Amorati, Ferroni, Pedulli & Valgimigli, 2003). Accordingly,
the *g*-factor = 2.0041 was typical of phenoxyl radicals and indicative of C/O coupling.

Since the reactivity of phenols like bakuchiol with radicals, hence the antioxidant activity, is dictated 358 359 by the bond dissociation enthalpy (BDE) of the O-H group, we measured it by the ReqEPR technique 360 (Johansson et al., 2010; Brigati et al., 2002), which consists in photolyzing the unknown phenol (^UPhOH = bakuchiol) in mixture with a reference phenol (^RPhOH) whose BDE(OH) is known, so to 361 establish their equilibration (eq. 14). Measurement of the corresponding equilibrium constant (eq. 15) 362 363 is achieved by numerical simulation of the EPR spectrum obtained from the mixture, due to 364 superimposition of the EPR signals of the two radicals, which affords their relative ratio (Figure 3B). 365 This affords the ΔH of the reaction via eq. 16 under the reasonable assumption that the entropy change is negligible ($\Delta H \approx \Delta G$) (Brigati et al., 2022). Using 2,4,6-tri-*tert*-butylphenol (TBP) as the reference, 366 367 the BDE(OH) of bakuchiol was determined (eq. 17) as 81.7±0.1 kcal/mol (Table 2), which well justifies its good antioxidant activity (vide infra). Interestingly, this value is similar to that of the 368 369 weakest OH group in (structurally related) resveratrol which, although not experimentally known, 370 can be estimated as ~ 81.4 Kcal/mol by averaging the calculated (DFT) value (80.2 Kcal/mol) and 371 the empirical value obtained by the group additivity rule (82.6 Kcal/mol) (Amorati et al., 2003).

$${}^{\text{R}}\text{PhOH} + {}^{\text{U}}\text{PhO} \bullet \rightleftharpoons {}^{\text{R}}\text{PhOH}$$
(14)

$$K_{eq} = \frac{[{}^{U}PhOH]}{[{}^{R}PhOH]} \times \frac{[{}^{R}PhO\bullet]}{[{}^{U}PhO\bullet]}$$
(15)

$$\Delta G^{\circ} = \Delta H^{\circ} - T \Delta S^{\circ} = -RT \ln K_{\rm eq}$$
(16)

$$BDE(^{U}PhOH) = BDE(^{R}PhOH) - \Delta H^{\circ}$$
(17)

376

377 *3.5 Antioxidant activity of bakuchiol in solution and in micelles*

378 The antioxidant activity of bakuchiol was investigated by monitoring oxygen consumption during the

controlled inhibited autoxidation of reference substrates, which is the golden standard in antioxidant 379 380 testing (Amorati & Valgimigli, 2018; Guo et al., 2021a; Guo et al., 2021b; Baschieri et al., 2019; 381 Amorati et al., 2016), both in homogenous organic solution and in heterogenous micellar system. In organic solution (PhCl, 30°C) we studied the inhibited autoxidation of two well established 382 oxidizable substrate, styrene and cumene, having largely different oxidation rates (k_p at 30°C is 41 383 and 0.34 M⁻¹s⁻¹, respectively) thereby affording complementary information (Baschieri et al., 2019). 384 385 In the autoxidation of styrene initiated by AIBN, bakuchiol produced a slow-down of the oxidation, without a detectable inhibition period, which was observed instead for reference antioxidant α -386 tocopherol (TOH), as shown in Figure 4A. This is due to the major difference in concentration 387 388 between the oxidizable substrate and the antioxidant (six orders of magnitude), which severely challenges the apparent performance of the antioxidant. Analysis of the oxygen uptake plots by eq. 5 389 afforded the inhibition rate constant – the rate constant for trapping alkylperoxyl radicals – k_{inh} of 390 2.20×10^5 M⁻¹s⁻¹ (Table 2), which was about one order of magnitude lower than that of TOH ($k_{inh} =$ 391 3.2×10^6 M⁻¹s⁻¹), nature's premiere lipid soluble antioxidant. The absence of a distinct inhibition 392 393 period did not produce information on the stoichiometric factor *n*, i.e. the number of peroxyl radicals 394 trapped by one molecule of antioxidant. This however was obtained by studying the inhibited autoxidation of cumene. Owing to the lower k_p , cumene gave clear inhibition periods in the presence 395 396 of micromolar bakuchiol, which were proportional to its concentration (Figure 4B), allowing to 397 determine n as 1.9 ± 0.1 , which is the typical value (n = 2) expected for a monophenolic antioxidant. The inhibition constant $k_{inh} = 1.42 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ is in good agreement with that measured with styrene, 398 despite the lower oxidative chain length during inhibition (Table 2).[§] The resulting averaged (styrene 399 and cumene) value for k_{inh} of bakuchiol in PhCl solution was $1.8 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. This value, although 400 401 lower that reference TOH, is identical within experimental error to that of well-established antioxidant resveratrol $(2.0 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \text{ at } 30^{\circ}\text{C} \text{ (Amorati et al., 2003))}.$ 402

