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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
29 tyrosinase inhibition by bakuchiol, by real-time oxygen sensing and UV-Vis monitoring (475nm),
30 showed competitive inhibition with average K_i constant (μM , 30°C , pH 6.8) of 6.71 ± 1.23 and
31 1.15 ± 0.34 for monophenolase and diphenolase reactions respectively, with respective IC_{50}
32 37.22 ± 5.18 and 6.91 ± 0.96 ~ at 1 mM substrate, compared to kojic acid IC_{50} 34.02 ± 5.51 and
33 16.86 ± 3.28 μM . Fluorescence quenching showed a single binding mode with formation constant K_a
34 $1.02 \times 10^6 \text{ M}^{-1}$. The antioxidant activity was studied by inhibited autooxidation of styrene and cumene
35 (PhCl, 30°C) affording inhibition constant $k_{\text{inh}} = 18.1 \pm 6.6$ ($10^4 \text{ M}^{-1} \text{ s}^{-1}$, 30°C) and of MeLin in Triton™
36 X-100 micelles giving $k_{\text{inh}} = 0.16 \pm 0.03$ ($10^4 \text{ M}^{-1} \text{ s}^{-1}$, 37°C). Stoichiometric factor was 1.9 ± 0.1 . ReqEPR
37 spectroscopy afforded the BDE(OH) as 81.7 ± 0.1 kcal/mol. Bakuchiol is a potent tyrosinase inhibitor
38 with good antioxidant activity having major potential as natural food preservative against oxidation
39 and food-browning.

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42 **Keywords:** bakuchiol; skin-whitening; melanin; food browning; antioxidant; peroxy radicals.

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47 **Compounds investigated in this study:**

48 (*S*)-Bakuchiol, CAS 10309-37-2 (PubChem CID: 5468522)

49 O-methylbakuchiol, CAS 10309-44-1 (PubChem CID: 14610678)

50 Kojic acid, CAS: 501-30-4 (PubChem CID: 3840)

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

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53 **1. Introduction**

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

61 Several studies have outlined important and diverse bioactivities of bakuchiol, such as
62 antiinflammatory, 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
66 (Krishna, Edachery & Athalathil, 2022), such as in anti-acne, anti-psoriasis and anti-age treatments
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).

70 Such efficacy is in part attributed to a reported antioxidant activity (Dhaliwal et al., 2019; Haraguchi,
71 Inoue, Tamura & Mizutani, 2000). The reactivity of bakuchiol with selected radicals was investigated
72 and its ability to protect rat brain homogenates from autoxidation was shown using the TBARS assay
73 (Adhikari et al., 2003); however, the absolute antioxidant activity and the trapping of most relevant
74 alkylperoxyl radicals, which represent the main mechanism sustaining direct antioxidant activity was
75 not determined, allowing no quantitative comparison with other antioxidants and no rational
76 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 μ M) (Cheng & Chen, 2017), appears too high to justify the depigmenting
86 efficacy outlined in clinical studies, leaving the matter essentially unsettled. Moreover, the inhibition
87 mechanism and the Michaelis-Menten related constants, which are most relevant in quantifying
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 &
93 Wang, 2021; Yang et al., 2021; Song, et al., 2021; Panzella & Napolitano, 2019), particularly for their
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
99 antioxidant activity of bakuchiol by state-of-the-art inhibited autoxidation studies both in
100 homogenous solution and in heterogeneous system (Amorati & Valgimigli, 2018; Guo, Baschieri,
101 Amorati & Valgimigli, 2021; Guo et al., 2021b), and by electron paramagnetic resonance (EPR)
102 (Amorati, Pedulli, Valgimigli, Johansson & Engman, 2010), so to complete the picture, affording the
103 mechanism and absolute kinetics and thermodynamics of peroxy radical trapping (Valgimigli & Pratt,
104 2015).

Our hypothesis was that bakuchiol's anti-tyrosinase potency is substantially higher than 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.

2. Materials and Methods

2.1 Materials

(*S*)-(+)-Bakuchiol (4-[(3*S*,1*E*)-3-ethenyl-3,7-dimethylocta-1,6-dien-1-yl]phenol; 99%) was from Cymit Quimica (Barcelona, Spain). L-Tyrosine ($\geq 98\%$), L-DOPA (3,4-dihydroxy-L-phenylalanine; $\geq 98\%$), kojic acid (5-hydroxy-2-hydroxymethyl-4H-pyranone; $\geq 98.5\%$) and mushroom tyrosinase (mTYR; EC 1.14.18.1, activity = 3130 units/mg) were purchased from Sigma-Aldrich and used without further purification. Fresh mTYR solutions were prepared every day and tyrosinase activity was analysed spectrophotometrically to adjust solutions to fixed tyrosinase Sigma units for consistent results. Briefly, one Sigma unit corresponds to the amount that will cause an increase in 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 units (I.U.) for monophenolase activity and to 2.24×10^{-2} I.U. for diphenolase activity, as defined by Fenoll et al. (2002). AAPH (2,2'-azobis(2-methylpropionamide) dihydrochloride), methyl linoleate ($\geq 98\%$) and TritonTM X-100, were used as received. AIBN (2,2'-Azobis(isobutyronitrile); 98%) was recrystallized from methanol, while 2,4,6-tri-*tert*-butylphenol (TBP, 98%) was recrystallized 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, to avoid significant decomposition. Cumene (98%) and styrene ($\geq 99\%$) were purified by double 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 ((3*S*,1*E*)-1-(3,7-dimethyl-3-vinylocta-1,6-dien-1-yl)-4-methoxybenzene) was prepared by
134 cautious addition of 0.2 mmol of bakuchiol (in dry DMF) to a suspension of 1.4 eq. NaH in dry DMF
135 at 0°C under N₂. The mixture was stirred for 30 min while allowing to reach r.t., then MeI (1.4 eq)
136 was added dropwise and the mixture was stirred for 4h at r.t. then quenched with brine and extracted
137 with hexane. The dried (Na₂SO₄) 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, OCH₃), 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;
143 in agreement with literature (Hu & Brenner-Moyer, 2022).

