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1 **The seed of the Amazonian fruit *Couepia bracteosa* exhibits higher scavenging capacity**
2 **against ROS and RNS than its shell and pulp extracts**

3

4 **Running head:** ROS and RNS scavenging capacity of *C. bracteosa* fruit extracts

5

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24 **Abstract**

25 Among the large number of scientifically oblivious fruits from Amazonia biome, *Couepia*
26 *bracteosa* figures as an interesting source of bioactive compounds, such as phenolic compounds
27 and carotenoids, which may be used for protecting human health against oxidative damage. For
28 the first time, the phenolic compounds and carotenoids of extracts obtained from the pulp, shell
29 and seeds of *C. bracteosa* fruits are reported, as well as its *in vitro* scavenging capacities against
30 some reactive oxygen species (ROS) and reactive nitrogen species (RNS). The shell extract
31 presented the highest phenolic compound and carotenoid contents (5540 and 328 μg/g extract,
32 dry basis, respectively), followed by the pulp and seed extracts. The major phenolic compound
33 was acacetin sulphate (one methoxy and two OH groups) (62%) in the shells; however, only
34 seeds presented apigenin sulphate (three OH groups), in which it was the major compound
35 (44%). The high content of apigenin sulphate may explain the highest scavenging efficiency of
36 the seed extract against all tested ROS/RNS among the studied extracts. Regarding carotenoids,
37 all-*trans*-neochrome (17%) and all-*trans*-β-carotene (16%) were the major carotenoids in the
38 pulp extracts, while all-*trans*-lutein (44%) was the major in shell and all-*trans*-α-carotene (32%)
39 and all-*trans*-β-carotene (29%) were the major ones in seed extracts.

40

41 **Keywords:** Phenolic compounds; carotenoids; antioxidant capacity; reactive oxygen species;
42 reactive nitrogen species.

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44 **1. Introduction**

45 Brazilian Amazonia holds a great biodiversity of fruit species, comprising
46 approximately 220 species of edible fruits that represent almost half of the diversity of native
47 fruits in Brazil. These fruits are considered excellent sources of bioactive compounds that may
48 be used for nutritional purposes and also for protecting human health against many diseases.
49 Thus, the knowledge of the species, their chemical composition and their biological properties is
50 one of the major challenges to improve their commercial value and rational exploitation.[1]

51 Recently, our research group has been directed some efforts to study different fruits
52 from Amazonia, concerning the prospection of bioactive compounds (phenolic compounds and
53 carotenoids) and also the *in vitro* antioxidant potential against some physiologically relevant
54 reactive oxygen (ROS) and nitrogen species (RNS) to understand its nutraceutical potential and
55 antioxidant benefits.[2-5] In biological systems, the production of ROS and RNS is important to
56 maintain homeostasis. However, in the eventuality of an imbalance between the production of
57 pro-oxidant reactive species and antioxidant defence capacity, like it happens during the ageing
58 process, the cellular components, such as lipids, proteins, DNA, even the tissue can be damaged
59 (oxidative stress), resulting in several diseases, such as diabetes, cancer, allergy, inflammation,
60 neurodegenerative and cardiovascular diseases.[6]

61 The *Couepia bracteosa* Benth species (Brazilian name: “pajurá”) belongs to the
62 Chrysobalanaceae R. Br. family. It is native to the Tropical Amazon and naturally found in the
63 following Brazilian States: Amazonas, Amapá, Pará and Rondônia. The *C. bracteosa* tree has
64 medium size (up to 25 m high); the fruits are globose drupes with 8 to 12 cm long by 8 to 15 cm
65 in diameter and 80-200 g of weight. The peel (exocarp) is dark-brown with rough surface,
66 covered with numerous white dots (lenticels). The pulp (mesocarp) is thick, fleshy and oily,
67 yellow-brown colour, with grainy consistency, sweetness flavour vaguely reminiscent of nuts
68 and a thick endocarp with rough surface, dark brown colour, abundant endosperm and just one
69 large seed. The pulp is traditionally consumed *in natura*, as well as used to prepare different
70 kind of sweets, such as jams.[7]

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71 To the best of our knowledge, no data related to the bioactive compounds or biological
72 potential of *C. bracteosa* fruits have been published in the literature. However, tocopherols,
73 flavonoids and derivatives and also triterpenes were reported for other species from *Couepia*
74 genus, such as *C. edulis*[8] and *C. paraensis*[9-11]. Furthermore, there are other reports in the
75 literature that support the presence of interesting bioactive compounds in *Couepia* genus. For
76 example, the chemopreventive activity (induction of quinone reductase activity) of *C. ulei*
77 compounds[12], the antibacterial, antioxidant and cytotoxicity activity against *Artemia salina* of *C.*
78 *grandiflora* extracts[13], and the anticancer activity (lyase inhibitors of DNA β-polymerase
79 activity) of *C. polyandra*[14].

80 In this paper, we are reporting, for the first time, the tentative identification and
81 quantification (HPLC-DAD-MS^n) of bioactive compounds (phenolic compounds and
82 carotenoids) of seed, shell and pulp extracts obtained from *C. bracteosa* fruits, and also the
83 antioxidant potential of each extract against some ROS and RNS with high relevance in
84 biological systems: superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hypochlorous acid
85 (HOCl), nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$). These results may help to stimulate the
86 rational exploitation of natural resources from the Amazonian biome due to the little number of
87 scientific studies about native fruits from that region, including the potential benefits not only
88 for the local people, but also for the food, pharmaceutical and cosmetic industries.