The antioxidant activity in heterogenous systems was investigated using the autoxidation of methyl
linoleate (MeLin, 2.74 mM) in Triton[™] X-100 micelles (8 mM) initiated by water soluble AAPH at

405 37°C, which is a well-validated kinetic model (Konopko & Litwinienko, 2022). Results summarized 406 in Figure 4C show that bakuchiol gave distinct inhibition of the autoxidation, whose duration was 407 proportional to its concentration. Comparison with TOH showed almost identical duration of the 408 inhibition which afforded (eq. 3) the stoichiometric factor as $n = 1.9 \pm 0.1$, in accordance with the 409 value recorded in organic solution.

The inhibition constant was obtained from the slope of the inhibited period by eq. 4, as $k_{inh} = (1.6 \pm 0.3) \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ (Table 2). For comparison, reference TOH under the same experimental settings afforded $k_{inh} = (1.5 \pm 0.2) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ in good agreement with literature using the same kinetic model (Konopko & Litwinienko, 2022). As also found in homogenous solution, bakuchiol traps peroxyl radical in micelles about one order of magnitude slower than TOH; however, it is interesting to note that its antioxidant activity in micelles is almost identical to that recently reported for resveratrol (1.5 $\times 10^3 \text{ M}^{-1}\text{s}^{-1}$ at 37° pH 7 (Konopko & Litwinienko, 2022)).

417

418 *3.6 Antioxidant activity of bakuchiol O-methyl derivative MeOBak*

419 Since a previous study suggested that bakuchiol antioxidant activity is due only in part to the phenolic 420 -OH group, while the terpenic chain in 4-position also contributes by trapping radicals (Adhikari et 421 al., 2003), we synthesized the protected MeOBak derivative by O-methylation of bakuchiol, and 422 tested it as an antioxidant in parallel autoxidation studies, using the oxidation of styrene in PhCl 423 solution and of MeLin in TritonTM X-100 micelles as model systems.

When tested under experimental settings identical to those used of native Bak, MeOBac showed no inhibition of the autoxidation of styrene (Figure 4A): similarly, no protection was observed in the autoxidation of MeLin in micelles, where oxygen consumption plots recorded in the presence of MeOBak were hardly distinguishable from those obtained without antioxidant (Figure 4C). This allowed to exclude any direct involvement of the terpene chain in the antioxidant activity of bakuchiol under our experimental settings (*vide infra*).

431 4. Discussion

432 Kinetic studies on tyrosinase inhibition performed in parallel both by real time O₂ sensing and by spectrophotometric monitoring of dopachrome afforded superimposable results which support each-433 434 other and demonstrate major inhibition efficacy by bakuchiol, already in the low micromolar range, at variance with a previous report that indicated very modest inhibition, even at concentration of 1 435 436 mg/mL (West et al., 2021). Inhibition is competitive both for monophenolase and diphenolase 437 reaction, with M-M related inhibition constant K_i of 6.71 \pm 1.23 μ M and 1.15 \pm 0.34 μ M, respectively, indicating higher inhibition potency for diphenolase reaction – it should be recalled that lower values 438 439 indicate higher activity. K_i values measured in kinetic studies, which represent the dissociation constant of the enzyme-inhibitor complex, are fully consistent with the $K_{\rm a}$ value of $1.02 \times 10^6 \ {
m M}^{-1}$ 440 determined by fluorescence quenching, which represents the apparent formation constant for such 441 442 complex. Interestingly, K_i values are lower than those we previously measured for reference inhibitor 443 kojic acid (10.91 µM and 9.91 µM for monophenolase and diphenolase inhibition respectively) (Guo 444 et al., 2022), indicating higher bioactivity of bakuchiol.

