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1 In vitro analysis of catalase and superoxide dismutase mimetic properties of blue tattoo ink

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16 **Abstract**

17 Tattoo inks are comprised of different combinations of bioactive chemicals with combined
18 biological effects that are insufficiently explored. Tattoos have been associated with oxidative
19 stress; however, a recent N-of-1 study suggested that blue tattoos may be associated with
20 suppressed local skin oxidative stress. The present study aimed to explore the attributes of the
21 blue tattoo ink (BTI) that may explain its possible effects on redox homeostasis, namely the
22 catalase (CAT) and superoxide dismutase (SOD)-mimetic properties that have been reported
23 for copper(II) phthalocyanine (CuPC) – the main BTI constituent. Intenze™ Persian blue (PB)
24 BTI has been used in the experiment. CAT and SOD-mimetic properties of PB and its pigment-
25 enriched fractions were analyzed using the carbonato-cobaltate (III) formation-derived H₂O₂
26 dissociation and 1,2,3-trihydroxybenzene autoxidation rate assays utilizing simple buffers and
27 biochemical matrix of normal skin tissue as chemical reaction environments. CuPC-based
28 tattoo ink PB and both its blue and white pigment-enriched fractions demonstrate CAT and
29 SOD-mimetic properties *in vitro* with effect sizes demonstrating a substantial dependence on
30 the biochemical environment. PB constituents act as inhibitors of CAT but potentiate its
31 activity in the biochemical matrix of the skin. CuPC-based BTI can mimic antioxidant
32 enzymes, however chemical constituents other than CuPC (e.g. the photoreactive TiO₂) seem
33 to be at least partially responsible for the BTI redox-modulating properties.

34

35 **Keywords:** tattoo; tattoo ink; oxidative stress; catalase; superoxide dismutase

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38 **Introduction:**

39 The art of tattooing dates back to the earliest stages of tribal communities and the oldest known
40 tattoo belongs to the famous mummy *Ötzi the Iceman* (3000 BC)[1]. Throughout history, the
41 prevalence of tattoos varied; however, in the last decades, the practice of tattooing spread
42 throughout the Western world and became a mainstream form of body art. Recent estimates
43 suggest that up to 25% of Europeans under the age of 20 and up to 38% of Americans under
44 the age of 29 bear at least one tattoo [2]. Despite the omnipresence of tattooing, the biomedical
45 effects of tattoos remain poorly explored, possibly because tattoo inks are comprised of
46 different combinations of many bioactive chemicals with combined biological effects that are
47 challenging to explore, let alone predict. In general, tattoo inks contain: i) organic (e.g. azo or
48 polycyclic aromatic) or inorganic pigments (e.g. titanium dioxide (TiO₂), barium sulfate
49 (BaSO₄), iron oxide, chromium oxide); ii) binders (e.g. polyethylene glycol,
50 polyvinylpyrrolidone); iii) solvents (e.g. water, alcohol); and iv) additives (preservatives,
51 surfactants)[2]. Additionally, impurities (e.g. nitrosamines or formaldehyde) may be present as
52 well, and the ink injected into the body may contain nickel and chromium particles shredded
53 from the needle during tattooing [3]. During the procedure of tattooing, all the constituents are
54 delivered into the dermis where they become 100% systemically bioavailable due to direct
55 contact with the blood and lymph. Although the kinetics of different tattoo ink constituents is
56 still unknown, it is assumed that soluble components undergo rapid systemic distribution, while
57 the insoluble pigments are mostly retained in the area of injection and in the draining lymph
58 nodes where they may exert biological effects [2].

59 Although substantial efforts have been made to better understand the biological effects of tattoo
60 inks utilizing *in vitro* and *in vivo* models, the results demonstrate that the observed effects are
61 strongly dependent on the chemical constitution of the ink and the toxicological model utilized
62 in the study. For example, Falconi et al. reported reduced viability and expression of the
63 procollagen $\alpha 1$ type I in primary human fibroblasts incubated with *Biolip 27* but not *Strong*
64 *black* [4]. Regensburger et al. reported substantial variability in the potency of 19 commercially
65 available tattoo inks in respect to their inhibitory effects on mitochondrial activity in primary
66 human dermal keratinocytes exposed to UVA radiation [5]. Arl et al. compared the effects of
67 blue, green, red, and black tattoo ink on cell viability and the generation of reactive oxygen
68 species (ROS) and reported that incubation with red and green tattoo inks induced the most
69 pronounced toxic effects on the human keratinocyte cell line [6]. Perplexing *in vivo* results
70 have also been reported. For example, in studies on tattoo ink carcinogenicity, it has been
71 reported that mice tattooed with red tattoo ink and exposed to ultraviolet radiation develop
72 tumors slightly faster (214 vs 224 days) and show an increased tumor growth rate in
73 comparison with sham-tattooed mice [7]. In contrast, black tattoo ink was protective against
74 ultraviolet radiation-induced squamous cell carcinoma, delaying the tumor onset by
75 approximately 50 days in tattooed mice [8]. Although it will be interesting to see the follow-
76 up studies on the interaction between exposure to different tattoo inks and the carcinogenic
77 potential of ultraviolet radiation (to address the uncertainty related to relatively small effects
78 reported in [7] and [8]), the apparently discrepant results provide a good illustration of the fact
79 that the biological effects of different tattoo inks seem to be too complex and specific to provide
80 foundations for inductive reasoning on the effects of tattoo inks in general. To better understand
81 the biomedical consequences of tattooing, a substantial effort should be made to i) elucidate
82 the biological effects of individual chemicals present in different tattoo inks, and ii) explore

83 the synergistic, additive, or antagonistic effects of chemical constituents of different tattoo inks
84 in model systems that resemble those found *in vivo*.

85 The present aim was to explore the properties of blue tattoo ink that may explain the recently
86 reported observation that a blue tattoo was able to suppress local skin oxidative stress [9].
87 Oxidative stress is a pathophysiological condition that ensues as a consequence of the inability
88 of a system to maintain the balance between the electrophilic and the nucleophilic arm of redox
89 homeostasis [10]. Considering redox homeostasis is critical for normal cellular functioning, it
90 is no surprise that oxidative stress has been recognized as an important etiopathogenetic factor
91 and a promising pharmacological target in pathophysiological conditions of the skin [11,12].
92 Tattoo inks have generally been associated with increased levels of oxidative stress (e.g.
93 [5,6,13–15]); however, this has so far only been supported by indirect findings from *in vitro*
94 experiments and there is currently no direct evidence for tattoo-induced oxidative stress in
95 humans. In contrast, there is some evidence indicating that blue tattoo ink may be able to reduce
96 oxidative stress. In an N-of-1 study, skin tattooed with blue tattoo ink demonstrated increased
97 surface reductive capacity and the interstitial and intracellular fluid-enriched capillary blood
98 from the tattoo had an increased content of protein sulfhydryls, reductive capacity, and catalase
99 (CAT) activity, and reduced lipid peroxidation in comparison with the sample obtained from
100 nontattooed skin [9]. Copper(II) phthalocyanine (CuPC), the main constituent of blue tattoo
101 inks, can both reduce and prevent lipid peroxidation in homogenates of the mouse brain,
102 kidney, and liver and exerts a substantial protective effect in the deoxyribose degradation assay
103 [16]. Furthermore, it has been reported that CuPC can act as a dual functional mimetic of CAT
104 and superoxide dismutase (SOD), two important antioxidant enzymes and that this property
105 may be responsible for its lipid peroxidation-suppressing effects [17].

106 The present study aimed to explore whether: i) a blue CuPC-based tattoo ink can act as a CAT
107 and SOD mimetic *in vitro*; ii) CAT and SOD mimetic properties of blue tattoo ink are primarily
108 present in the CuPC-enriched ink fraction; iii) blue tattoo ink and its CuPC-enriched and
109 residual fractions potentiate or inhibit the effects of CAT and SOD in the complex biochemical
110 matrix of normal skin tissue (i.e. in the presence of endogenous CAT, SOD, and regulators of
111 their activity); iv) components of the tattoo ink may directly interact with components of the
112 skin.