89

90 **2. Experimental**

91 **2.1. Chemicals**

92 Nitroblue tetrazolium chloride (NBT), β-nicotinamide adenine dinucleotide (NADH),
93 phenazine methosulphate (PMS), lucigenin, 30% hydrogen peroxide, sodium hypochlorite
94 solution (4% available chlorine), dihydrorhodamine 123 (DHR), 4,5-diaminofluorescein (DAF-
95 2), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), quercetin, acacetin,
96 apigenin, all-*trans*-lutein, all-*trans*-zeaxanthin, all-*trans*-β-cryptoxanthin, all-*trans*-β-carotene,
97 dimethyl sulfoxide (DMSO), ethanol, methanol, methyl *tert*-butyl ether (MTBE), acetonitrile

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98 and all other chemical salts and solvents of analytical grade were obtained from Sigma-Aldrich
99 (St. Louis, USA). Ultrapure water was obtained from the arium® pro system (Sartorius,
100 Germany). All phenolic compounds and carotenoids standards showed at least 95% of purity, as
101 determined by HPLC-DAD.

102

103 **2.2. *C. bracteosa* samples and extract preparation**

104 The *C. bracteosa* fruits were acquired in three street markets (≈ 1 kg) in Manaus,
105 Amazonas, Brazil (03º06'07"S and 60º01'30"W). All the fresh and ripe fruits (≈ 3 kg) were
106 combined, washed with distilled water and the pulp, shell and seeds were manually separated to
107 prepare three different extracts. Approximately 50 g of pulp or shell or seeds was submitted to
108 extraction with absolute ethanol in a mass/solvent ration of 1:10 (w/v), for 4 h at room
109 temperature (25 °C), protected from light incidence and under agitation (≈ 80 rpm) using
110 magnetic stirrer. The extracts were vacuum-filtered (Whatman filter paper nº 4) and the solvent
111 was evaporated under reduced pressure (T< 40°C).[4] All concentrated extracts were freeze-dried,
112 transferred to amber glass bottles and stored at -20 °C for further analysis.

113

114 **2.3. HPLC-DAD-MS^n analysis of phenolic compounds and carotenoids**

115 **2.3.1. Equipments**

116 The identification and quantification of phenolic compounds in all extracts was
117 performed in an Accela HPLC system (Thermo Fisher Scientific, San Jose, CA) equipped with a
118 quaternary pump (Accela 600), a DAD detector and an auto-sampler cooled to 5 ºC. The
119 equipment was also connected in series to a LTQ Obritrap™ XL mass spectrometer (MS)
120 (Thermo Fisher Scientific, San Jose, CA) with electrospray ionization source (ESI), and a
121 hybrid system combining a linear ion-trap and the Orbitrap as the *m/z* analyzer. The
122 identification of carotenoids was performed in a Shimadzu HPLC (Kyoto, Japan) equipped with
123 a quaternary pump (LC-20AD), a degasser unit (DGU-20A5), a Rheodyne injection valve with

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124 a 20 µL loop, a DAD detector (SPD-M20A), and connected in series to a MS from Bruker
125 Daltonics (AmaZon speed ETD, Bremen, Germany) with atmospheric pressure chemical
126 ionization (APCI) and an ion-trap as the *m/z* analyzer. The quantification of carotenoids was
127 carried out in a LaChrom HPLC system (D-700, Merck Hitachi Ltd., Tokyo, Japan) equipped
128 with a quaternary pump (L-7100) and DAD detector (L-7455). For all the chromatographic
129 analysis, samples and solvents were filtered using, respectively, membranes of 0.22 and 0.45
130 µm, both from Millipore (Billerica, MA, USA).
131

132 **2.3.2. Determination of phenolic compounds and carotenoids from *C. bracteosa* extracts**

133 The phenolic compounds were analysed after solubilising 50 mg of the freeze-dried
134 extract from each fruit part in methanol/water (80:20, v/v) and the compounds were separated
135 on a C_{18} Synergi Hydro column (4 µm, 250 x 4.6 mm, Phenomenex), at 0.9 mL/min, column
136 temperature at 29 °C and mobile phase consisting of water/formic acid (99.5:0.5, v/v)
137 acetonitrile/formic acid (99.5:0.5, v/v) in a linear gradient.[15] The column eluate was split to
138 allow only 0.3 mL/min to enter the ESI interface. The UV-Vis spectra were obtained between
139 200 and 600 nm, and the chromatograms were processed at 280, 320 and 360 nm. Mass spectra
140 were obtained after ionization in an ESI source in the negative ion mode, with a scan range from
141 *m/z* 100 to 1000, and the MS parameters were set at the same conditions as described in our
142 previous work.[4] Phenolic compounds were tentatively identified based on the following data:
143 elution order, retention time of peaks and characteristics of the UV-visible and mass spectra in
144 comparison with authentic standards (data not shown) analysed under the same conditions and
145 data available in the literature[3-5, 15, 16]. The quantification was carried out by comparison to
146 external analytical curves (1 to 100 µg/mL, in duplicate) using five-point for the standards
147 apigenin (at 339 nm, $r^2 \geq 0.99$) and acacetin (at 327 nm, $r^2 \geq 0.99$).

148 For carotenoid analysis, 50 mg of each freeze-dried extract of *C. bracteosa* were
149 solubilised in acetone and directed to the steps of exhaustive extraction, liquid-liquid partition,
150 saponification and drying under N_2 flow, following the same procedures described in details by