445 Although less robust in quantifying inhibition potency, owing to their dependence on substrate and 446 enzyme concentration, IC₅₀ values also support high anti-tyrosinase activity of bakuchiol. Values measured by O₂ sensing ranged 12-33 µM for monophenolase inhibition and 2-18 µM for diphenolase 447 448 inhibition in the tested concentration range (see Appendix), while values obtained by spectrophotometry nicely matched the above (see Appendix). Taking 1 mM substrate as reference 449 setting for comparison and using averaged results from O₂ sensing and spectrophotometry, IC₅₀ 450 451 values measured here for kojic acid indicate similar activity for monophenolase inhibition (34.02 µM 452 vs 37.22 μ M), while for diphenolase inhibition bakuchiol (IC₅₀ = 6.76 μ M) was sensibly more effective than kojic acid (IC₅₀ = 16.86 μ M). This is at variance with a previous study reporting IC₅₀ 453 454 values of bakuchiol 18-folds higher than kojic acid for diphenolase inhibition (Cheng & Chen, 2017), and proves a sensibly higher anti-tyrosinase activity of bakuchiol than previously expected. 455

456 It was recently shown by Kang et al. (2020). that a phytoestract of *P. corylifolia* containing 77% 457 bakuchiol reduced melanin biosynthesis in normal human epidermal melanocytes and the activity was attributed to reduction of tyrosinase enzyme expression and to reduction of TRP-1, TRP-2 and 458 459 SOX-9 supporting proteins in melanocytes, without significant toxicity. The study showed also a 460 downregulation of melanocyte dendrites formation, necessary to the transfer of melanosomes to 461 neighboring keratinocytes. Our current data highlight an additional complementary mechanism for 462 the depigmenting activity outlined in that (Kang et al., 2020) and other studies (Dhaliwal et al., 2019; 463 West et al., 2021): the potent inhibition of tyrosinase reaction. This hopefully completes the picture allowing full rationalization of bakuchiol's depigmenting activity. Most important, it opens to new 464 465 applications of bakuchiol based on its interference with tyrosinase chemistry (vide infra).

Being a phenolic compound, the antioxidant activity of bakuchiol is expectedly dictated by its ability to quench chain-carrying peroxyl radicals to the corresponding hydroperoxide, by formal H-atom transfer from the phenolic OH (18) (Amorati & Valgimigli, 2018). Inhibited autoxidation studies indicated a stoichiometric factor $n \sim 2$ (1.9±0.1) both in organic solution and in aqueous micelles; therefore, a second peroxyl radical is trapped by the resulting phenoxyl radical, most typically by addition to the aromatic ring in conjugated positions (arrows in eq. 19) (Valgimigli & Pratt, 2015), or possibly by styryl-type addition (19).

473
$$ROO \cdot + HO \longrightarrow ROOH + \cdot O \longrightarrow R$$
(18)

$$ROO \cdot + \cdot O \xrightarrow{\frown} R \xrightarrow{diff.} ROO \cdot + O \xrightarrow{\frown} OOR$$
(19)

474

Wherever the second ROO• trapping occurs, it is not regulating the antioxidant activity, which is
dictated by rate-determining reaction 18 (Amorati & Valgimigli, 2018). Since the BDE(OH) of the
alkylhydroperoxides is 88.6 kcal/mol (Amorati et al., 2011; Amorati, Menichetti, Mileo, Pedulli &
Viglianisi, 2009), our present measurement of the BDE(OH) of bakuchiol by ReqEPR as 81.7

479 kcal/mol affords the ΔH° of reaction 18 as -6.9 kcal/mol, which justifies bakuchiol's efficient trapping 480 of ROO• radicals ($k_{inh} = 1.8 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ in PhCl solution at 30°C).