113

114 **Materials and methods:**

115 **Sample preparation**

116 Intenze™ Persian blue tattoo ink (PB) (Intenze, USA) was used in the experiment. The
117 ingredients declared on the official material safety data sheet included: H₂O (The European
118 Community number (EC): 231-791-2), BaSO₄ (EC: 231-784-4), TiO₂ (EC: 215-280-1), CuPC
119 (EC: 205-685-1), glycerine (EC: 200-289-5), isopropyl alcohol (EC: 200-661-7), *Hamamelis*
120 *Virginiana* L. extract (EC: 283-637-9). Diluted PB samples were obtained by v/v dilution in
121 pre-defined ratios in double-distilled H₂O (ddH₂O; 0.055 μS/cm). Dye fractionation was done
122 by differential centrifugation. PB was first spun down for 30 minutes at a relative centrifugal
123 force (RCF) of 12879 x g, and then the same process was repeated twice with both the
124 supernatant (blue fraction) and the pellet (white fraction). The supernatant of the blue pigment-
125 enriched fraction and the pellet of the white pigment-enriched fraction were used for
126 subsequent analyses.

127 UV-Vis spectrophotometry

128 UV-Vis spectra were obtained by scanning the absorbance in the wavelength range from 220
129 nm to 750 nm using The NanoDrop® ND-1000 (Thermo Fisher Scientific, USA).

130 Catalase-like activity

131 CAT solution was prepared by dissolving 1 mg of lyophilized bovine liver CAT powder (Sigma
132 Aldrich, USA) in 10 ml of phosphate-buffered saline (PBS) (pH 7.4). CAT activity was
133 measured using the method first described by Hadwan [18] and adapted in [19]. Briefly, the
134 samples were incubated with 50 µl of the substrate solution (2-10 mM H₂O₂ in PBS) and the
135 reaction was stopped by adding 150 µl of the Co(NO₃)₂ stop solution (5 mL Co(NO₃)₂ x 6 H₂O
136 (0.2 g in 10 mL ddH₂O) + 5 mL (NaPO₃)₆ (0.1 g in 10 mL ddH₂O) added to 90 mL of NaHCO₃
137 (9 g in 100 mL ddH₂O)). The concentration of H₂O₂ was determined indirectly by measuring
138 the absorbance of the carbonato-cobaltate (III) complex ([Co(CO₃)₃]Co) at 450 nm using the
139 Infinite F200 PRO multimodal microplate reader (Tecan, Switzerland). Due to interference, a
140 unique baseline model was established for each sample by simultaneous incubation with
141 substrate solutions of graded nominal concentrations between 1 and 10 mM H₂O₂ and the
142 Co(NO₃)₂ stop solution. The amount of residual H₂O₂ was estimated from the model for each
143 sample and each time-point. CAT activity was assessed indirectly based on permutation-
144 derived estimates of the baseline values (t = 0 s) and final values (t₁ = 60 s or 300 s)[20].

145 Superoxide dismutase-like activity

146 The SOD-like activity was measured by assessing the inhibition of 1,2,3-trihydroxybenzene
147 (THB) autoxidation rate [21,22]. Briefly, 5 µl of each sample was placed in a 96 well-plate and
148 incubated with freshly pre-mixed THB working solution (64 µl of 60 mM THB dissolved in 1
149 mM HCl mixed with 3400 µl of 0.05 M Tris-HCl and 1 mM Na₂EDTA adjusted to pH 8.2).
150 THB autoxidation was measured by assessing the absorbance increment at 450 nm with
151 repeated measurements obtained by the Infinite F200 PRO multimodal microplate reader
152 (Tecan, Switzerland).

153 Preparation of the skin tissue constituents as the reaction matrix

154 To test whether the effects observed *in vitro* would be affected by the presence of skin tissue
155 constituents present *in vivo*, a rat skin homogenate was prepared. Briefly, a piece of skin from
156 a single rat euthanized in deep anesthesia (70 mg/kg ketamine; 7 mg/kg xylazine) was dissected
157 and stored at -80 °C. The animal was in the control (untreated) group of another experiment
158 and the tissue was dissected after decapitation in concordance with the 3Rs concept [23] in
159 order not to interfere with the experimental protocol. The animal study from which the tissue
160 was obtained was approved by the Ethics Committee of The University of Zagreb School of
161 Medicine (380-59-10106-18-111/173) and the Croatian Ministry of Agriculture (EP 186/2018).
162 The skin was rapidly dissected from the surrounding adnexa and placed in 1000 µl of lysis
163 buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, 1% sodium
164 deoxycholate, 0.1% SDS, 1 mM PMSF, protease inhibitor cocktail (Sigma-Aldrich, USA) and
165 PhosSTOP phosphatase inhibitor (Roche, Switzerland) adjusted to pH 7.5) on ice. The tissue
166 was homogenized using Microson Ultrasonic Cell Disruptor (Misonix, SAD), centrifuged for
167 10 min at 4 °C, and RCF of 12 879 × g, and the supernatant was stored at -80 °C. For the acute
168 experiments, 5 µl of the tissue homogenate was used per well, and for the pretreatment
169 experiments, 45 µl of the skin homogenate was incubated with either 5 µl of dd H₂O, or 5 µl of
170 the sample (1:10 PB, 1:10 blue, and 1:10 white fraction) for 180 min at 37°C. Bradford's

171 analysis of the protein concentration (using the bovine serum albumin (BSA) standard)
172 indicated the biochemical matrix of the skin contained 4.37 µg protein per µl.

173 Lateral flow assay for the assessment of the interaction between the constituents of the tattoo 174 ink and components of the skin, albumin, and catalase

175 A lateral flow assay (LFA) was conducted to assess the interaction between chemical
176 constituents of the blue tattoo ink and biochemical components of the skin. Additionally, the
177 LFA was employed to test the interaction of tattoo ink with catalase (to address the possibility
178 of direct interaction as a mediator of biological effects), and BSA as a standard protein with a
179 large intrinsic binding potential for a large diversity of small molecules. All samples (skin
180 homogenate, CAT, BSA) were spotted onto the nitrocellulose strips (0.45 µm pore size;
181 Amersham Protran; GE Healthcare Life Sciences, USA) in a way that each strip contained the
182 free route for the uninterrupted analyte flow (control lane) and the sample-capturing lane
183 (experimental lane). The membranes were left to air-dry for 15 minutes. Once dry, the strips
184 were fixed in the glass holder in a way that the proximal end was available for the
185 administration of the analyte (tattoo ink) followed by administration of the same volume of
186 vehicle (ddH₂O) to remove excess PB. An additional LFA experiment was conducted using
187 nitrocellulose spotted PB and its blue and white fractions as the stationary samples and ddH₂O
188 as the mobile phase. In both experiments, analyte mobility was recorded and the signal density
189 line profiles of the control and experimental lane for each sample were subsequently extracted
190 for 5 time-points using Fiji (NIH, USA).

191 Tattoo ink interaction electrophoretic mobility shift assays

192 Electrophoretic mobility shift assays (EMSA) were conducted to complement the LFA and
193 provide additional information on the nature of the interaction between skin components and
194 the tattoo ink constituents. Sample pre-incubation EMSA (SP-EMSA) was done by analyzing
195 the mobility interference using parallel electrophoretic separation of the tattoo ink-treated and
196 “naïve” skin homogenates and CAT. Pre-treated samples were incubated with PB to achieve a
197 1:100 dilution of the ink, while the control samples were incubated with an equal volume of
198 vehicle (ddH₂O). The samples were mixed with the modified bromophenol blue-free sample
199 buffer 4x stock containing 40% glycerol, 8% SDS, 200 mM Tris HCl (so that bromophenol blue
200 does not interfere with the CuPC color), incubated with the sample buffer for 10 minutes at
201 95°C, and loaded onto the TGX Stain-Free FastCast 12% polyacrylamide gels (Bio-Rad, USA)
202 for electrophoretic separation. Spectral analysis of gels was done by ChemiDoc MP Imaging
203 System (Bio-Rad, USA). Transfer onto the nitrocellulose was done using the Trans-Blot Turbo
204 semi-dry transfer system (Bio-Rad, USA). The elution of CAT from the nitrocellulose (for
205 subsequent spectral analysis) was done by incubating cut-out proteins in the 50% pyridine in
206 0.1 M ammonium acetate (v/v; pH 8.9) for 120 min at 37 °C [24]. In addition to SP-EMSA,
207 the interaction of the electrophoretically immobile CuPC with skin constituents and CAT was
208 studied using the CuPC-capturing gradient electrophoretic separation (CCG-EMSA). Briefly,
209 a capturing gel containing gradient concentrations of 1:850 PB mixed with a standard stacking
210 polyacrylamide matrix was placed on top of the separating gel, and the skin and CAT samples
211 were subjected to the electrophoretic separation to analyze the interaction of increased
212 electrophoretic CuPC exposure (increased CuPC matrix path) and sample mobility.