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151 Ribeiro *et al.*[4] The dried saponified carotenoid extracts were re-suspended in methanol/MTBE
152 (70:30, v/v) and injected into the chromatographic systems. The carotenoids were separated on a
153 C_{30} YMC column (5 µm, 250 mm x 4.6 mm) with a linear gradient of methanol and MTBE at
154 0.9 mL/min and temperature column set at 29 °C.[15] The UV-Vis spectra were recorded between
155 200 and 600 nm and the chromatograms were processed at 450 nm. The column eluate was
156 directed to the APCI interface and the mass spectra were obtained after ionization in the positive
157 ion mode, with a scan range from *m/z* 100 to 800 and MS parameters set as described by Chisté
158 and Mercadante[15]. The carotenoids were tentatively identified according to the following
159 combined information: elution order, retention time, co-chromatography with authentic
160 standards, UV-visible spectrum (λ_{max}, spectral fine structure (%III/II), peak *cis* intensity
161 (%A_B/A_{II})) compared with data available in the literature[4, 15, 17]. The characterization of each *cis*-
162 isomers of carotenoids was based on the observed decrease in the %III/II values and increase
163 %A_B/A_{II} values (≈ 7-11% = 9-*cis*; ≈ 45% = 13-*cis* e ≈ 56% = 15-*cis* carotenoid) as the cis
164 double bond moves from the end to the centre of the molecule.[17] The carotenoids were
165 quantified by HPLC-DAD by comparison to standards using five-point external analytical
166 curves (0.5-30 µg/mL, in duplicate) for all-*trans*-lutein, all-*trans*-zeaxanthin, all-*trans*-β-
167 cryptoxanthin and all-*trans*-β-carotene. All other carotenoids (including epoxy and *cis* isomers)
168 were estimated using the curve of the corresponding all-*trans*-carotenoid.

169 The contents of phenolic compounds and carotenoids of all extracts, determined by
170 HPLC-DAD, were expressed as µg/g of extract (dry basis), considering three independent
171 extraction procedures (n=3).

172

173 **2.4. ROS- and RNS-scavenging assays**

174 The scavenging assays against all ROS and RNS were carried out in a microplate reader
175 (Synergy HT, Biotek, Vermont, USA) equipped with thermostat and detection systems for the
176 measurement of fluorescence, UV-Vis and chemiluminescence. Quercetin was used as positive
177 control in all assays and its IC_{50} values were similar to those already reported by our research

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178 group.[2-4] DMSO was used to solubilise all extracts of *C. bracteosa* fruit in all assays, excepting 179 for the HOCl-scavenging assay (ethanol). Additional experiments were performed with all 180 extracts to ensure the results are not flawed by any interference of solvents or 181 fluorescence/chemiluminescence/absorbance response of extracts (data not shown). The IC_{50} 182 values (*in vitro* inhibitory concentration of the extract which is able to reduce, by 50%, the 183 effect of ROS or RNS) were calculated from the curves of percentage of inhibition *versus* 184 antioxidant concentration using GraphPad Prism 6 software. For each assay, four independent 185 experiments were performed, in duplicate, using six different concentrations.

186

187 **2.4.1. $O_2^{\bullet-}$-scavenging assay**

188 The non-enzymatic system NADH/PMS/O_2 was used to generate $O_2^{\bullet-}$, which promotes 189 the reduction of NBT into a purple coloured diformazan compound. This reaction was followed 190 by spectrophotometry, at 560 nm, for 2 minutes, by monitoring the effect of each *C. bracteosa* 191 extract and the positive control against the $O_2^{\bullet-}$-induced reduction of NBT.[3] The scavenging 192 capacities were expressed as the percentage of inhibition of the NBT reduction to diformazan.

193

194 **2.4.2. H_2O_2-scavenging assay**

195 The effect of each *C. bracteosa* extract and the positive control against the H_2O_2- 196 induced oxidation of lucigenin was monitored by chemiluminescence, at 37 °C and the signal 197 was detected immediately after the introduction of the plate in the reader.[3] The scavenging 198 capacities were expressed as percentage of inhibition of H_2O_2-induced oxidation of lucigenin.

199

200 **2.4.3. HOCl-scavenging assay**

201 HOCl was immediately prepared before the assay using a NaOCl solution 1% (w/v) and 202 adjusting to pH 6.2 with a diluted solution of H_2SO_4. The concentration of HOCl obtained after

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203 the reaction was determined by spectrophotometry at 235 nm using a molar absorption
204 coefficient of 100 $M^{-1}cm^{-1}$. The capacity of each extract and the positive control to scavenge
205 HOCl was determined by monitoring the HOCl-induced oxidation of DHR (non-fluorescent) to
206 rhodamine 123 (fluorescent).[3] The scavenging capacities were expressed as percentage of
207 inhibition of HOCl-induced oxidation of DHR.

208

209 **2.4.4. •NO-scavenging assay**

210 The antioxidant effect of *C. bracteosa* extracts and the positive control was measured by
211 monitoring the oxidation of DAF-2 to the fluorescent triazolofluorescein (DAF-2T) induced by
212 •NO, which was generated by the decomposition of NOC-5. The fluorescence signal was
213 followed during 30 minutes of incubation at 37 °C.[3] The scavenging capacities were expressed
214 as the percentage of inhibition of •NO-induced oxidation of DAF-2.

215

216 **2.4.5. $ONOO^-$-scavenging assay**

217 The $ONOO^-$ was synthesized as previously described by Fernandes, Gomes, Costa &
218 Lima.[18] The capacity of each extract and the positive control in scavenging $ONOO^-$ was
219 determined by monitoring the $ONOO^-$-induced oxidation of non-fluorescent DHR to the
220 fluorescent rhodamine 123.[3] Parallel experiments simulating physiological concentrations of
221 CO_2 were performed using 25 mM $NaHCO_3$. The scavenging capacities were expressed as
222 percentage of inhibition of $ONOO^-$- induced oxidation of DHR.