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

148 Kinetic evaluation of tyrosinase reaction with or without inhibitor was carried out using UV-Vis
149 spectrophotometry similarly to previous methods (Copeland, 2000; Song et al., 2021; Yu & Fan,
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
152 double-beam spectrophotometer. L-Tyrosine and L-Dopa (5 levels, 0.125-2 mM) were used as the
153 substrate of mushroom tyrosinase (mTYR, 0.5-5.0 U/ml) for monophenolase and diphenolase
154 reactions, respectively. Bakuchiol (0 to 12 μM) and kojic acid (0 to 50 μM) were comparatively tested
155 as inhibitors. Absorbance of dopachrome was measured at 475 nm for a time-period of 20 to 60 min

156 reading the solution against a reference cuvette containing all reaction components except the
 157 substrate. Absorbance variation *vs* time at different substrate concentration allowed to obtain initial
 158 velocity ($V = \Delta A / \Delta \text{min}$) which was converted in $\mu\text{M}/\text{min}$ according to Lambert-Beer law as follows:
 159 $V (\mu\text{M}/\text{min}) = V (\Delta A / \Delta \text{min}) \times 10^6 / \epsilon_{\lambda_{\text{max}}} \times l$. The molar extinction coefficient (ϵ) for dopachrome at
 160 $\lambda_{\text{max}} = 475 \text{ nm}$ is $3700 \text{ M}^{-1} \text{ cm}^{-1}$. Michaelis-Menten parameters (K_m e V_{max}) were obtained by
 161 processing initial velocity *vs* substrate concentration data by nonlinear fitting to M-M equation (1),
 162 using Sigmaplot 11.0 (Systat Software Inc., San Jose, California) (Guo et al., 2022). Linearized
 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)
 166 and the M-M constant. (the substrate concentration yielding half-maximum reaction rate).[†]

$$167 \quad V = \frac{V_{\text{max}} [S]}{K_m + [S]} \quad (1)$$

$$168 \quad \frac{1}{V} = \frac{K_m}{V_{\text{max}} [S]} + \frac{1}{V_{\text{max}}} \quad (2)$$

169 *2.4 mTYR kinetic studies by oximetry*

170 Evaluation of tyrosinase monophenolase and diphenolase kinetics and inhibition by bakuchiol or
 171 kojic acid was carried out by monitoring the oxygen consumption, at 30°C , by miniaturized oxygen
 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
 177 sample, corresponding to 0.236 mM at 30°C which reflects in the sensor reading as 20% (P_0). Thus,
 178 the oxygen concentration during the enzymatic oxidation of L-DOPA or L-tyrosine were converted
 179 into mM by the equation: $[\text{O}_2] (\text{mM}) = P \times 0.236 (\text{mM}) / 20\%$. The initial rate of oxygen consumption
 180 was obtained as (ΔP) *vs* time (in seconds) by regression of the initial data range of oxygen

181 consumption, and it was converted by equation: $V \text{ (}\mu\text{M/min)} = V \text{ (}\Delta P/\Delta t) \times 0.236 \times 10^3 \times 60 / P_0$
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 quartz 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 air-
199 saturated solution of the oxidizable substrate containing AIBN (0.01-0.1M) was equilibrated at 30 °C
200 with an identical reference solution containing excess 2,2,5,7,8-pentamethyl-6-hydroxychromane
201 (PMHC, 25 mM). After equilibration, and when a constant O₂ consumption was reached, a stock
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 μM 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

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

208 The inhibition rate constant for peroxy radical trapping k_{inh} was obtained from oxygen consumption
 209 plots by eqs. 4 or 5, where k_p , and $2k_t$, are the rate constants for chain propagation and termination of
 210 the substrate, R_0 and R_{inh} are respectively the rate of oxygen consumption ($-d[O_2]/dt$) in the absence
 211 and presence of the antioxidant, while n is the stoichiometric factor, *i.e.* the number of peroxy
 212 radicals trapped by one molecule of antioxidant (Amorati & Valgimigli, 2018; Amorati et al., 2016).
 213 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 ($k_p = 0.34 \text{ M}^{-1}\text{s}^{-1}$; $2k_t = 4.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, at 30°C) were used as the oxidizable substrates (Amorati et al., 2011; Baschieri et al., 2019).

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

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

$$217 \quad \frac{R_0}{R_{inh}} - \frac{R_{inh}}{R_0} = \frac{nk_{inh}[Antiox]}{\sqrt{2k_tR_i}} \quad (5)$$

218 2.7 Autoxidation studies in aqueous micelles

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 Triton™ X-100 (final concentration
 222 8 mM) micelles were prepared by vortex mixing, then adding a freshly prepared stock solution of
 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 O_2 consumption was recorded. Oxygen
 228 concentration was monitored with the same equipment described for mTYR kinetics and data were
 229 similarly treated by the equation: $V \text{ (M/s)} = V (\Delta P/\Delta t) \times 0.210 \times 10^{-3} / P_0$, where P_0 is the initial 20%