213

214 Data analysis

215 Data were analyzed in R (4.1.0). In the experiments where multiple substrate concentrations
216 and multiple substrate exposure times were used, CAT-like activity was analyzed using linear
217 regression with enzymatic activity (permutation-derived estimates) defined as the dependent
218 variable and sample, substrate concentration, and time defined as independent variables. Model
219 assumptions were checked by visual inspection of the residual and fitted value plots. Model
220 outputs were reported as point estimates of least-square means with accompanying 95%
221 confidence intervals. Comparisons of the samples from the model were reported as effect sizes
222 (differences of estimated marginal means with accompanying 95% confidence intervals).
223 Alpha was set at 5% and p-values were adjusted using the Tukey method.

224 Results:

225 Persian blue tattoo ink acts as a weak catalase and superoxide dismutase mimetic *in vitro*

226 PB demonstrated dilution-dependent CAT-like activity *in vitro*, with the 1:10 dilution showing
227 the ability to dissociate ~0.9 mM of H₂O₂ in 300 s on average, in substrate concentrations
228 ranging from 6 to 10 mM (**Fig 1A**). PB (1:10) dissociated 0.6 mM of H₂O₂ in the presence of
229 10 mM H₂O₂ and 1 mM when incubated with 8 and 6 mM, indicating lower efficacy at high
230 substrate concentrations. The 1:100 dilution of PB demonstrated no CAT-like activity. The
231 largest tested PB concentration (1:10) exerted SOD-like activity as well, by reducing the rate
232 of THB autoxidation in the first 300 s of the assay. After 300 s, the maximum suppressive
233 capacity of 1:10 PB was reached and PB potentiated autoxidation (**Fig 1B**). Lower PB
234 concentrations (1:100; 1:1000; 1:10 000) showed no SOD-like activity.

235

236 Blue and white fractions of the Persian blue tattoo ink show no catalase mimetic properties, 237 but demonstrate divergent superoxide dismutase-like behavior

238 Centrifugation-based fractionation of PB yielded a blue CuPC-enriched fraction and a white
239 fraction most likely enriched with TiO₂ and BaSO₄ (**Fig 2A**). UV-Vis spectra of fractionated
240 samples suggested that CuPC was primarily present in the blue fraction, as evident by the
241 presence of the Soret peak (B-band) and the Q-band with the Davydov splitting characteristic
242 for the phthalocyanine derivatives [25,26] (**Fig 2B**). Neither the blue nor the white fraction
243 demonstrated CAT-like activity *in vitro* (**Fig 2C-E**). Interestingly, „negative estimates of the
244 activity“ were obtained for the 1:10 dilution of both fractions indicating possible photocatalytic
245 generation of H₂O₂ [27]. The observed effect was substrate concentration-dependent and more
246 pronounced for the white fraction, which also demonstrated a pronounced time-dependence
247 (**Fig 2C-E**). While the CuPC-enriched blue fraction showed no SOD-like activity at $t < 300$ s,
248 the white fraction (1:10) potentiated THB autoxidation. At $t > 300$ s, the blue PB fraction (1:10)
249 demonstrated SOD-like activity (**Fig 2F**).

250 Persian blue tattoo ink and its blue and white pigment-enriched fractions inhibit catalase *in* 251 *vitro* but potentiate its action in the presence of biochemical constituents of the skin

252 Apart from acting as CAT/SOD mimetics, PB constituents may modulate redox balance by
253 affecting the activity of endogenous enzymes. In the presence of bovine liver CAT, the CuPC-
254 enriched PB fraction acted as a weak CAT inhibitor with no evident dose-response, while PB
255 and the white fraction exhibited a pronounced dose-dependent inhibition of the enzyme (**Fig**
256 **3A, B**). A different pattern was observed in the presence of biochemical constituents of the
257 skin, where PB and its blue and white pigment-enriched fractions acted as potentiators of

258 endogenous CAT, with the largest effect observed in the presence of the white fraction (**Fig**
259 **3C, D**). As the acute effects may not faithfully represent biochemical effects that may take
260 place *in vivo*, an additional experiment was conducted in which the tested samples were first
261 incubated with the biochemical constituents of the skin. Interestingly, following prolonged
262 incubation (180 min at 37 °C), the pronounced effect of the white fraction was substantially
263 attenuated, and the CuPC-enriched fraction induced the most pronounced effect on the activity
264 of endogenous CAT (**Fig 3E, F**). In the presence of tissue constituents, both the blue and white
265 fractions acted as SOD mimetics at $t < 300$ s, while there was no difference between the effect
266 of PB and the control condition (**Fig 3G**). After the 300 s time-point, the CuPC-enriched
267 fraction demonstrated stable SOD mimetic activity, while the white fraction potentiated THB
268 autoxidation (**Fig 3G**). Following the prolonged incubation at 37 °C, SOD potentiating effects
269 of PB and its blue and white fractions were completely lost (**Fig 3H**).

270 Constituents of the blue tattoo ink interact with components of the biochemical matrix of the 271 skin

272 To better understand the nature of the observed effects chemical interaction between
273 constituents of the tattoo ink and the components of the biochemical matrix of the skin was
274 evaluated using the LFA, SP-EMSA, and CCG-EMSA. LFA indicated that skin homogenate,
275 CAT, and BSA can all interact with constituents of PB that exhibit nitrocellulose lateral flow
276 (Fig 4A). BSA demonstrated the largest PB flow-disrupting capacity both in terms of binding
277 the flow front and resistance to subsequent elution by vehicle. Both CAT and the biochemical
278 matrix of the skin were also able to bind the PB front and resist ddH₂O elution (Fig 4A).
279 Interestingly, upon elution, the skin homogenate demonstrated a wave pattern suggesting the
280 presence of interaction with several separate components (Fig 4B). The CCG-EMSA and SP-
281 EMSA were used as complementary methods to better understand the nature of the observed
282 interaction. The CCG-EMSA revealed that the observed CAT-binding properties of PB were
283 not able to resist the electrophoretic mobility of the enzyme indicating that the nature of the
284 interaction was i) too weak to affect the electrophoretic flow, or ii) incompatible with the
285 unfolded linear structure and/or negative charge introduced by the reductive environment and
286 SDS (Fig 4C). Interestingly, although there was no evident electrophoretic mobility shift
287 introduced by increasing path length through the PB-enriched stacking polyacrylamide for
288 CAT this was not the case with skin homogenate where CCG-EMSA revealed a pronounced
289 dose-response electrophoretic mobility shift regardless of the reducing environment and high
290 SDS concentration (Fig 4C). The SP-EMSA confirmed the aforementioned findings as CAT
291 electrophoretic mobility was relatively resistant to the effects of PB, while tattoo ink
292 decelerated mobility of some biochemical constituents of the skin – speaking in favor of
293 binding even in the reductive environment and in the presence of high SDS (Fig 4D). The exact
294 chemical component (or components) of the ink responsible for the electrophoretic mobility
295 shift, as well as primary components of the skin responsible for the observed interaction,
296 remain to be further explored. Nevertheless, spectral analysis of the electrophoretic and lateral
297 flow mobility membranes/gels provided additional information that may lay the groundwork
298 for a better understanding of the observed interaction in the future. The SP-EMSA experiments
299 suggest that the blue pigment (CuPC) demonstrates electrophoretic mobility only in the
300 stacking polyacrylamide matrix (Fig 5A). Such a pattern indicates that CuPC either i) moves
301 on its own in the electrical field achieved in the polyacrylamide gel electrophoresis (PAGE)
302 setup (i.e. exerts electrophoretic mobility), or ii) moves together with CAT and some
303 components of the skin as a result of molecular interactions that cannot withstand the resistance