481 Indeed, the BDE(OH) value of bakuchiol can be correlated to its reactivity via linear Evans-Polanyi 482 relationships between $log(k_{inh})$ and BDE(OH), which are well established for phenolic antioxidants 483 Amorati, et al., 2009). When such a plot is built for differently substituted phenols using literature 484 data in apolar organic solution (Johansson et al., 2010; Amorati, et al., 2009), parallel correlations 485 lines are observed depending on the steric hindrance in ortho-position to the reactive OH group 486 (Figure 4D). It should be noted that bakuchiol perfectly fits in the correlation line for phenols lacking 487 ortho substituents, implying that its reactivity with peroxyl radicals stems entirely from reaction of 488 the phenolic OH group, while the terpenoid chain would influence its reactivity only due to its electronic properties, *i.e.* by stabilizing the phenoxyl radical and lowering the BDE(OH). 489

490 Our findings are at variance with a previous study which attributed part of the antioxidant activity of 491 bakuchiol to the terpenoid chain (Adhikari, et al., 2003). This conclusion was drawn mainly for the 492 finding that the O-methylated derivative (MeOBak) reacted with thivl radicals (by H-abstraction in 493 the allyl positions) forming detectable transient species. However, it should be noted that the reaction 494 of the side chain with some radical does not imply its antioxidant action. Indeed, the formed C-495 centered radical (R•) would rapidly react with oxygen forming an alkylperoxyl radical ROO• that likely propagates the oxidative chain (Amorati & Valgimigli, 2018). As a proof of concept, we 496 497 prepared MeOBak and tested it as an antioxidant both in the autoxidation of styrene in solution and 498 of MeLin in micelles. Our results indicated no antioxidant activity of MeOBak in both systems under 499 our testing conditions, confirming that the antioxidant activity of bakuchiol stems entirely from the 500 phenolic OH, while the side chain would contribute by increasing its reactivity. However, it is 501 possible that at much higher concentration and depending on the exact experimental settings, the 502 terpenic chain would have chain termination-enhancing behavior, and show some minor antioxidant 503 activity via a different mechanism we previously disclosed for non-phenolic terpenoids like linalool 504 (Baschieri, Ajvazi, Tonfack, Valgimigli, & Amorati, 2017).

The lower antioxidant activity of bakuchiol in micelles compared to apolar organic solution (k_{inh} of 1.6 × 10³ M⁻¹s⁻¹ vs 1.8 × 10⁵ M⁻¹s⁻¹) follows the well-known behavior of any phenolic antioxidant and it pairs with a similar reduction in activity of reference TOH (k_{inh} from 3.2 × 10⁶ M⁻¹s⁻¹ to 1.5 × 10⁴ M⁻¹s⁻¹). It is partly due to H-bonding of the phenolic OH group at the water-lipid interface and partly due to rate-limiting exchange of radicals and antioxidants among micellar particles (Amorati et al., 2016).

511 It is interesting to note that, both in apolar organic solution and in aqueous micelles, the antioxidant 512 activity of bakuchiol perfectly matches that of well-established antioxidant resveratrol, which boosts 513 the interest for the potential applications of bakuchiol.

The co-existence of good antioxidant activity and high tyrosinase inhibiting activity fully justifies the
interest for bakuchiol in topical skin-care treatments, *e.g.* against photoaging (Chaudhuri et al., 2014;
Dhaliwal et al., 2019; West et al. 2021; Mahdavi et al., 2022; Zhu et al., 2022), which meets a growing
demand for plant-derived compounds with such bioactivity (Panzella & Napolitano, 2019).