223

224 **3. Results and Discussion**

225 **3.1. Phenolic compounds and carotenoids from *C. bracteosa* extracts**

226 The phenolic compounds (Fig. 1) and carotenoids (Fig. 2) of all *C. bracteosa* extracts in
227 this study were separated, identified and quantified by HPLC-DAD-MS^n. In relation to the

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228 phenolic compounds identification, according to Table 1, peak 1 and 4 were assigned as
229 acacetin sulphate, probably positional isomers, since both peaks showed $[M-H]^-$ at *m/z* 363 and
230 exhibited neutral loss of 80 u (*m/z* 283) in the MS^2 spectra, which indicated loss of a sulphate
231 moiety esterified to acacetin $[M-H-SO_3]^-$, and fragments at *m/z* 268 $[M-H-SO_3-CH_3]^-$, indicating
232 loss of a methyl group after losing the sulphate moiety. The identity was confirmed by the same
233 MS characteristics observed after analyzing acacetin standard (data not shown). Peak 2 showed
234 $[M-H]^-$ at *m/z* 349 with a high intense loss of a sulphate moiety $[M-H-SO_3]^-$ (*m/z* 269) and was
235 tentatively identified as apigenin sulphate, since the fragmentation of *m/z* 269 (MS^3), presented
236 the same fragmentation pattern of the authentic standard of apigenin [neutral losses of 28u (CO)
237 and 44u (CO_2)] (data not shown). Peak 3 presented $[M-H]^-$ at *m/z* 377 and was tentatively
238 identified as oleuropein derivative after comparing the MS^2 and MS^3 features with those data
239 already well described in the literature.[16, 19]

240 Sulphate esters of flavonoids are relatively rare compounds and their functional
241 significance in plant tissues is not clear. They are found mainly in species occurring in coastal
242 and swampy areas rich in mineral salts, as well as in plants occurring in arid habitats.[20, 21] In
243 such plants, bind reaction of inorganic sulphate to flavonoids is probably one of the mechanisms
244 connected with biochemical adaptation of species to environment.[21] Although the phenolic
245 profile of *C. bracteosa* fruits was reported for the first time in this study, there is another report
246 available, in which two active compounds were identified in the ethyl acetate extracts of *C. ulei*
247 stems: erythro-2,3-bis(4-hydroxy-3-methoxyphenyl)-3-ethoxypropan-l-ol and a known
248 compound, evofolin-B, along with five inactive compounds (betulinic acid, oleanolic acid,
249 pomolic acid, (+)-syringaresinol and ursolic acid).[12]

250 Regarding the carotenoid profile, 18 compounds were separated and tentatively
251 identified and quantified (Table 2). The MS^2 experiments confirmed the assignment of the
252 protonated molecule ($[M+H]^+$) of all identified peaks through the fragments expected for the
253 carotenoid polyene chain and functional groups, along with the UV-Vis spectra features.[15, 17]
254 The carotenoid composition was slightly different for each extract, with predominance of
255 xanthophylls with one to three hydroxyl groups (OH), mostly with one or two epoxide groups.

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256 The identification of all-*trans*-lutein (peak 9), all-*trans*-zeaxanthin (peak 10), all-*trans*-β-
257 cryptoxanthin (peak 13) and all-*trans*-β-carotene (peak 17) was positively confirmed through
258 co-elution with authentic standards, as well as by comparison of their UV-vis and MS spectra
259 features with standards. Peaks 2, 4 and 5 (Table 2) presented the same MS and MS^2 spectra
260 characteristics: $[M+H]^+$ at *m/z* 601 and three consecutive neutral losses of water from the
261 protonated molecule were observed at *m/z* 583 $[M+H-18]^+$, *m/z* 565 $[M+H-18-18]^+$ and *m/z* 547
262 $[M+H-18-18-18]^+$, as well as a fragment at *m/z* 491 $[M+H-18-92]^+$ resulting from an additional
263 loss of toluene moiety (92 u) from the polyene chain. Peak 6 also presented $[M+H]^+$ at *m/z* 601,
264 but only two consecutive losses of water were observed in the MS^2 spectrum (*m/z* 583 and *m/z*
265 565). Peaks 7, 8, and 11 showed $[M+H]^+$ at *m/z* 585 and the indication of two OH attached to
266 the carotenoid molecules was demonstrated by the consecutive losses of two water moieties in
267 its MS^2 spectra (*m/z* 567 and *m/z* 549). In addition, the fragment at *m/z* 221 was observed in all
268 these peaks that correspond to an epoxy substituent in a β-ring with a OH group.[17] Moreover, all
269 these previous peaks showed a hypsochromic shift of 10 nm (peak11) and 25-30 nm (peaks 7
270 and 8) in relation to β-carotene (450 nm, peak 17), which indicates the presence of a 5,6-epoxy
271 or 5,8-furanoid groups in the carotenoid structures. Although 5,8-epoxides were already found
272 in other Amazonian fruits, such as buriti, marimari, palm oil, peach palm, physalis and
273 tucuma[17], it is not possible to assure that 5,6-epoxy to 5,8-furanoid rearrangement did not occur
274 during preparation and storage of the extracts obtained from *C. bracteosa*. However, peaks 7, 8
275 and 11 were assigned as “not identified” due to the lack of visible fragments at *m/z* 205 (β-ring
276 with an epoxy group) in their MS^2 spectra. In the same sense, other minor peaks (peaks 3, 12
277 and 14) were also assigned as “not identified” due to the lack of consistent data between its UV-
278 visible and MS spectra features as compared with data available in the literature to ensure its
279 tentative identification.

280 Peaks 15 to 18 belong to the carotene group since all presented $[M+H]^+$ at *m/z* 537 with
281 a characteristic neutral loss of toluene at *m/z* 444 $[M+H-92]^+$. The presence of fragments at *m/z*
282 481 and *m/z* 444 (peaks 15 and 16) corresponds to the respective losses of ε-ring and toluene as

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283 in α-carotene and its isomers.[22] Additionally, the assignment of all *cis*-isomers considered that
284 the spectral fine structure (%III/II) decreases and the intensity of *cis*-peak (% A_B/A_{II}) increases
285 as the *cis*-double bond is getting closer to the centre of the molecule.