304 of mobility through the dense portion of the resolving polyacrylamide. Electrophoretic mobility
305 experiments with PB gradient stacking gel support latter as penetrance of the blue pigment
306 didn't increase (or possibly even decreased) as a result of increased vertical exposure (Fig 5B)
307 suggesting that the mobility shift of the skin homogenates may have been the result of the
308 interactions at the level of capturing-adapted stacking polyacrylamide. Nevertheless, it is
309 possible that i) some CuPC remained bound to proteins and caused electrophoretic deceleration
310 in quantities that are too small to be detected by simple visual inspection, and/or ii) some other
311 constituents of the tattoo ink demonstrated protein binding and caused deceleration in the
312 resolving fraction of the gel. To test this, spectral analysis of LFA membranes and
313 electrophoretic mobility gels was conducted. Spectral analysis of the LFA membranes revealed
314 that the LFA mobility pattern of PB and both its blue and white fractions demonstrate at least
315 3 general patterns: the sample pool (the area where the sample was deposited); the middle
316 mobile phase (largely absent in the white fraction); and the mobile front (present in all
317 samples)(Fig 5C). The sample pool and the middle mobile phase demonstrated a satisfactory
318 quantum yield upon excitation (EX) at 302 nm combined with the wide 535-645 nm emission
319 (EM) filter. All 3 LFA patterns (the sample pool and both mobile phases) for PB, blue, and
320 white fractions were successfully visualized under 755-777 nm EX with the 810-860 nm EM
321 filter, and a variety of conditions (e.g. EX/EM(nm): 460-490/518-546; 520-545/577-613; 625-
322 650/675-725; 650-675/700-730) were found to enable a good representation of the mobile front
323 (Fig 5C). Spectral analysis of the LFA membranes from the experiment with spotted CAT and
324 biochemical matrix of the skin revealed that, in addition to the interaction with the blue fraction
325 of the ink, both samples also captured components of the ink most likely representing the
326 mobile front from the LFA experiment in which PB was used as the mobile phase (with CAT
327 sample demonstrating greater mobile front binding capacity than the skin sample)(Fig 5D).
328 Although treatment-naïve CAT has been shown to emit in the close spectral range (control
329 CAT; Fig 5D), the comet pattern indicates the observed signal was most likely from the
330 component of the mobile phase and not an endogenous signal from the spotted sample. Spectral
331 analysis of the SP-EMSA polyacrylamide gels provides further evidence supporting the
332 hypothesis that, in addition to CuPC, other components of the tattoo ink (primarily present in
333 the white fraction) may interact with CAT and constituents of the skin (and affect their
334 electrophoretic mobility)(Fig 5E). Spectral analysis of the SP-EMSA polyacrylamide gels
335 confirms the observations from the LFA and indicates that another PB component (most likely
336 originating from the white fraction) enters the resolving polyacrylamide (alone or bound to
337 sample constituents) and possibly affects the sample electrophoretic mobility (even after
338 accounting for the baseline spectral properties of PB and its blue and white fractions and the
339 effects of the tattoo ink concentration)(Fig 5E, F). Interestingly, the presence of the component
340 of the white fraction seems to have functional consequences as the ink-exposed sample shows
341 the ability to potentiate the chemiluminescent reaction of luminol and H₂O₂ (Fig 5G). To ensure
342 that the observed effect was not due to the presence of CuPC present in quantities that are too
343 small to be detected by visual inspection, both CAT samples were eluted from the membrane
344 and subjected to spectral analysis (Fig 5H). To further ensure no CuPC was present, UV-Vis
345 spectra of the eluates were measured and compared to the spectra of the same samples before
346 PAGE showing a clear disappearance of the Q-band with the Davydov splitting characteristic
347 for the phthalocyanine derivatives (Fig 5I).

348

349 **Discussion:**

350 The presented results support the hypothesis that PB, a blue CuPC-based tattoo ink, can act as
351 a mimetic of CAT and SOD and provide a possible mechanistic explanation for the reduced
352 levels of oxidative stress in the skin with a blue tattoo [9]. Although CuPC can act as a dual
353 functional mimetic of CAT and SOD and suppress lipid peroxidation *in vitro* [17], it was
354 hypothesized that the *a priori* assumption that PB would necessarily reflect the properties of
355 its main component (CuPC) would be unjustified considering the unspecified concentration of
356 CuPC and the presence of other chemicals that may theoretically annihilate or even reverse its
357 potential antioxidant effects. The results indicate the caution was reasonable. Although PB
358 demonstrated CAT and SOD-like activity, the observed H₂O₂ dissociation capacity was
359 relatively modest, and SOD-like activity was present only in the first part of the assay and
360 demonstrated high variability across trials (**Fig 1**). Furthermore, the CAT-mimetic action was
361 not persuasive once PB was fractionated (**Fig 2C-E**), while the SOD-like activity of the
362 fractions (**Fig 2F**) suggested that the large variability and time-dependence observed in the first
363 experiment (**Fig 1B**) may have reflected the opposing effects of chemical constituents on THB
364 autoxidation. Despite the initial hypothesis that CuPC may be the main chemical constituent
365 of tattoo ink responsible for the effects of a blue tattoo on skin redox homeostasis [9],
366 potentiation of THB autoxidation by the white PB fraction (**Fig 2F**) indicated that there are
367 likely at least two chemical mediators with possibly opposing actions. Although it was not
368 possible to confirm the presence of individual chemical constituents in PB fractions, it is highly
369 likely that BaSO₄ and TiO₂ were the main constituents of the white fraction, and that they may
370 be responsible for the observed potentiation of THB autoxidation. Although both BaSO₄ and
371 TiO₂ can induce oxidative stress in different models [28,29], TiO₂ may be a more likely
372 mediator of the observed effect, as it stimulates the expression of antioxidant defense systems
373 to a greater extent *in vitro* [30]. In addition, TiO₂ can generate superoxide radicals and other
374 ROS by reducing oxygen, due to an increased number of conduction band electrons following
375 light exposure [27,31]. In the context of the effects of blue and white PB fractions on SOD
376 activity, the reported time-dependence of SOD-mimetic properties of PB (**Fig 1B**) may be
377 related to the limited ability of blue fraction constituents to suppress THB autoxidation,
378 potentiated by the chemicals present in the white fraction of the ink.

379 In addition to the observed CAT/SOD-mimetic activity *in vitro*, in order to affect redox balance
380 *in vivo*, tattoo ink should be able to exert the effect in the presence of the complex biochemical
381 matrix of normal skin tissue (i.e in the presence of endogenous CAT, SOD, and regulators of
382 their activity). In the presence of CAT, PB inhibited the H₂O₂ dissociation rate with the most
383 pronounced inhibition observed with the white ink fraction (**Fig 3A, B**). Although little is
384 known about the effects of BaSO₄ on CAT activity, it has been shown that TiO₂ can bind to
385 CAT via electrostatic and hydrogen bonding forces, destabilize its structure and affect its
386 activity in a dose-dependent manner [32]. Interestingly, the effects of tattoo ink on CAT were
387 drastically altered in the presence of the biochemical constituents of normal skin, as all tested
388 samples potentiated the relatively low endogenous H₂O₂ dissociation potential (**Fig 3C, D**).
389 The white fraction of the ink induced the most pronounced effect, increasing the activity 78-
390 fold, while the blue fraction induced only „a modest” ~5-fold increment. A substantial
391 increment in the dissociation potential observed with the white fraction is in line with the SP-
392 EMSA results indicating that a constituent of the ink that does not contain CuPC (most likely
393 originating from the white fraction) has a pronounced ability to degrade H₂O₂ (Fig 5G-I).
394 Interestingly, the potentiation of CAT observed with the unfractionated ink was somewhere in

395 between (~17-fold), indicating that chemical constituents of tattoo ink might either act as
396 competitive activators or engage in some other form of interaction that is reflected in CAT
397 activity. A similar pattern was observed regarding the SOD-mimetic action, where the
398 constituents from the blue and white fractions exhibited SOD-like properties on their own but
399 canceled each other out when added to the tissue homogenate together (PB) (**Fig 3G**). Why
400 were both ink fractions able to potentiate SOD activity, and whether the observed effect was
401 mediated by the intrinsic SOD-mimetic properties of the chemical constituents or their action
402 on the endogenous enzyme, defies a simple explanation and remains to be further explored.
403 Nevertheless, considering that TiO₂ can potentiate the activity of SOD [33], one possibility is
404 that TiO₂ may exert a dose-dependent modulatory effect on SOD similarly as has been shown
405 for CAT [32].