Most interestingly, our results also suggest a previously overlooked potential of bakuchiol: its use as natural food preservative. Not only bakuchiol outperforms reference kojic acid as tyrosinase inhibitor, it also outperforms by over one order of magnitude the efficacy in peroxyl radical trapping of ubiquitous food preservative butylated hydroxytoluene (BHT, $k_{inh} \sim 1 \times 10^4$ M⁻¹s⁻¹ at 30°C in PhCl) (Amorati et al., 2003), promising much improved protection against oxidative damage combined with excellent protection from enzymatic food-browning.

524

525 5. Conclusions

This study addresses for the first time on quantitative grounds the kinetics of tyrosinase inhibition and of peroxyl radical trapping by bakuchiol, highlighting anti-tyrosinase activity significantly higher than previously expected, with competitive mechanism both toward monophenolase and diphenolase reactions and K_i values of 6.71 ± 1.23 µM and 1.15 ± 0.34 µM respectively. These values are significantly lower than the values for reference kojic acid implying higher potency, confirmed also

by the measured IC₅₀ values (1 mM substrate) of 37.22 µM, and 6.76 µM for monophenolase and 531 532 diphenolase inhibition by bakuchiol vs 34.02 µM and 16.86 µM for kojic acid. This verifies our initial hypothesis. At the same time, the rate constant of peroxyl radical trapping k_{inh} of $1.8 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ 533 (PhCl solution) and the corresponding value in aqueous micelles are indistinguishable from those of 534 well-established antioxidant resveratrol (Konopko & Litwinienko, 2022) and over 10-folds larger 535 536 than those of ubiquitous food preservative BHT (Amorati et al., 2003). They stem entirely from the 537 phenolic function and justify the interest for this food-borne molecule. While our quantitative data help rationalize the activity shown in skin-care treatments (Dhaliwal et al., 2019; West et al. 2021;), 538 539 the combination of such two properties (anti-tyrosinase and antioxidant) offers full rational for a 540 previously overlooked application as natural food preservative, potentially able to contrast food spoilage arising both from air oxidation and from enzymatic browning, which are regarded in the 541 542 food industry as the main undesired events in post-harvest processing and preservation of fresh food 543 (Mahdavi et al., 2022). The lack of significant toxicity arising from previous studies (Dhaliwal et al., 2019; Kang et al., 2020) and the reported antimicrobial/antifungal activities (Alam et al., 2018) make 544 545 this phytochemical even more interesting in this regard. Thus, our current results call for further 546 research to fully explore the previously overlooked potential of bakuchiol in the food industry.

547

548 Abbreviations

AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride; AIBN, 2,2'-azobis(isobutyronitrile); Bak,
bakuchiol; L-DOPA, levo-dihydroxyphenylalanine; MeLin, methyl linoleate; mTYR, mushroom
tyrosinase; OSS, oxidative stress status; ROS, reactive oxygen species; SOX9, 9th transcription factor
of the Sry high-mobility-group-box family; TBP, 2,4,6-tri-*tert*-butylphenol; α-TOH, alphatocopherol; TRP-1, tyrosinase related protein 1; TRP-2, tyrosinase related protein 2; TYR, tyrosinase.

554

555 Declaration of interest

556 The authors declare no competing financial interest.

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562 Appendix A. Supplementary data

563 GC-MS analysis of bakuchiol and MeOBak, kinetic plots of tyrosinase inhibition monitored by

spectrophotometry, tables of IC_{50} values of bakuchiol, additional plots of fluorescence quenching

and additional ReqEPR spectra, oxygen uptake during anzymatic vs. spontaneous oxidation of

566 Dopa.

- 567 Notes
- [†] DOPA in aqueous solution undergoes spontaneous autocatalytic oxidation (Roginsky, Barsukova,

569 Bruchelt, & Stegmann, 1997); however, this was estimated to account for less than 2% of our

570 measured rates of mTYR-catalyzed reaction, having non-significant influence on the reported

- 571 kinetics (see Appendix, Figure S8).
- [§] In cumene TOH produced complete inhibition (not shown) *i.e.* the chain length v_{inh} was close to 1,

therefore it was used as a reference only for measuring the rate of initiation R_i via eq 3.