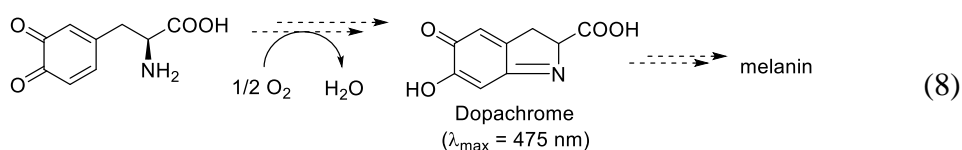
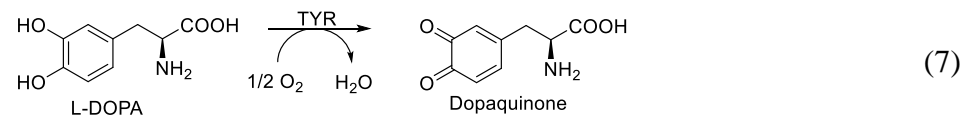
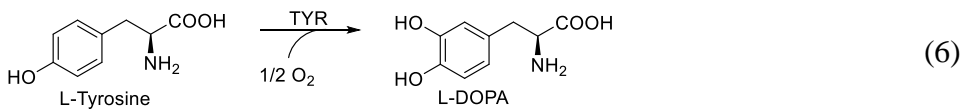
230 O₂ saturation reading that corresponds to 0.210 mM at 37°C. Oxygen consumption in the presence of
231 the antioxidant was compared with that recorded with identical reference mixtures lacking the
232 antioxidant. The inhibition rate constant was obtained from oxygen consumption plots by eqs. 3-5,
233 as described for autoxidation studies in homogenous solution, using $k_p = 36 \text{ M}^{-1}\text{s}^{-1}$ for MeLin in
234 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
237 peroxide (10% v/v) were sealed under nitrogen in a suprasil quartz EPR tube. The sample was inserted
238 in the thermostatted (30°C) cavity of an X-band EPR spectrometer and photolyzed with a mercury-
239 xenon lamp (240-400 nm, max 4500 mW/cm²). Spectra were recorded with the following settings:
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 H₂SO₄ ($g = 2.00258$) (Amorati et al.,
243 2011; Valgimigli et al., 2000). When using mixtures of TBP and bakuchiol in ReqEPR experiments,
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

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



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3. Results

3.1 Kinetics of mTYR inhibition

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

Concerning the inhibition mode, both for monophenolase and diphenolase reactions, regression lines 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 saturating substrate concentration – on increasing the concentration of the inhibitor, which is

278 indicative of a reversible competitive inhibition (Copeland, 2000). Competitive inhibition is
279 confirmed by analysis of V_{\max} and K_m values obtained by fitting M-M curves (Figure 2A, C), collected
280 in Table 1. While V_{\max} remains constant within experimental error, the M-M constant K_m – the
281 concentration of substrate required to produce half-maximum reaction rate – increases linearly with
282 the concentration of the inhibitors (Guo et al., 2022; Copeland, 2000). Confirmation of this behavior,
283 typical of competitive inhibitors, is given by the secondary M-M plots of K_m vs [bakuchiol] (Figures
284 S4, S5 in Appendix).

285 To further confirm the above findings, the reactions were also investigated in parallel by the
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
288 agreement and the corresponding V_{\max} and K_m are compared to those obtained by oxygen sensing in
289 Table 1. Average inhibition constant K_i – the dissociation constant of the enzyme-inhibitor complex
290 – was $6.71 \pm 1.23 \mu\text{M}$ for monophenolase and $1.15 \pm 0.34 \mu\text{M}$ for diphenolase reactions. Such low
291 values indicate high inhibition potency and should be compared with the higher K_i values of 10.91
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

296 Although K_i is the most reliable parameter to quantify and compare the potency of enzyme inhibitors,
297 as it does not depend on enzyme and substrate concentrations (Copeland, 2000), it is most common
298 in the literature to find activity quantified by the IC_{50} values: the concentration of the inhibitor
299 affording 50% reduction of the enzyme reaction rate. Hence IC_{50} values for bakuchiol were measured
300 by comparing the rate of reaction at fixed enzyme activity in the absence (V_0) and presence (V_i) of
301 increasing inhibitor concentration $[I]$, according to Langmuir equation (9) (Guo et al., 2022; Copeland,
302 2000).

$$\frac{V_I}{V_0} = \frac{1}{1 + \frac{[I]}{IC_{50}}} \quad (9)$$

The resulting values obtained both by O₂ 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.

Our values for monophenolase and diphenolase reaction, averaged between O₂ uptake and spectrophotometry, were $37.22 \pm 5.18 \mu\text{M}$ and 6.76 ± 0.73 , respectively (Table 1). Reference kojic 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-tyrosine and L-DOPA, respectively, in excellent agreement with previous studies by our group (Guo et al., 2022),²² and by others (He et al., 2021).

313

3.3 Fluorescence quenching study

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 of mTYR in 50 mM phosphate buffer (pH 6.8) in the range 290-500 nm (λ_{max} 338 nm) upon excitation at 280 nm was recorded either in the absence of inhibitor (F_0) and in the presence of growing concentration of bakuchiol (Figure 2E), in the absence of substrate. On increasing the concentration of bakuchiol fluorescence intensity (F) progressively decreased, without significant shifting of the 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 [Q], was used to analyze the quenching type, either static or dynamic.

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

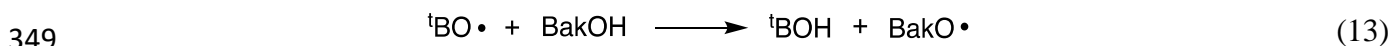
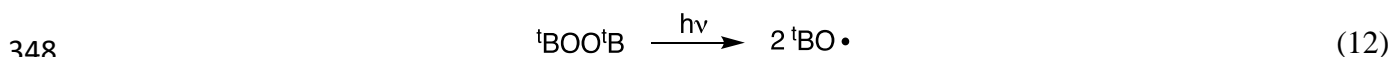
In eq. 10 and k_q and K_{SV} are respectively the quenching rate constant and the Stern-Volmer constant, while τ_0 ($=10^{-8}$ s) is the typical lifetime of the fluorophore in the absence of the quencher (Mátyus, Szöllösi & Jenei, 2006). Figure 2F shows that the plot was linear only at low quencher concentration,

while it shows upward curvature for higher Bak levels. This behavior is often encountered in case of 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 $K_{SV} = 4.32 \times 10^5 \text{ M}^{-1}$ and $k_p = 4.32 \times 10^{13} \text{ M}^{-1}\text{s}^{-1}$. The bimolecular quenching rate constant k_p for bakuchiol 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, implying the formation of a stable complex with mTYR (Yu & Fan, 2021). Dominance of the static quenching mechanism is also confirmed by fitting the data to the modified Stern-Volmer plot for static quenching (see Figure S6 in Appendix) (Yu & Fan, 2021; Mátyus et al., 2006). The apparent 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). 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 complex formation, fully consistent with the results of enzyme inhibition.

$$\text{Log} [(F_0 - F)/F] = \text{Log} K_a + n \text{Log}[Q] \quad (11)$$

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.