406 Finally, the effects of PB and its fractions have been tested upon prolonged (180 min)
407 incubation with skin homogenates at homeothermic temperature (37 °C), to assess whether
408 some of the effects may be transient (e.g. due to dependence on an endogenous substrate) and
409 affected by physiological temperature. Interestingly, both CAT and SOD-mimetic properties
410 were dramatically altered by pre-incubation and the most pronounced H₂O₂ dissociation rate
411 was observed for the blue ink fraction followed by PB (**Fig 3E, F**). The SOD-mimicking effect
412 was annihilated by the pre-incubation procedure (**Fig 3H**). The exact nature of the observed
413 phenomenon and whether the homeothermic pre-incubation more faithfully reflects the fate of
414 the tattoo ink constituents in the human skin remains to be elucidated. On one hand, prolonged
415 incubation at physiological temperature may provide more accurate environmental conditions
416 for the biochemical reactions that may take place in the human body. On the other hand, the
417 observed potentiation of the enzymatic activity may be dependent on a particular substrate
418 present in the biochemical matrix of the tissue homogenate in limited quantities (in contrast to
419 its continuous influx *in vivo*). Another possible explanation for the observed discrepancy might
420 be a temperature-induced change in physicochemical properties of the tattoo ink constituents.
421 Tattoo inks contain nanoparticles and both CuPC and TiO₂ can be found in the nanoparticle
422 form in the blue tattoo ink (with the mean diameter of the CuPC/TiO₂ being 167 nm for the
423 Intenze™ blue tattoo ink)[34,35]. Nanoparticles have an intrinsic potential to generate ROS,
424 which has been recognized as a key mediator of nanotoxicity [36]. Considering that toxicity,
425 photoreactivity, and ROS-generating potential depend on the particle size, shape, surface
426 characteristics, and the crystal structure [31], a hypothetical reaction between the tattoo ink and
427 either the tissue homogenate or the microtiter plate that may alter the structure of its
428 nanoparticle components may provide an explanation for the observed alteration of the
429 modulatory activity on CAT and SOD (**Fig 3**).

430 To further elucidate the nature of the CAT and SOD-mimetic actions of the blue tattoo ink a
431 series of experiments was done to explore whether the observed functional alterations are
432 accompanied by the ability of ink constituents to bind to CAT and the components of the skin
433 matrix (**Fig 4,5**). Specific studies focused on the interaction of individual components are yet
434 to be done. Nevertheless, preliminary experiments assessing a more general overview of the
435 possibility that two complex and heterogeneous samples (tattoo ink and skin) contain
436 molecules that show the ability to interact directly presented here (**Fig 4,5**) support the notion
437 that the observed changes in the enzymatic activity may be a consequence of the interaction of
438 at least several separate molecular entities from the skin, and definitely more than one chemical
439 constituent of the tattoo ink.

440 In the context of previously reported redox-related changes in the skin with a blue tattoo, the
441 results presented here support the hypothesis that the suppression of oxidative stress in the N-
442 of-1 study may be related to the antioxidant properties of some constituents present in blue
443 tattoo ink [9]. In the N-of-1 study, a 15% reduction in lipid peroxidation in the blue tattoo was
444 associated with 11.8% greater CAT activity [9]. In concordance with this, in this study, the
445 constituents of blue tattoo ink were able to potentiate CAT activity in the presence of the
446 biochemical components of the skin (**Fig 3C-F**). Interestingly, in the same study, SOD activity
447 was slightly higher in the tattoo sample; however, this result was taken as highly uncertain
448 considering the difference was in the range of the coefficient of variation of the method [9]. In
449 the context of the *in vitro* results presented here, it can be assumed that apart from the limited
450 precision of the utilized method, SOD activity in the blue tattoo may have been unchanged due
451 to the opposing action of different constituents of the tattoo ink (**Fig 3G**) or due to the same
452 phenomenon responsible for the loss of SOD-mimetic activity following prolonged incubation
453 at 37°C (**Fig 3H**).

454 **Conclusion:**

455 The presented results confirm the hypothesis that blue, CuPC-based tattoo ink can act as a CAT
456 and SOD mimetic *in vitro* and provide evidence that the antioxidant effects of a blue tattoo *in*
457 *vivo* [9] may be mediated by the ability of CuPC and other chemical constituents of the blue
458 tattoo ink to mimic the activity of endogenous antioxidant enzymes. In contrast to the
459 assumption that the CAT and SOD-mimetic properties of the tattoo ink would be primarily
460 explained by the presence of CuPC, the results suggest that both the CuPC-enriched blue and
461 the residual white fraction may exert CAT and SOD-mimetic properties and affect redox
462 balance, indicating that other chemical constituents (e.g. TiO₂) may also be involved in
463 modulation of redox homeostasis. Finally, it has been demonstrated that the ability of different
464 constituents of the tattoo ink to potentiate and/or inhibit the activity of CAT and SOD depends
465 on numerous factors (e.g. the presence of other constituents that exert synergistic, additive, or
466 antagonistic effects; the concentration of the constituents and/or the substrate; the presence of
467 compounds from the biochemical matrix of the skin; the incubation time and temperature) that
468 should be taken in account.

469 **Limitations:**

470 Several important limitations should be emphasized. First, it was not possible to analyze the
471 presence, or the quantity of individual chemical constituents of the tattoo ink used in the
472 experiment, and the presence of different chemicals was assumed based on the official material
473 safety data sheet of the product. Apart from the CuPC that was most likely present based on
474 the UV-Vis spectrum characterized by the Soret peak (B-band) and the Q-band with the
475 Davydov splitting characteristic for the phthalocyanine derivatives, the existence of other
476 chemicals (and possibly also impurities) could not have been confirmed experimentally.
477 Furthermore, the presence of CuPC and TiO₂ nanoparticles was assumed based on the
478 experimental data for the Intenze™ blue tattoo ink reported by Høgsberg et al. [35], however,
479 the existence or the size of nanoparticles was not confirmed experimentally and the potential
480 influence of the experimental conditions on the particle aggregation, size, shape, surface
481 characteristics, or the crystal structure (important for the redox-related effects) was not
482 assessed. Finally, the possibility that the chemical reaction with some of the reagents may have
483 introduced bias in some measurements can never be completely ruled out. For example, it has
484 been observed that 450 nm absorbance of some of the dilutions of some fractions was affected

485 by spectrophotometric measurements (possibly due to light exposure as some chemical
486 constituents such as TiO₂ act as well-known photocatalysts [27])(**Supplement**). Nevertheless,
487 precautionary steps were taken to prevent the chemical bias that may have been introduced due
488 to unforeseen chemical reactions of samples and reagents (e.g. baseline validation model was
489 established and analyzed for each sample individually to ensure that the expected changes such
490 as the dissociation of H₂O₂ can be assumed and quantified without the risk of chemical
491 interaction) (**Supplement**).

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493 None.

494 **Conflict of interest statement:**

495 No conflict of interest to disclose.

496 **Data availability statement:**

497 Data can be obtained from the author's GitHub repository (<https://github.com/janhomolak>).

498 **Author's contributions:**

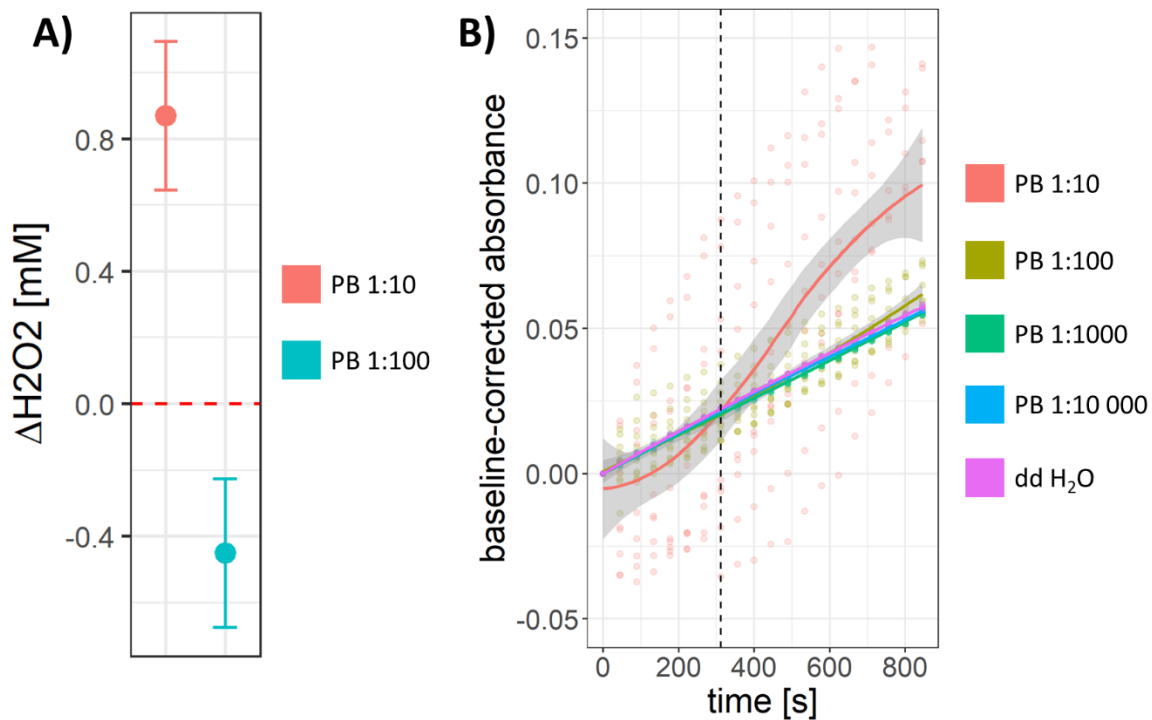
499 **JH** conceived the study, conducted the experiments, analyzed data, and wrote the manuscript.