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- 696 Zhu, Y.-Z., Chen, K., Chen, Y.-L., Zhang, C., Xie, Y.-Y., Hider, R. C. & Zhou, T. (2022) Design
- and synthesis of novel stilbene-hydroxypyridinone hybrids as tyrosinase inhibitors and their
- application in the anti-browning of freshly-cut apples. *Food Chemistry*, *3*85, 132730. DOI:
- 699 10.1016/j.foodchem.2022.132730.

- 701 **Table 1.** Kinetic parameters of tyrosinase inhibition by bakuchiol at 30° C (pH = 6.8). Kinetic data
- 702 were obtained by non-linear fitting of M-M plots at different bakuchiol concentrations, by O₂

	Μ	onophenolase (su	bstrate = L-t	yrosine)	
	ann	$O_2 U$	ptake		
Bakuchiol (µ <i>M</i>)	K_m or K_m^{upp} (mM)	V_m or V_m^{upp} (μ M/min)	<i>K</i> _{<i>I</i>} (μM)	Average <i>K_I</i> (µM)	<i>IC</i> ₅₀ (μM) 1 mM subs
0	0.19 ± 0.02	4.20 ± 0.10	-		1 11111 5 4005
1	0.21 ± 0.01	3.98 ± 0.25	8.04	_	33.35 ±3.41
2	0.26 ± 0.03	3.96 ± 0.05	5.80	- 6.93	
4	0.30 ± 0.02	4.02 ± 0.10	7.11	$- \pm 0.93$	
8	0.41 ± 0.04	4.00 ± 0.17	6.76	_	
	UV-Vis	Spectrophotomet	ry (dopachror	ne formation)	
Bakuchiol (µ <i>M</i>)	K_m or K_m^{app} (mM)	V_m or V_m^{app} (μ M/min)	<i>KI</i> (μM)	Average <i>K_I</i> (µM)	<i>IC</i> ₅₀ (μM) 1 mM subs
0	0.19 ± 0.02	4.11 ± 0.14	-		
1	0.22 ± 0.02	4.13 ± 0.16	5.92	6.49	41.09
4	0.30 ± 0.01	4.12 ± 0.07	6.48	- ±0.58	±3.12
8	0.40 ± 0.03	4.16 ± 0.13	7.07	_	
1	Averaged value	s (O ₂ and UV-Vis))	6.71 ± 1.23	37.22 ± 5.1
		Diphenolase (sub	strate = L-D	OPA)	
		$O_2 U$	ptake		
Bakuchiol (µM)	$K_m \text{ or } K_m^{app}$ (mM)	V_m or V_m^{app} (μ M/min)	<i>K</i> _{<i>I</i>} (μM)	Average <i>K_I</i> (µM)	<i>IC</i> ₅₀ (μM) 1 mM subst
0	0.20 ± 0.01	9.84 ± 0.07	-		
0.5	0.32 ± 0.02	9.73 ± 0.17	0.81		
1	0.38 ± 0.02	9.62 ± 0.16	1.07	1.16	7.06
2	0.49 ± 0.03	9.75 ± 0.20	1.33	±0.28	±0.42
4	0.75 ± 0.09	9.82 ± 0.42	1.44	_	
	UV-Vis	Spectrophotometr	ry (dopachron	ne formation)	
Bakuchiol	K_m or K_m^{app} (mM)	V_m or V_m^{app}	K_I (uM)	Average K_I (µM)	<i>IC</i> ₅₀ (µ M)

-

1.03

1.26

1.11

1.13

 ± 0.12

 1.15 ± 0.34

sensing and UV-Vis spectrophotometry.^a 703

^a V_{max} and K_{m} or $V_{\text{max}}^{\text{app}}$ and $K_{\text{m}}^{\text{app}}$ refer to not inhibited and inhibited assays, respectively.[†] 704

 10.26 ± 0.25

 9.98 ± 0.24

 10.12 ± 0.38

 9.73 ± 0.51

Averaged values (O₂ and UV-Vis)

(mM)

 0.20 ± 0.02

 0.30 ± 0.03

 0.36 ± 0.05

 0.56 ± 0.09

(**µM**)

0

0.5

1

2

1 mM substr.