286 The shell extract of *C. bracteosa* fruit presented the highest phenolic compound and
287 carotenoid contents (5540 and 328 µg/g extract, respectively) (Tables 1 and 2), followed by the
288 pulp and seed extracts. The major phenolic compound identified in the pulp and shell extracts
289 was acacetin sulphate, accounting for 62 and 48% of total sum of the identified phenolic
290 compounds, followed by the oleuropein derivative compound (28% in both cases), while
291 apigenin sulphate was only found in the seed extracts and it was the major compound (336 µg/g
292 extract), accounting for 44% of the total sum of phenolic compounds. Regarding carotenoids,
293 all-*trans*-neochrome and all-*trans*-β-carotene were the major compounds identified in the pulp
294 extracts (22 and 21 µg/g extract, respectively), while all-*trans*-lutein (146 µg/g extract) was the
295 major compound in shell and all-*trans*-α-carotene and all-*trans*-β-carotene were the major ones
296 in seed extracts (11 and 10 µg/g extract, respectively).

297

298 **3.2. Scavenging capacities of *C. bracteosa* extracts against ROS and RNS**

299 According to Table 3, all *C. bracteosa* extracts were able to scavenge the tested ROS
300 and RNS in a concentration-dependent manner (Figure 3). However, its scavenging efficiencies
301 did not seem directly related to the total yield of phenolic compounds or carotenoids found in
302 each extract (Tables 1 and 2).

303 The seed extract was the most efficient one against all tested ROS and RNS
304 notwithstanding its lower contents of phenolic compounds (763 µg/g) and carotenoids (34 µg/g)
305 compared to the amounts found in the shell and pulp extracts (Tables 1 and 2). The high
306 scavenging capacity of the seed extract of *C. bracteosa* fruits may be probably attributed to the
307 presence of apigenin sulphate, even at low concentration (Fig. 1, Table 1), since this compounds
308 was only detected in the extracts obtained from the seeds. Some studies have already reported

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309 the high antioxidant properties of apigenin.[23-25] Additionally, the other identified phenolic
310 compounds (acacetin and oleuropein) are also promising bioactive compounds and they have
311 also been studied due to its beneficial effects to human health.[26-28]

312 The seed extracts showed high scavenging capacity against $O_2^{\bullet-}$ (Fig. 3a) with an IC_{50}
313 of 11.5 μg/mL, while the pulp and shell extracts showed no activity against this ROS, at the
314 highest tested concentration (1000 μg/mL). Although $O_2^{\bullet-}$ is not considered as a potent pro-
315 oxidant species *per se*, it represents a key point in the oxidative stress as a primary generated
316 ROS. The $O_2^{\bullet-}$ production plays an important role in cellular signalling and in the development
317 of pathophysiological conditions, as hypertension, ischemia-reperfusion, inflammation, and
318 atherosclerosis.[6] In our study, the scavenging capacity of *C. bracteosa* seed extract against $O_2^{\bullet-}$
319 was higher than that found for quercetin (positive control) (IC_{50} = 14.2 μg/mL) and also higher
320 than those reported for water and ethanol/water extracts of *Caryocar vilosum* fruit pulp[3], another
321 Amazonian fruit, pulp and peel extracts of *Psidium cattleianum* fruits[4], as well as than infusion
322 and decoction extracts obtained from artichoke leaves[29].

323 Once formed, $O_2^{\bullet-}$ can be physiologically dismutated to H_2O_2 by the action of the
324 superoxide dismutase (SOD) enzyme, or spontaneously at acid conditions. H_2O_2, although not a
325 free radical, presents high reactive potential, since it has a long lifetime, it is able to cross cell
326 membranes and therefore may be potentially cytotoxic, mainly due to its participation in $^{\bullet}OH$
327 generation by the reactions catalysed by iron and/or copper ions (Fenton and Haber-Weiss
328 reactions).[30] Again, the seed extract of *C. bracteosa* was the most efficient extract against H_2O_2
329 (Fig. 3b), with an IC_{50} at high μg/mL level (426 μg/mL), followed by the shell extract (894
330 μg/mL), while the pulp extract could decrease the oxidizing effect of H_2O_2 only by 29%, at the
331 highest tested concentration (1000 μg/mL) (Table 3). The seed extract also exhibited higher
332 scavenging efficiency against H_2O_2 than quercetin (509 μg/mL) and peel extract of *P.*
333 *cattleianum* fruit[4], but lower than hydrophilic extracts of murici (228 μg/mL)[5] and *V. cauliflora*
334 plant (medicinal plant from Amazonia) (IC_{50} from 106 to 401 μg/mL)[31].

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335 Most part of H_2O_2 produced by phagocytes (neutrophils and monocytes) is used by the
336 myeloperoxidase (MPO) enzyme to catalyse the oxidation of Cl^-, yielding HOCl. HOCl has
337 been considered as a strong pro-inflammatory agent, and consequently it has been implicated in
338 several diseases associated to chronic inflammation, such as atherosclerosis, ischemia-
339 reperfusion renal injury, multiple sclerosis, disease Alzheimer's and some cancers.[32, 33] This
340 reactive specie presents a very fast reaction rate with various compounds in biological systems,
341 such as sulfhydryl, polyunsaturated fatty acids, DNA pyridine nucleotides and aminoacids, and
342 its toxicity has been referred between 100 to 1000 times higher than $O_2^{\bullet-}$ and H_2O_2.[34] Our
343 results suggest that all *C. bracteosa* extracts have high potential to scavenge HOCl (Table 3 and
344 Figure 3c) with the seed extract as the most efficient one (IC_{50} = 0.39 µg/mL), followed by the
345 shell and pulp extracts. All the extracts presented higher scavenging capacity against HOCl than
346 the freeze-dried extracts of *Cytisus scoparius* (56 to 60 µg/mL)[35] and the ethanol or ethyl
347 acetate/ethanol extracts of *C. villosum* pulp (199 and 299 µg/mL, respectively)[3], but lower
348 activity than quercetin (0.10 µg/mL).