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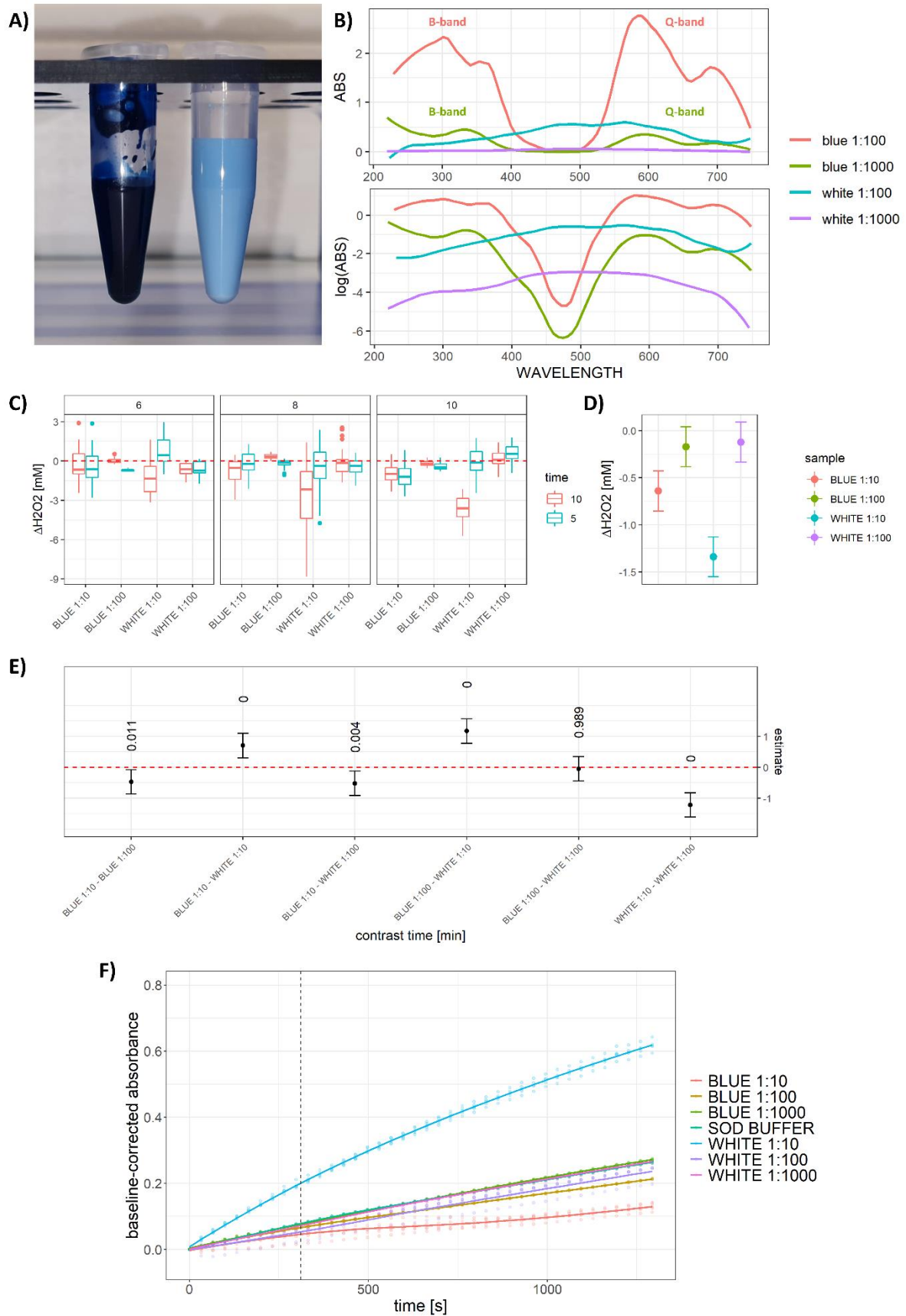
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- 590



591

592 **Fig 1.** Catalase (CAT) and superoxide dismutase (SOD)-like activity of Persian Blue tattoo ink
 593 (PB) *in vitro*. A) Output of the model including the tested dilutions and substrate concentrations
 594 demonstrating dilution-dependent CAT-like activity of PB, with the 1:10 PB dilution acting as
 595 CAT mimetic (substrate concentrations: 6, 8, and 10 mM H₂O₂; t = 300 s). B) Dilution-
 596 dependent SOD-like activity of PB, with 1:10 PB demonstrating SOD-like properties at t < 300
 597 s, and potentiation of 1,2,3-trihydroxybenzene autooxidation at t > 300 s. 1:100, 1:1000 and
 598 1:10 000 dilutions show no SOD-mimetic activity.

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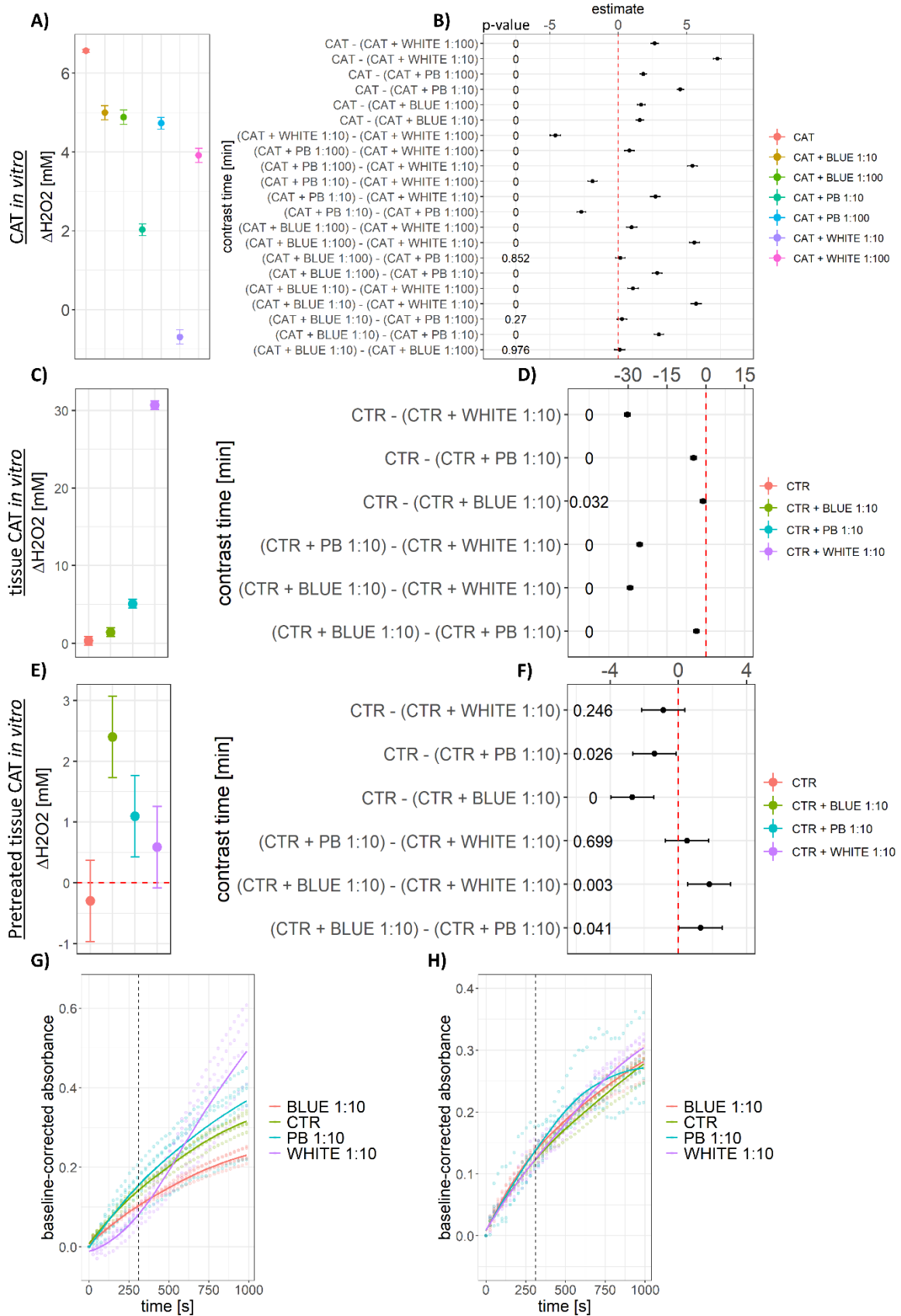