Photolysis of di-*tert*-butylperoxide in the presence of bakuchiol in the cavity of the EPR spectrometer generated alkoxy 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 by the bond dissociation enthalpy (BDE) of the O-H group, we measured it by the ReqEPR technique (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 establish their equilibration (eq. 14). Measurement of the corresponding equilibrium constant (eq. 15) is achieved by numerical simulation of the EPR spectrum obtained from the mixture, due to superimposition of the EPR signals of the two radicals, which affords their relative ratio (Figure 3B). 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, 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 weakest OH group in (structurally related) resveratrol which, although not experimentally known, can be estimated as ~ 81.4 Kcal/mol by averaging the calculated (DFT) value (80.2 Kcal/mol) and the empirical value obtained by the group additivity rule (82.6 Kcal/mol) (Amorati et al., 2003).



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

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

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

3.5 Antioxidant activity of bakuchiol in solution and in micelles

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

379 controlled inhibited autoxidation of reference substrates, which is the golden standard in antioxidant
 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.
 382 In organic solution (PhCl, 30°C) we studied the inhibited autoxidation of two well established
 383 oxidizable substrate, styrene and cumene, having largely different oxidation rates (k_p at 30°C is 41
 384 and $0.34 \text{ M}^{-1}\text{s}^{-1}$, respectively) thereby affording complementary information (Baschieri et al., 2019).
 385 In the autoxidation of styrene initiated by AIBN, bakuchiol produced a slow-down of the oxidation,
 386 without a detectable inhibition period, which was observed instead for reference antioxidant α -
 387 tocopherol (TOH), as shown in Figure 4A. This is due to the major difference in concentration
 388 between the oxidizable substrate and the antioxidant (six orders of magnitude), which severely
 389 challenges the apparent performance of the antioxidant. Analysis of the oxygen uptake plots by eq. 5
 390 afforded the inhibition rate constant – the rate constant for trapping alkylperoxyl radicals – k_{inh} of
 391 $2.20 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (Table 2), which was about one order of magnitude lower than that of TOH ($k_{inh} =$
 392 $3.2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$), nature's premiere lipid soluble antioxidant. The absence of a distinct inhibition
 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
 395 autoxidation of cumene. Owing to the lower k_p , cumene gave clear inhibition periods in the presence
 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.
 398 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,
 399 despite the lower oxidative chain length during inhibition (Table 2).[§] The resulting averaged (styrene
 400 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
 401 lower than reference TOH, is identical within experimental error to that of well-established
 402 antioxidant resveratrol ($2.0 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ at 30°C (Amorati et al., 2003)).
 403 The antioxidant activity in heterogenous systems was investigated using the autoxidation of methyl
 404 linoleate (MeLin, 2.74 mM) in Triton™ X-100 micelles (8 mM) initiated by water soluble AAPH at

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

The inhibition constant was obtained from the slope of the inhibited period by eq. 4, as $k_{\text{inh}} = (1.6 \pm 0.3) \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ (Table 2). For comparison, reference TOH under the same experimental settings afforded $k_{\text{inh}} = (1.5 \pm 0.2) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ in good agreement with literature using the same kinetic model (Konopko & Litwinienko, 2022). As also found in homogenous solution, bakuchiol traps peroxy 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)).

3.6 Antioxidant activity of bakuchiol O-methyl derivative MeOBak

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

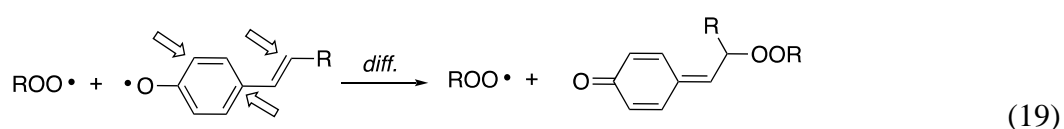
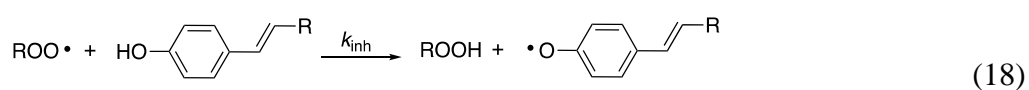
When tested under experimental settings identical to those used of native Bak, MeOBac showed no inhibition of the autoxidation of styrene (Figure 4A): similarly, no protection was observed in the autoxidation of MeLin in micelles, where oxygen consumption plots recorded in the presence of MeOBak were 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*).

4. Discussion

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 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 mg/mL (West et al., 2021). Inhibition is competitive both for monophenolase and diphenolase reaction, with M-M related inhibition constant K_i of $6.71 \pm 1.23 \mu\text{M}$ and $1.15 \pm 0.34 \mu\text{M}$, respectively, indicating higher inhibition potency for diphenolase reaction – it should be recalled that lower values 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_a value of $1.02 \times 10^6 \text{ M}^{-1}$ determined by fluorescence quenching, which represents the apparent formation constant for such complex. Interestingly, K_i values are lower than those we previously measured for reference inhibitor kojic acid ($10.91 \mu\text{M}$ and $9.91 \mu\text{M}$ for monophenolase and diphenolase inhibition respectively) (Guo et al., 2022), indicating higher bioactivity of bakuchiol.