349 Not only ROS are involved in the oxidative stress, but RNS are also known to interfere
350 with the biological activity of several molecules, which may affect the shelf-life and the quality
351 of food[36], as well as being implicated in several human diseases.[37] Therefore, research strategies
352 directed for searching isolated compounds or plant extracts that act as natural antioxidants
353 against RNS has been assumed an important role in the modern science. Regarding this issue,
354 $^{\bullet}$NO is produced by the nitric oxide synthase (NOS) enzyme, throughout by the conversion of L-
355 arginine to L-citrulline[37], and at low concentrations $^{\bullet}$NO exhibit important activity in
356 physiological conditions. However, if the production exceeds normal levels, it can cause
357 harmful effects in the tissues leading to serious inflammatory conditions, as well as being
358 involved in endotoxin shock.[37] As can be seen in Table 3, among all *C. bracteosa* extracts, the
359 seeds extract was, by far, the most active against $^{\bullet}$NO (IC_{50} = 18 µg/mL) (Fig. 3d), with higher
360 scavenging capacity than seed extracts of sesame (*Sesamun indicum*) (98-238 µg/mL), α-
361 tocopherol (57 µg/mL)[38], extracts of some fruits used in traditional Indian medicine *(Terminalia*

362 *chebula*, *Terminalia belerica* and *Emblica officinalis*) (33-41 µg/mL) and curcumin (91
363 µg/mL)[39]. However, the •NO scavenging capacity of *C. bracteosa* seed extracts was lower than
364 that found for quercetin (0.15 µg/mL), and also less efficient than *V. cauliflora* extracts (0.9 to
365 3.6 µg/mL)[31], extracts of *P. cattleianum* fruit (2-7 µg/mL)[4] and infusion, decoction and
366 hydroalcoholic extracts of artichoke leaves (5.5-11 µg/mL)[29].

367 The toxicity of •NO is related to the high concentration in the biological systems and in
368 the presence of $O_2^{\bullet-}$ a highly oxidant species is formed: $ONOO^-$. This RNS has been shown to
369 oxidize a variety of biomolecules including thiols, lipids, proteins, carbohydrates, DNA and has
370 been implicated in the development of some diseases, including arteriosclerosis, cardiovascular
371 diseases, inflammation, ischemia-reperfusion, cancer, diabetes and neurodegenerative disorders,
372 such as Alzheimer's or Parkinson's diseases.[37] The $ONOO^-$-scavenging capacity of *C. bracteosa*
373 seed extract, in absence (2.64 µg/mL) or in presence of $NaHCO_3$ (4.9 µg/mL), was superior to
374 that found for the shell and pulp extracts (Figures 3 e and f). The evaluation of the scavenging
375 capacity of $ONOO^-$ in the presence of $NaHCO_3$ is important because, under physiological
376 conditions, the reaction between $ONOO^-$ and CO_2 is predominant[40] and may lead the formation
377 of further reactive species that are also responsible for the nitration and oxidation reactions
378 observed *in vivo*. These reactive species have the ability to oxidize a variety of biomolecules
379 (thiols, lipids, proteins, carbohydrates, DNA, among others) via complex mechanisms of
380 oxidation reaction and which are strongly pH dependent.[37] An interesting effect was observed in
381 the $ONOO^-$-scavenging effect of both the shell and pulp extracts of *C. bracteosa,* where they
382 were more efficient in scavenging $ONOO^-$ in the presence of $NaHCO_3$ (20.6 and 35 µg/mL,
383 respectively) than in its absence (53 µg/mL and 167 µg/mL, respectively). The efficiency of
384 seed extracts, in absence and in presence of $NaHCO_3$, was higher than extracts of *P. cattleianum*
385 fruit[4], *V. cauliflora* fruit[31], artichoke laves[29] and a hydrophilic extract of *B. crassifolia* (Mariutti
386 et al., 2014). In contrast, quercetin (Table 3) showed higher $ONOO^-$-scavenging capacity than
387 all *C. bracteosa* extracts, in absence or in presence of $NaHCO_3$. Therefore, as the *C. bracteosa*

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388 extracts could scavenge $ONOO^-$, both in absence and in presence of $NaHCO_3$, they are also
389 supposed to exhibit scavenging capacity against other reactive species, such as $^{\bullet}NO_2$ and $CO_3^{\bullet-}$.
390

391 **4. Conclusion**

392 For the first time, the profiles of phenolic compounds and carotenoid of extracts
393 obtained from *C. bracteosa* fruits were reported, as well as its antioxidant capacities against the
394 oxidizing effect of ROS and RNS of physiological importance. The seed extract was the most
395 efficient one against all ROS and RNS probably due to the presence of apigenin sulphate, which
396 was not detected in the other extracts. Noteworthy, although all extracts have presented
397 scavenging capacity against the tested ROS and RNS, in a concentration-dependent manner,
398 they presented the highest efficiency against $^{\bullet}NO$ and $ONOO^-$ with IC_{50} values at low µg/mL
399 range. Thus, the extracts of *C. bracteosa* fruits may be considered as a promising source of
400 bioactive compounds with high antioxidant properties exhibiting great potential for the
401 application in the pharmaceutical, cosmetic and food industries.
402

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483 **Figure captions**

484 **Figure 1**. HPLC-DAD chromatogram of phenolic compounds of pulp, shell and seeds extracts
485 of *Couepia bracteosa* fruits. Peak characterization is given in **Table 1**.

486

487 **Figure 2.** HPLC-DAD chromatogram of carotenoids of pulp, shell and seed extracts of *Couepia*
488 *bracteosa* fruits. Peak characterization is given in **Table 2**.

489

490 **Figure 3.** Scavenging capacities of pulp, shell and seed extracts of *Couepia bracteosa* fruits
491 against **(a)** superoxide radical ($O_2^{\bullet -}$), **(b)** hydrogen peroxide (H_2O_2), **(c)** hypochlorous acid
492 (HOCl), **(d)** nitric oxide ($^{\bullet}NO$), and **(e)** peroxynitrite ($ONOO^-$) in the absence and **(f)** presence
493 of $NaHCO_3$. Each point shows the standard error of the mean (SEM) bars and represents the
494 values from four experiments, performed in duplicate.