6.76

 ± 0.73

 6.91 ± 0.96

Table 2. EPR spectral parameters of bakuchiol phenoxyl radical, BDE(OH) determined by ReqEPR radical equilibration at 30°C in *tert*-butyl-benzene (n = 10), rate constants for trapping peroxyl radicals (k_{inh}) and stoichiometric factor (n) measured in the autoxidation of styrene and cumene in homogenous solution (PhCl, 30°C), and of MeLin dispersed in Triton X-100 micelles (37°C), all inhibited by bakuchiol.

Thermodynamics of the -OH group									
Radical		g-factor							
Bak(•)	6.67 (2H _{ortho})	; 1.94 (2H _{meta}); 7.05	5 (H _{para}); 3.27	(H _{vinyl})	2.0041				
TBP(•)		2.0046							
^U PhOH	^R PhOH	Keq		BDE (Kc	al/mol)				
Bak	Bak TBP 14.25 ± 2.07 81.7		81.7 ±	± 0.1					
Kinetics of ROO• trapping									
Substrate (medium)	$k_{\rm inh} (10^4{ m M}^{-1}{ m s}^{-1})$	n ^b		Vinh ^c				
Styrene (so	olution)	22.0 ± 3.0	n.d.		172				
Cumene (s	olution)	14.2 ± 2.2	1.9 ± 0.1		7.5				
Average in	solution	18.1 ± 6.6	1.9 ± 0.1						
MeLin (mi	icelles)	0.16 ± 0.03	1.9 ± 0.1		10.6				

^a Hyperfine splitting constants in Gauss (= 0.1 Tesla). ^b Stoichiometric factor = number of peroxyl radical trapped by one molecule of antioxidant. ^c Kinetic chain length $v_{inh} = R_{inh}/R$

727 FIGURE CAPTIONS

Figure 1. Structure of investigated bakuchiol and the methyl ether compared to retinol.

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730 Figure 2. (A-C) Kinetics of mTYR reaction (30°C, pH 6.8) monitored by O₂ uptake, inhibited by bakuchiol, for (A, B) monophenolase reaction (substrate = L-Tyrosine; mTYR 1.6 U/mL) and (C, D) 731 732 diphenolase reaction (substrate = L-DOPA, mTYR 0.8 U/mL). Graphs represent Michaelis-Menten 733 hyperbolic plots (A, C) and Lineweaver-Burk linear plots (B, D) of the same experiments. (E-G) 734 Fluorescence emission spectra of mTYR (20 U/mL) in the absence (a) and the presence (b-k) of increasing concentration of bakuchiol up to 1.320 µM (E), the corresponding Stern-Volmer plot (F), 735 736 and the log-log plot (eq. 11) relating fluorescence quenching to the number of binding sites (*n*) and 737 complex formation constant K_a (G).

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Figure 3. EPR (X-band) spectrum recorded by photolyzing in the cavity of the spectrometer (in *tert*butylbenzene/^tBOO^tB 9:1, at 30°C): (A) bakuchiol, (B) a mixture of bakuchiol and TBP 20:1.
Simulations were obtained by Monte Carlo method using the parameters in Table 2. The spectral lines
due to TBP• radical are indicated by an arrow (B): the resulting radical ratio was 1.26:1 (Bak•/TBP•).

Figure 4. Oxygen consumption plots recorded during the autoxidation of 4.3 M styrene in PhCl (A), and 3.6 M cumene in PhCl (B), both initiated by AIBN (0.05 M) at 30°C, or of 2.74 mM MeLin in 8 mM TritonTM X-100 micelles initiated by 5 mM AAPH at 37°C, pH 7 (C), without inhibitors (dashed line) or in the presence of bakuchiol, or MeOBak, or TOH, as indicated. Thin lines represent the regression of the inhibited periods. In panel (D) Evans-Polanyi correlation of the rate constant k_{inh} (at 30°C) for trapping ROO• radicals by phenols with 2,6 (*ortho*) substituents of different size *vs* their BDE(OH). The data point of bakuchiol is indicated by a full star (\star).



Figure 1. Structures of investigated bakuchiol and the methyl ether compared to retinol.



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