Although less robust in quantifying inhibition potency, owing to their dependence on substrate and 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 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 setting for comparison and using averaged results from O₂ sensing and spectrophotometry, IC₅₀ values measured here for kojic acid indicate similar activity for monophenolase inhibition ($34.02 \mu\text{M}$ vs $37.22 \mu\text{M}$), while for diphenolase inhibition bakuchiol (IC₅₀ = $6.76 \mu\text{M}$) was sensibly more effective than kojic acid (IC₅₀ = $16.86 \mu\text{M}$). This is at variance with a previous study reporting IC₅₀ 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.

456 It was recently shown by Kang et al. (2020). that a phytoextract of *P. corylifolia* containing 77%
 457 bakuchiol reduced melanin biosynthesis in normal human epidermal melanocytes and the activity
 458 was attributed to reduction of tyrosinase enzyme expression and to reduction of TRP-1, TRP-2 and
 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
 464 allowing full rationalization of bakuchiol's depigmenting activity. Most important, it opens to new
 465 applications of bakuchiol based on its interference with tyrosinase chemistry (*vide infra*).
 466 Being a phenolic compound, the antioxidant activity of bakuchiol is expectedly dictated by its ability
 467 to quench chain-carrying peroxy radicals to the corresponding hydroperoxide, by formal H-atom
 468 transfer from the phenolic OH (18) (Amorati & Valgimigli, 2018). Inhibited autoxidation studies
 469 indicated a stoichiometric factor $n \sim 2$ (1.9 ± 0.1) both in organic solution and in aqueous micelles;
 470 therefore, a second peroxy radical is trapped by the resulting phenoxyl radical, most typically by
 471 addition to the aromatic ring in conjugated positions (arrows in eq. 19) (Valgimigli & Pratt, 2015),
 472 or possibly by styryl-type addition (19).



475 Wherever the second $\text{ROO}\cdot$ trapping occurs, it is not regulating the antioxidant activity, which is
 476 dictated by rate-determining reaction 18 (Amorati & Valgimigli, 2018). Since the BDE(OH) of the
 477 alkylhydroperoxides is 88.6 kcal/mol (Amorati et al., 2011; Amorati, Menichetti, Mileo, Pedulli &
 478 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 ($k_{\text{inh}} = 1.8 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ 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 $\log(k_{\text{inh}})$ 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 peroxy radicals stems entirely from reaction of
488 the phenolic OH group, while the terpenoid chain would influence its reactivity only due to its
489 electronic properties, *i.e.* by stabilizing the phenoxyl radical and lowering the BDE(OH).

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 thiyl 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 alkylperoxy radical ROO• that
496 likely propagates the oxidative chain (Amorati & Valgimigli, 2018). As a proof of concept, we
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).

505 The lower antioxidant activity of bakuchiol in micelles compared to apolar organic solution (k_{inh} of
506 $1.6 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ vs $1.8 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$) follows the well-known behavior of any phenolic antioxidant and
507 it pairs with a similar reduction in activity of reference TOH (k_{inh} from $3.2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ to 1.5×10^4
508 $\text{M}^{-1}\text{s}^{-1}$). It is partly due to H-bonding of the phenolic OH group at the water-lipid interface and partly
509 due to rate-limiting exchange of radicals and antioxidants among micellar particles (Amorati et al.,
510 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.

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

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

524

525 **5. Conclusions**

526 This study addresses for the first time on quantitative grounds the kinetics of tyrosinase inhibition
527 and of peroxy radical trapping by bakuchiol, highlighting anti-tyrosinase activity significantly higher
528 than previously expected, with competitive mechanism both toward monophenolase and diphenolase
529 reactions and K_i values of $6.71 \pm 1.23 \text{ }\mu\text{M}$ and $1.15 \pm 0.34 \text{ }\mu\text{M}$ respectively. These values are
530 significantly lower than the values for reference kojic acid implying higher potency, confirmed also

531 by the measured IC₅₀ values (1 mM substrate) of 37.22 μ M, and 6.76 μ M for monophenolase and
532 diphenolase inhibition by bakuchiol vs 34.02 μ M and 16.86 μ M for kojic acid. This verifies our initial
533 hypothesis. At the same time, the rate constant of peroxy radical trapping k_{inh} of $1.8 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$
534 (PhCl solution) and the corresponding value in aqueous micelles are indistinguishable from those of
535 well-established antioxidant resveratrol (Konopko & Litwinienko, 2022) and over 10-folds larger
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
538 help rationalize the activity shown in skin-care treatments (Dhaliwal et al., 2019; West et al. 2021;),
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
541 spoilage arising both from air oxidation and from enzymatic browning, which are regarded in the
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.,
544 2019; Kang et al., 2020) and the reported antimicrobial/antifungal activities (Alam et al., 2018) make
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**

549 AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride; AIBN, 2,2'-azobis(isobutyronitrile); Bak,
550 bakuchiol; L-DOPA, levo-dihydroxyphenylalanine; MeLin, methyl linoleate; mTYR, mushroom
551 tyrosinase; OSS, oxidative stress status; ROS, reactive oxygen species; SOX9, 9th transcription factor
552 of the Sry high-mobility-group-box family; TBP, 2,4,6-tri-*tert*-butylphenol; α -TOH, alpha-
553 tocopherol; 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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559 discussion.

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562 **Appendix A. Supplementary data**

563 GC-MS analysis of bakuchiol and MeOBak, kinetic plots of tyrosinase inhibition monitored by
564 spectrophotometry, tables of IC₅₀ values of bakuchiol, additional plots of fluorescence quenching
565 and additional ReqEPR spectra, oxygen uptake during enzymatic vs. spontaneous oxidation of
566 Dopa.

567 **Notes**

568 † 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).

572 § In cumene TOH produced complete inhibition (not shown) *i.e.* the chain length ν_{inh} was close to 1,
573 therefore it was used as a reference only for measuring the rate of initiation R_i via eq 3.