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Table 1. Phenolic compounds of pulp, shell and seed extracts obtained from *Couepia bracteosa* fruits, as tentatively identified by its chromatographic, UV-Vis and mass spectroscopy characteristics (HPLC-DAD-ESI-MS/MS).

Peaks	t_R range (min)[a]	λ_{max} (nm)[b]	$[M-H]^-$ (*m/z*)	Fragments (*m/z*)[c]	Compounds	Concentration (µg/g extract)[d]		
						pulp	shell	seed
1	23.4-23.6	270, 330, 338	363.01749	MS^2 [363]: 348, 320, **283**, 268 MS^3 [363→283]: **268**, 255, 239, 165	Acacetin sulphate[e]	4461 ± 195	7958 ± 287	2289 ± 52
2	30.6-30.8	268, 320(sh), 344	349.00427	MS^2 [349]: 331, 283, **269**, 239, 211 MS^3 [349→269]: 241, **225**, 197, 149	Apigenin sulphate[e]	nd	nd	5058 ± 107
3	31.7-31.9	270, 330	377.03336	MS^2 [377]: **362**, 334, 297, 282, 252 MS^3 [377→362]: 333, 298, 281, **252**	Oleuropein aglycon[f]	2037 ± 45	4749 ± 159	1393 ± 74
4	39.7-39.9	270, 330, 342	363.01874	MS^2 [363]: **348**, 283, 268, 253, 225 MS^3 [363→348]: 330, 320, **268**, 238	Acacetin sulphate[e]	654 ± 16	3,914 ± 37	2702 ± 81
					Sum of phenolic compounds	**7152 ± 1385**	**16621 ± 1612**	**11443 ±1099**

[a]Retention time on the C_{18} Synergi Hydro (4µm) column. [b]Solvent: gradient of 0.5% formic acid in water and acetonitrile with 0.5% formic acid. [c]In the MS² and MS^3, the most abundant ions are shown in boldface. [d]Mean ± standard deviation (n = 3, dry basis). The peaks were quantified as equivalent of acacetin[e] and apigenin[f]. nd = not detected.

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Table 2. Chromatographic, UV-Vis, mass spectroscopy characteristics (HPLC-DAD-MS^n) and contents of carotenoids of pulp, peel and seed extracts obtained from *Couepia bracteosa* fruit.

Peak	Carotenoid	HPLC-DAD-APCI-MS^n						Concentration (µg/g extract)^c		
		t_R (min)^a	λ_{max} (nm)^b	%III/II	%A_B/A_{II}	$[M+H]^+$ (*m/z*)	MS^2 (*m/z*)	pulp	shell	seed
1	*cis*-Neochrome[1]	5.6-5.9	300, 390, 417, 441	50	39	nd	nd	8.3 ± 0.7	nd	nd
2	*cis*-Neochrome [1]	6.2-6.5	300, 390, 417, 442	75	23	601	583, 565, 547, 491, 221	7 ± 1	nd	nd
3	Not identified[1]	6.4-6.6	420, 448	nc	0	nd	nd	nd	30 ± 1	nd
4	all-*trans*-Neochrome[1]	6.6-6.9	399, 421, 448	94	0	601	583, 565, 547, 491, 221	22 ± 3	nd	nd
5	9-*cis*-Neochrome[1]	7.1-7.3	304, 398, 421, 448	89	7	601	583, 565, 547, 491, 221	14 ± 2	20 ± 1	nd
6	all-*trans*-Luteoxanthin[1]	10.1-10.3	399, 421, 447	100	0	601	583, 565, 491, 221	9.5 ± 0.6	24 ± 2	1.7 ± 0.5
7	Not identified[1]	11.6-11.8	400, 427, 451	70	0	585	567, 549, 493, 221	7.4 ± 0.6	nd	nd
8	Not identified[1]	12.1-12.3	313, 400, 427, 452	60	7	585	567, 549, 493, 221	18 ± 1	nd	nd
9	all-*trans*-Lutein[2]	12.0-12.3	420, 444, 472	50	0	569	551, 533, 477	nd	146 ± 11	2.5 ± 0.6
10	all-*trans*-Zeaxanthin[3]	14.2-14.4	420, 450, 476	12	10	569	551, 533, 477	3.3 ± 0.2	20 ± 2	nd
11	Not identified[1]	14.9-15.1	324, 410, 440, 468	54	11	585	567, 549, 493, 475, 221	6.2 ± 0.2	nd	nd
12	Not identified[1]	18.5-18.7	321, 420, 445, 472	50	23	553	535, 517, 497, 461	1.34 ± 0.03	nd	1.60 ± 0.08
13	all-*trans*-β-Cryptoxanthin[4]	21.8-22.0	420, 450, 475	0	0	553	535, 473, 461	3.62 ± 0.03	7.4 ± 0.4	2.3 ± 0.3
14	Not identified[1]	22.6-23.0	400, 425, 450	nc	0	553	535, 473, 461	nd	nd	2.3 ± 0.3
15	*cis*-α-Carotene[1]	23.7-24.0	330, 418, 438, 468	54	16	537	481, 444, 413	nd	nd	2.4 ± 0.6
16	all-*trans*-α-Carotene[1]	27.4-27.7	420, 445, 473	50	0	537	481, 444, 413	5.7 ± 0.2	23 ± 3	11 ± 2
17	all-*trans*-β-Carotene[1]	31.4-31.9	420, 450, 477	28	0	537	457, 444, 413	20.7 ± 0.6	52 ± 4	10 ± 3
18	9-*cis*-β-Carotene[1]	33.0-33.7	328, 420, 446, 470	nc	11	537	457, 444, 413	2.64 ± 0.07	6 ± 1	1.1 ± 0.2
							Total carotenoids (µg/g)	**130 ± 7**	**328 ± 41**	**34 ± 4**

^{a}Retention time on the C_{30} column. ^{b}Linear gradient of methanol/MTBE. ^{c}Mean ± standard deviation (n = 3, dry basis). nc = not calculated. nd = not detected. The peaks were quantified as equivalent to all-*trans*-β-carotene[1], all-*trans*-lutein[2], all-*trans*-zeaxanthin[3] and all-*trans*-β-cryptoxanthin[4]. RAE = retinol activity equivalent

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Table 3. Scavenging capacities of pulp, shell and seed extracts obtained from *Couepia bracteosa* fruits against superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$).