600

601 **Fig 2.** Catalase (CAT) and superoxide dismutase (SOD)-like activity of blue and white
 602 fractions of the Persian Blue tattoo ink (PB) *in vitro*. A) A representative image of the blue

603 (left) and white (right) fractions of PB. B) Absorption spectra of the 1:100 (red) and 1:1000
604 (green) dilution of the blue fraction, and the 1:100 (turquoise) and 1:1000 (purple) dilution of
605 the white fraction of PB. The absorption spectrum of the blue fractions demonstrates the Soret
606 peak (B-band) and Q-band, strongly indicating that copper phthalocyanine was only present in
607 the blue fraction of PB. C) Results demonstrating no CAT-like activity of different dilutions of
608 either fraction *in vitro* (substrate concentrations: 6, 8, 10 mM H₂O₂; t₁= 300 s; t₂= 600 s). D)
609 Output of the model including tested dilutions of the blue and white fraction, time, and substrate
610 concentration, demonstrating no observed CAT-like activity. E) Comparison of the observed
611 CAT-like activities of two dilutions of the blue and white fraction at different substrate
612 concentrations. P-values are reported above estimates of differences of estimated marginal
613 means accompanied by 95% confidence intervals. F) Dilution-dependent SOD-like activity of
614 PB fractions. The 1:10 dilution of the white fraction potentiates autooxidation of 1,2,3-
615 trihydroxybenzene at t < 300 s, while all other dilutions of both fractions show no pronounced
616 effects. At t > 300 s, the 1:10 dilution of the blue fraction demonstrates pronounced, while the
617 1:100 dilution shows slightly less pronounced SOD-like activity. The 1:10 dilution of the white
618 fraction shows a strong 1,2,3-trihydroxybenzene autooxidation potentiating effect.

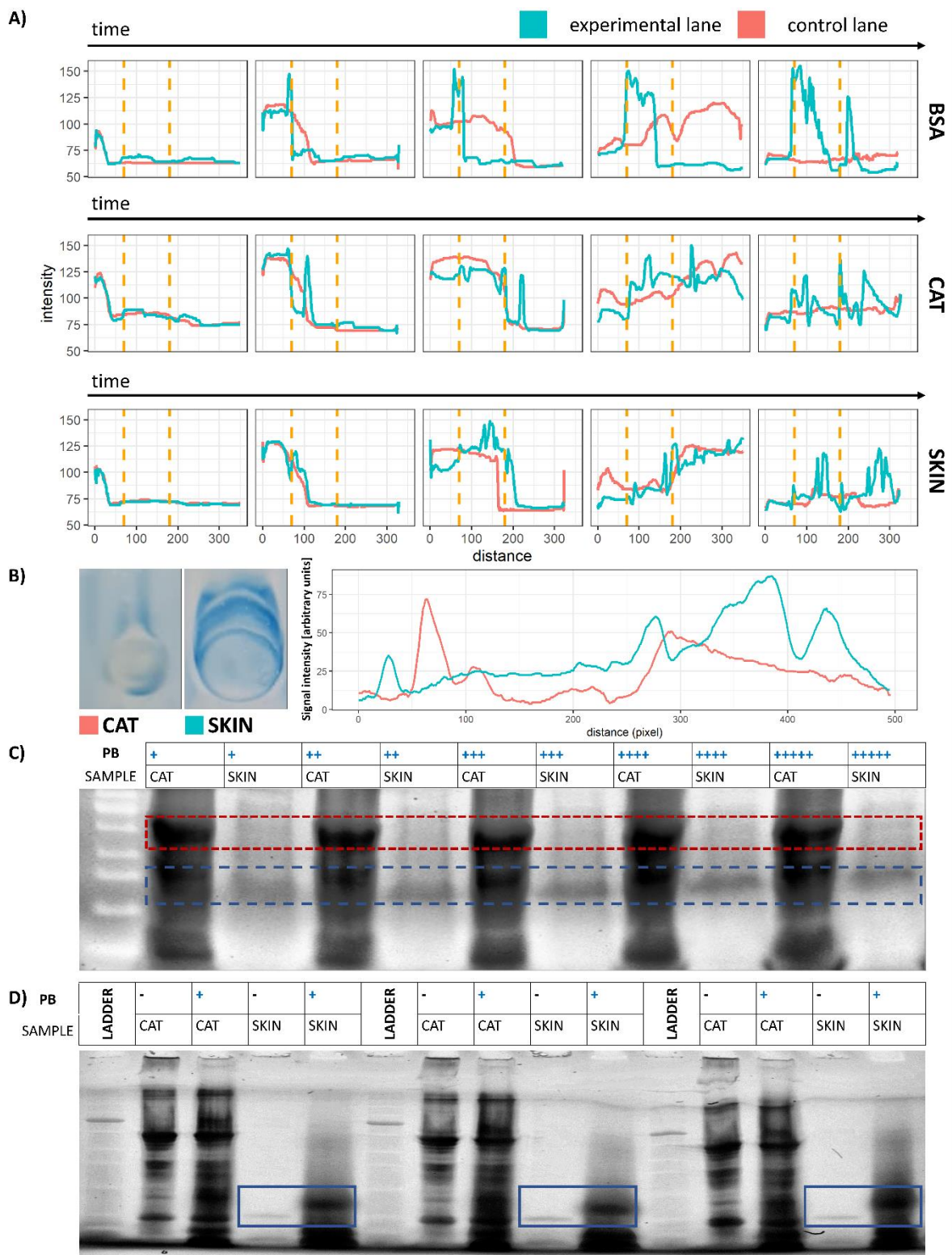
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620

621 **Fig 3.** Catalase (CAT) and superoxide dismutase (SOD)-like activity of the Persian Blue tattoo
 622 ink (PB) and its blue and white fractions *in vitro* in the presence of biochemical constituents of
 623 the skin. A) Output of the model including tested dilutions of PB and its blue and white

624 fractions and substrate concentrations (6, 8, and 10 mM H₂O₂), demonstrating the ability of PB
625 and its fractions to inhibit CAT *in vitro* (t = 60 s). While the effect was not dose-dependent for
626 the blue fraction, the white fraction and PB showed a pronounced dose-dependent inhibition
627 of the enzyme. B) Model results presented as point estimates of differences of estimated
628 marginal means with 95 % confidence intervals for the model presented in A. C) Output of the
629 model including tested dilutions of PB and the blue and white PB fractions demonstrating the
630 ability of the samples to potentiate CAT activity in the complex biochemical matrix of normal
631 skin tissue (substrate concentration: 10 mM H₂O₂; t = 600 s). D) Model results presented as
632 point estimates of differences of estimated marginal means with 95 % confidence intervals for
633 the model presented in C. E) Output of the model including tested dilutions of PB and the blue
634 and white PB fractions demonstrating the effect of the samples on CAT activity in the complex
635 biochemical matrix of normal skin tissue following pre-incubation of the dyes with the tissue
636 samples (pre-incubation time: 180 min; pre-incubation temperature: 37 °C; substrate
637 concentration: 10 mM H₂O₂; t = 600 s). F) Model results presented as point estimates of
638 differences of estimated marginal means with 95 % confidence intervals for the model
639 presented in E. G) SOD mimetic activity of PB and the blue and white PB fractions in the
640 complex biochemical matrix of normal skin tissue. At t < 300 s, PB demonstrated no SOD-like
641 activity, while both blue and white fractions acted as SOD mimetics. At t > 300 s, PB was
642 associated with slight potentiation, while the white PB fraction induced pronounced
643 autooxidation of 1,2,3-trihydroxybenzene. Conversely, the blue PB fraction demonstrated
644 SOD-mimetic activity. H) SOD mimetic activity of PB and the blue and white PB fractions in
645 the complex biochemical matrix of normal skin tissue following incubation of the dyes with
646 skin tissue for 180 min at 37 °C. Pre-incubation of the dyes with the skin tissue constituents
647 alleviated the effects observed in F.