574

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698 application in the anti-browning of freshly-cut apples. *Food Chemistry*, 385, 132730. DOI:
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700

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
703 sensing and UV-Vis spectrophotometry.^a

Monophenolase (substrate = L-tyrosine)					
O ₂ Uptake					
Bakuchiol (μM)	K_m or K_m^{app} (mM)	V_m or V_m^{app} ($\mu\text{M}/\text{min}$)	K_I (μM)	Average K_I (μM)	IC_{50} (μM) 1 mM substr.
0	0.19 ± 0.02	4.20 ± 0.10	-		
1	0.21 ± 0.01	3.98 ± 0.25	8.04		
2	0.26 ± 0.03	3.96 ± 0.05	5.80	6.93	33.35
4	0.30 ± 0.02	4.02 ± 0.10	7.11	± 0.93	± 3.41
8	0.41 ± 0.04	4.00 ± 0.17	6.76		
UV-Vis Spectrophotometry (dopachrome formation)					
Bakuchiol (μM)	K_m or K_m^{app} (mM)	V_m or V_m^{app} ($\mu\text{M}/\text{min}$)	K_I (μM)	Average K_I (μM)	IC_{50} (μM) 1 mM substr.
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		
Averaged values (O ₂ and UV-Vis)				6.71 ± 1.23	37.22 ± 5.18
Diphenolase (substrate = L-DOPA)					
O ₂ Uptake					
Bakuchiol (μM)	K_m or K_m^{app} (mM)	V_m or V_m^{app} ($\mu\text{M}/\text{min}$)	K_I (μM)	Average K_I (μM)	IC_{50} (μM) 1 mM substr.
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 Spectrophotometry (dopachrome formation)					
Bakuchiol (μM)	K_m or K_m^{app} (mM)	V_m or V_m^{app} ($\mu\text{M}/\text{min}$)	K_I (μM)	Average K_I (μM)	IC_{50} (μM) 1 mM substr.
0	0.20 ± 0.02	10.26 ± 0.25	-		
0.5	0.30 ± 0.03	9.98 ± 0.24	1.03	1.13	6.76
1	0.36 ± 0.05	10.12 ± 0.38	1.26	± 0.12	± 0.73
2	0.56 ± 0.09	9.73 ± 0.51	1.11		
Averaged values (O ₂ and UV-Vis)				1.15 ± 0.34	6.91 ± 0.96

704 ^a V_{max} and K_m or V_{max}^{app} and K_m^{app} refer to not inhibited and inhibited assays, respectively. [†]

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 peroxy 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.

<i>Thermodynamics of the -OH group</i>			
Radical	hfsc / Gauss ^a		<i>g</i> -factor
Bak(•)	6.67 (2H _{ortho}); 1.94 (2H _{meta}); 7.05 (H _{para}); 3.27 (H _{vinyl})		2.0041
TBP(•)	1.77 (2H _{meta}); 0.18 (18H)		2.0046
^U PhOH	^R PhOH	<i>K</i> _{eq}	BDE (Kcal/mol)
Bak	TBP	14.25 ± 2.07	81.7 ± 0.1
<i>Kinetics of ROO• trapping</i>			
Substrate (medium)	<i>k</i> _{inh} (10 ⁴ M ⁻¹ s ⁻¹)	<i>n</i> ^b	<i>v</i> _{inh} ^c
Styrene (solution)	22.0 ± 3.0	n.d.	172
Cumene (solution)	14.2 ± 2.2	1.9 ± 0.1	7.5
<i>Average in solution</i>	18.1 ± 6.6	1.9 ± 0.1	
MeLin (micelles)	0.16 ± 0.03	1.9 ± 0.1	10.6

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

727 FIGURE CAPTIONS

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

729

730 **Figure 2.** (A-C) Kinetics of mTYR reaction (30°C, pH 6.8) monitored by O₂ uptake, inhibited by
731 bakuchiol, for (A, B) monophenolase reaction (substrate = L-Tyrosine; mTYR 1.6 U/mL) and (C, D)
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
735 increasing concentration of bakuchiol up to 1.320 µM (E), the corresponding Stern-Volmer plot (F),
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).

738

739 **Figure 3.** EPR (X-band) spectrum recorded by photolyzing in the cavity of the spectrometer (in *tert*-
740 butylbenzene/¹BOO¹B 9:1, at 30°C): (A) bakuchiol, (B) a mixture of bakuchiol and TBP 20:1.
741 Simulations were obtained by Monte Carlo method using the parameters in Table 2. The spectral lines
742 due to TBP• radical are indicated by an arrow (B): the resulting radical ratio was 1.26:1 (Bak•/TBP•).

743

744 **Figure 4.** Oxygen consumption plots recorded during the autoxidation of 4.3 M styrene in PhCl (A),
745 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
746 mM TritonTM X-100 micelles initiated by 5 mM AAPH at 37°C, pH 7 (C), without inhibitors (dashed
747 line) or in the presence of bakuchiol, or MeOBak, or TOH, as indicated. Thin lines represent the
748 regression of the inhibited periods. In panel (D) Evans-Polanyi correlation of the rate constant *k_{inh}* (at
749 30°C) for trapping ROO• radicals by phenols with 2,6 (*ortho*) substituents of different size *vs* their
750 BDE(OH). The data point of bakuchiol is indicated by a full star (★).

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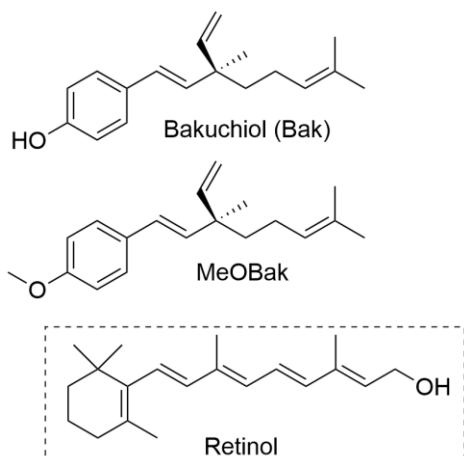


Figure 1. Structures of investigated bakuchiol and the methyl ether compared to retinol.

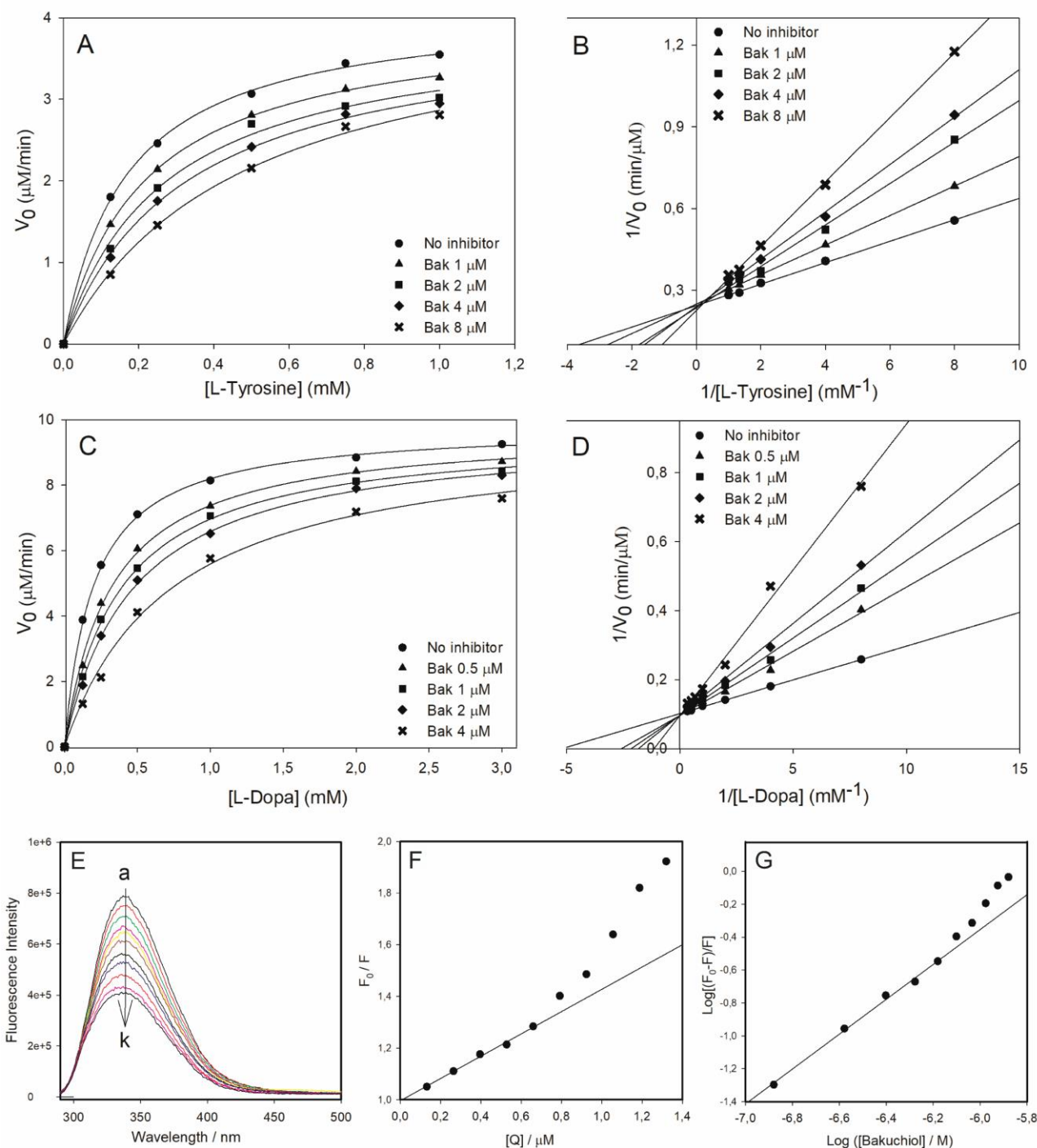
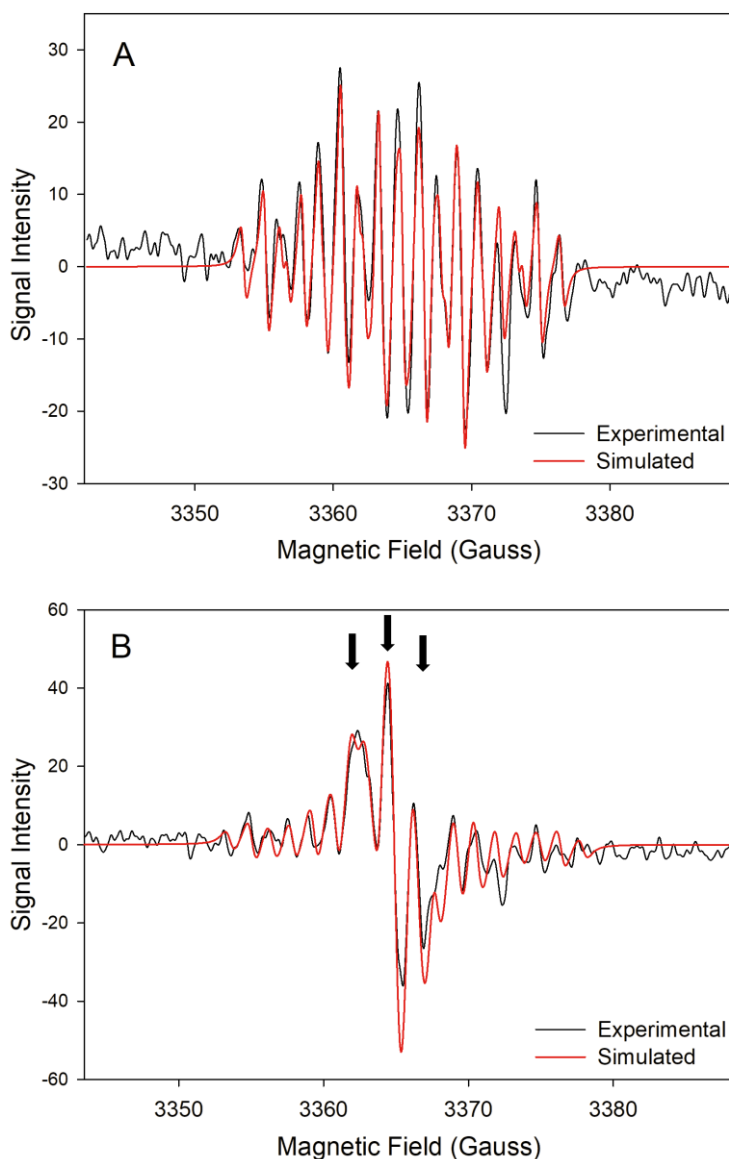