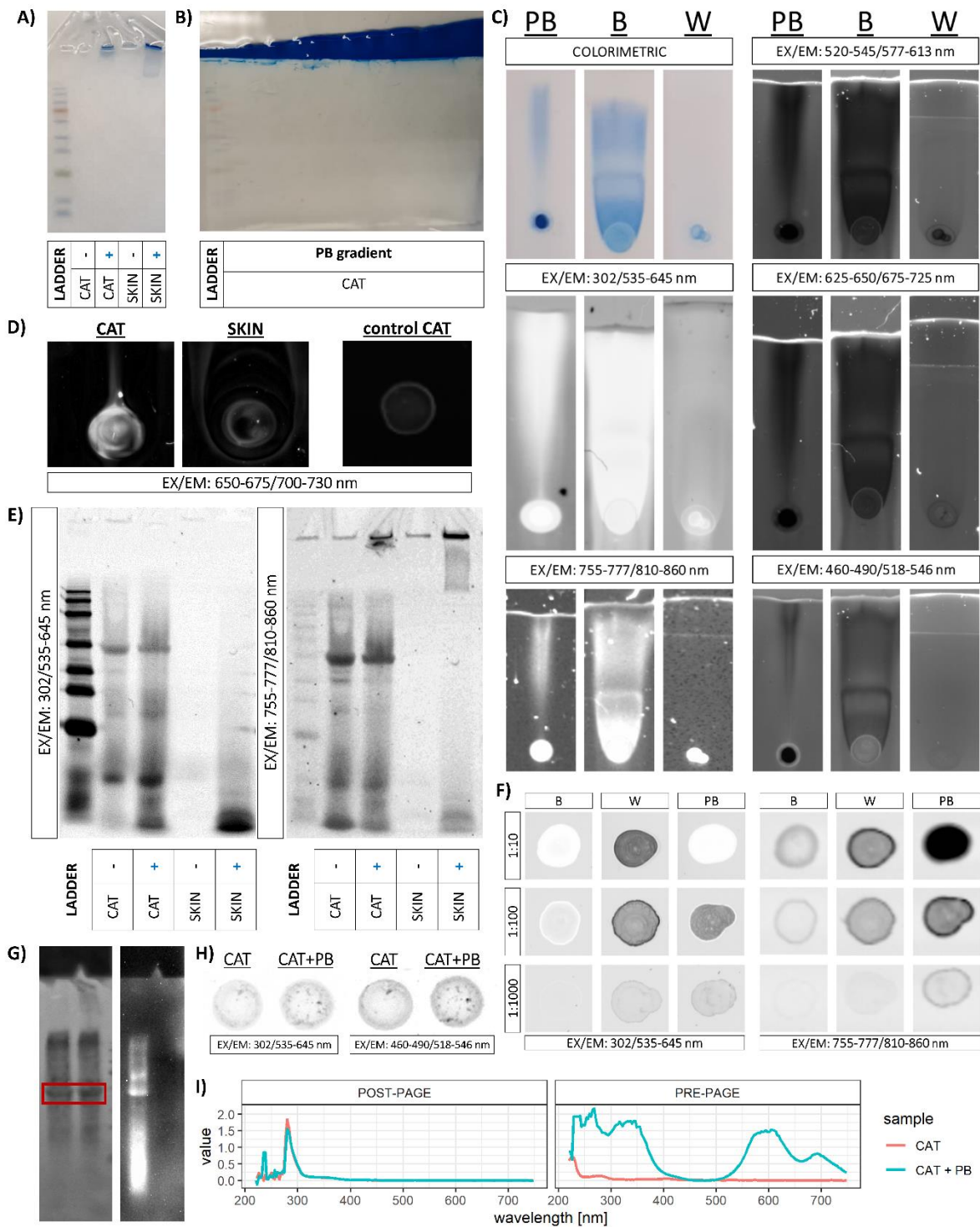


648

649 **Fig 4.** Interaction of the blue tattoo ink with constituents of the skin homogenate assessed by
 650 the lateral flow assay (LFA), CuPC-capturing gradient electrophoretic mobility shift assay
 651 (CCG-EMSA), and sample pre-incubation electrophoretic mobility shift assay (SP-EMSA). A)
 652 Line profiles obtained from LFA with the Persian Blue (PB) tattoo ink used as a mobile phase.
 653 Profiles obtained from the free route for the uninterrupted analyte flow (control lane) and the
 654 sample-capturing lane containing two capturing pools (experimental lane; capturing pools are
 655 marked with yellow lines) are shown for the control high binding capacity protein – bovine

656 serum albumin (BSA; top), catalase (CAT; middle), and the skin homogenate (SKIN; bottom).
657 Both time (5 time-points) and the direction of the flow are aligned with the X-axis. B) CAT
658 and skin homogenate after LFA with PB mobile phase followed by ddH₂O (left). The skin
659 sample shows a wave pattern suggesting the presence of interaction with several separate
660 components. Line profiles of the samples presented on the left with 3 peaks associated with the
661 skin sample suggest heterogeneous interaction patterns. C) The polyacrylamide gel from the
662 CCG-EMSA experiment demonstrates the absence of the electrophoretic mobility shift for the
663 CAT sample (red), and dose-dependent electrophoretic deceleration for the skin sample (blue).
664 The trichalo-containing polyacrylamide gel was visualized using 302 nm excitation and 535-
665 645 nm emission. A negative image is presented. D) The gel from the SP-EMSA experiment
666 showing 3 technical replicates of pre-treated and untreated CAT and skin samples with no
667 apparent deceleration in the CAT sample, and a prominent reduction in mobility of the
668 prominent peak in the skin sample (blue). The trichalo-containing polyacrylamide gel was
669 visualized using 302 nm excitation and 535-645 nm emission. A negative image is presented.

670



671

672 **Fig 5.** Interaction of the blue tattoo ink with constituents of the skin homogenate assessed by
 673 the lateral flow assay (LFA), CuPC-capturing gradient electrophoretic mobility shift assay
 674 (CCG-EMSA), and sample pre-incubation electrophoretic mobility shift assay (SP-EMSA)
 675 spectral analysis. A) Native image of the polyacrylamide gel from the SP-EMSA experiment
 676 after electrophoretic separation of catalase (CAT) and skin samples. B) Native image of the
 677 polyacrylamide gel from the CCG-EMSA experiment after electrophoretic separation. C)
 678 Spectral analysis of the LFA membranes using different excitation and emission wavelengths

679 demonstrates 3 general patterns: the sample pool (the area where the sample was deposited –
680 lower portion of the images); the middle mobile phase (largely absent in the white fraction –
681 the middle portion of the images); and the mobile front (present in all samples – upper portion
682 of the images). D) CAT and the skin sample exposed to the Persian Blue tattoo ink (PB) mobile
683 phase, and the control CAT sample (not exposed to the PB mobile phase to control for baseline
684 spectral properties) emission at 700-730 nm upon excitation at 650-675 nm. A substantial
685 increment in emission and the smearing pattern speak in favor of the interaction of CAT with
686 the PB mobile front. E) Spectral analysis of the gel from the SP-EMSA experiment shows that
687 both the PB-pretreated skin sample and PB-pretreated CAT emit a signal in concordance with
688 the presence of constituents from the white fraction (see F). Notice the difference in the signal
689 from the stacking gel and the resolving gel in the context of the ink fractions emission signals
690 (presented in F). Negative images are presented. F) The effect of PB fractions and
691 concentrations in two different spectral planes. Negative images are presented. G) Ponceau S
692 staining of the nitrocellulose membrane with the PB-treated and PB-untreated CAT from the
693 SP-EMSA experiment (left), and the corresponding membrane exposed to luminol and H₂O₂
694 demonstrating a substantial H₂O₂ dissociation potential only in the PB-treated CAT sample
695 (right). The main CAT fraction is emphasized in red. The pattern indicates that both the PB-
696 pretreated CAT and the front have the ability to promote H₂O₂ dissociation. H) Negative
697 images of the signal obtained from the PB-treated and PB-untreated CAT eluted from the
698 membrane shown in G in two different spectral planes indicating the presence of the
699 constituents associated with the white fraction. I) Confirmation of the absence of the CuPC
700 from the samples containing CAT eluted from the membrane shown in G. UV-Vis spectra both
701 CAT and PB-pretreated CAT before the polyacrylamide gel electrophoresis (PRE-PAGE) and
702 after elution from the membrane (POST-PAGE).

703