Figure 2. (A-C) Kinetics of mTYR reaction (30°C, pH 6.8) monitored by O₂ uptake, inhibited by bakuchiol, for (A, B) monophenolase reaction (substrate = L-Tyrosine; mTYR 1.6 U/mL) and (C, D) diphenolase reaction (substrate = L-DOPA, mTYR 0.8 U/mL). Graphs represent Michaelis-Menten hyperbolic plots (A, C) and Lineweaver-Burk linear plots (B, D) of the same experiments. (E-G) 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), and the log-log plot (eq. 11) relating fluorescence quenching to the number of binding sites (n) and complex formation constant K_a (G).



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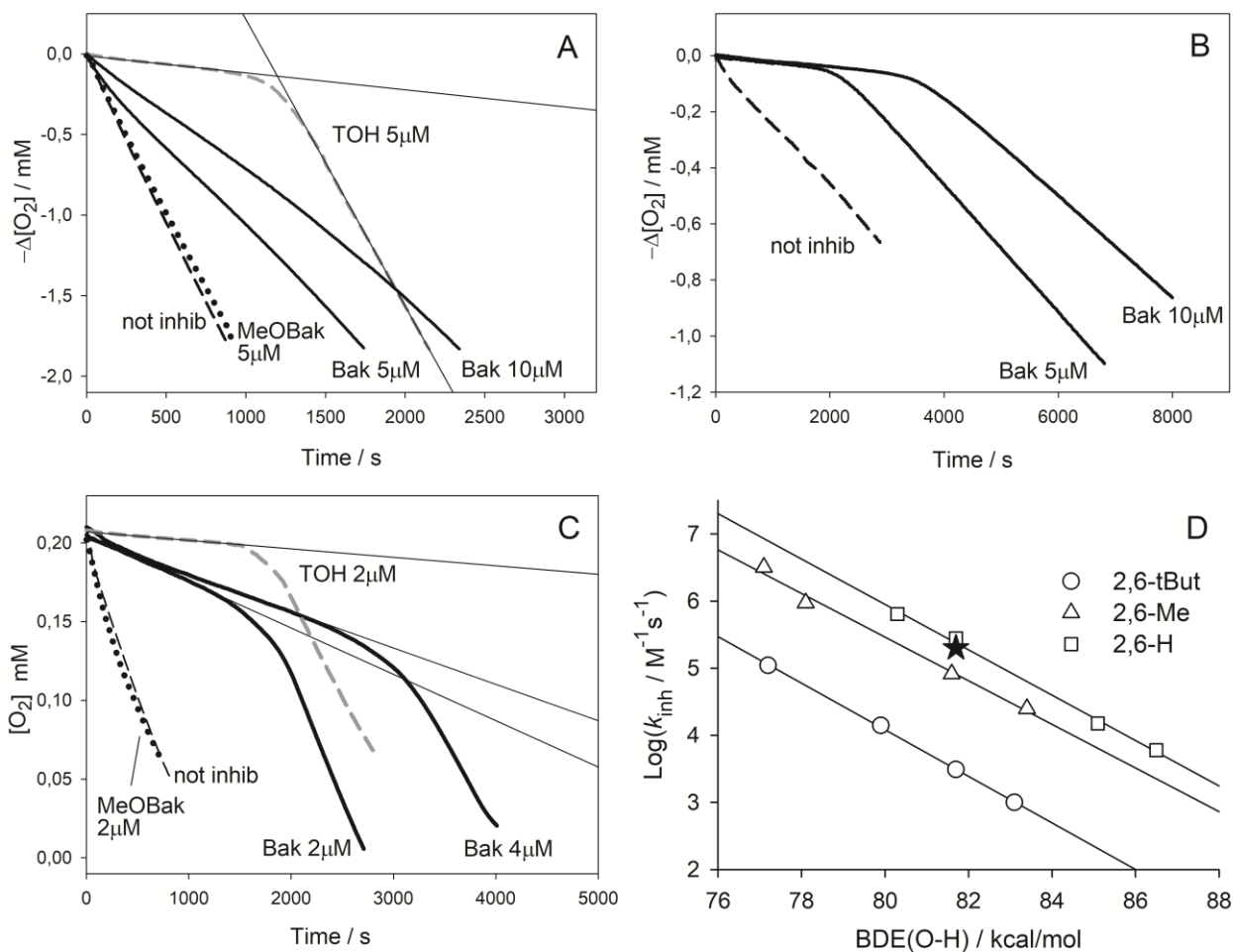
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799 **Figure 4.** Oxygen consumption plots recorded during the autoxidation of 4.3 M styrene in PhCl (A),
800 and 3.6 M cumene in PhCl (B), both initiated by AIBN (0.05 M) at 30°C, or of 2.74 mM MeLin in 8
801 mM Triton X-100 micelles initiated by 5 mM AAPH at 37°C, pH 7 (C), without inhibitors (dashed
802 line) or in the presence of bakuchiol, or MeOBak, or TOH as indicated. Thin lines represent the
803 regression of the inhibited periods. In panel (D) Evans-Polanyi correlation of the rate constant k_{inh} (at
804 30°C) for trapping ROO• radicals by phenols with 2,6 (*ortho*) substituents of different size vs their
805 BDE(OH). The data point of bakuchiol is indicated by a full star (★).

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Graphical abstract