Reactive species	IC_{50} (µg/mL) (n = 4)			
	Couepia bracteosa extracts			Positive control
	pulp	shell	seed	quercetin
ROS				
$O_2^{\bullet -}$	NA	NA	11.5 ± 0.6	14.2 ± 0.4
H_2O_2	29.4 ± 0.2 %*	894 ± 3	426 ± 7	509 ± 6
HOCl	47.1 ± 0.6	25.3 ± 0.4	0.39 ± 0.01	0.10 ± 0.01
RNS				
$^{\bullet}NO$	36.1 ± 0.3%*	485 ± 2	18 ± 1	0.15 ± 0.01
$ONOO^-$	167 ± 5	53 ± 1	2.64 ± 0.06	0.122 ± 0.004
$ONOO^-$ **	35 ± 1	20.6 ± 0.3	4.9 ± 0.1	0.121 ± 0.005

IC_{50} = inhibitory concentration, *in vitro*, to decrease by 50% the oxidizing effect of each reactive species (mean ± standard error of the mean, SEM). NA = IC_{50} no activity was found up to the highest tested concentration (1000 µg/mL).

*Scavenging effect (%) (mean ± standard error of the mean, SEM) at 1000 µg/mL.

**Assay carried out in the presence of $NaHCO_3$ (25 mM) to simulate physiological concentration of CO_2.

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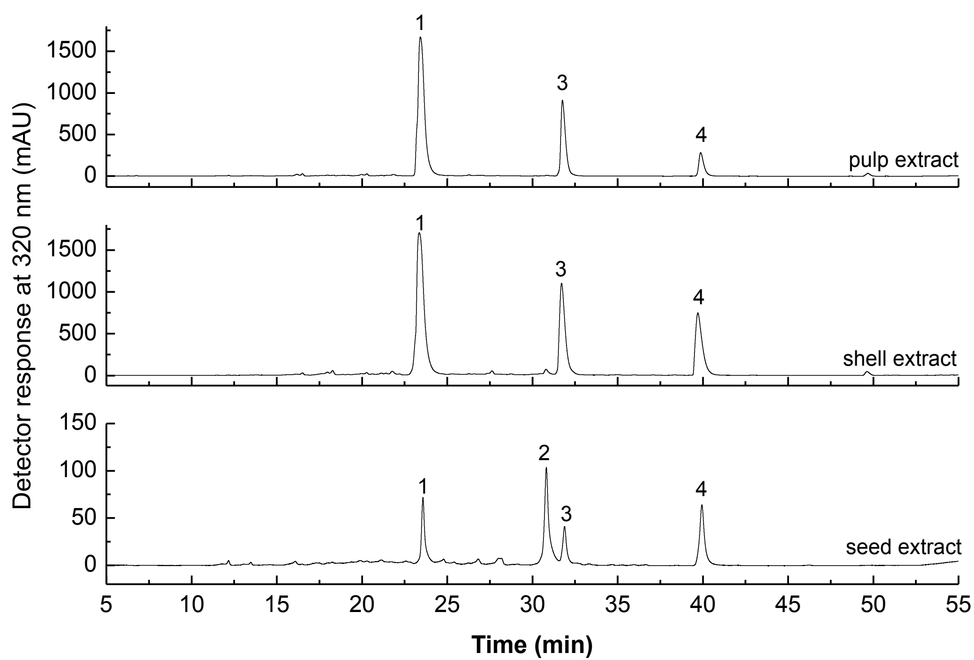

Figure 1

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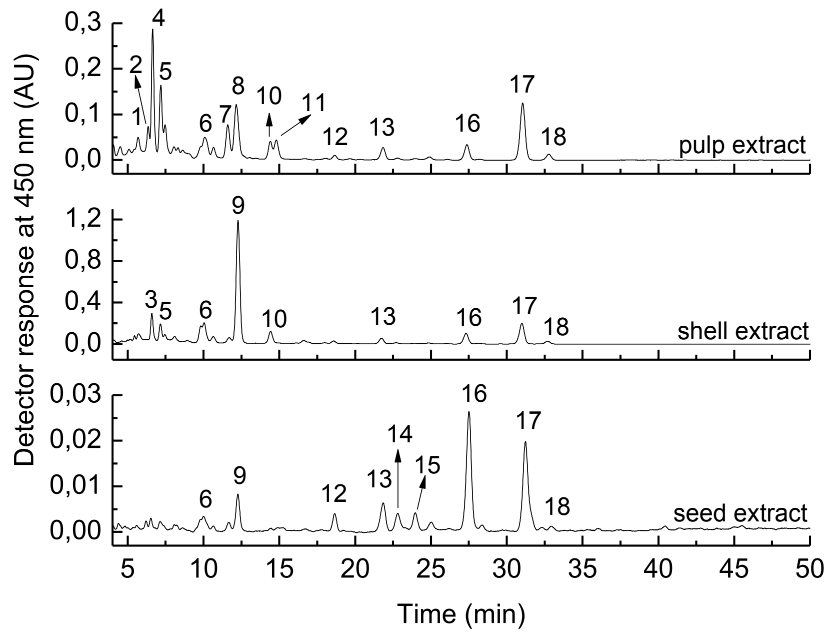

Figure 2

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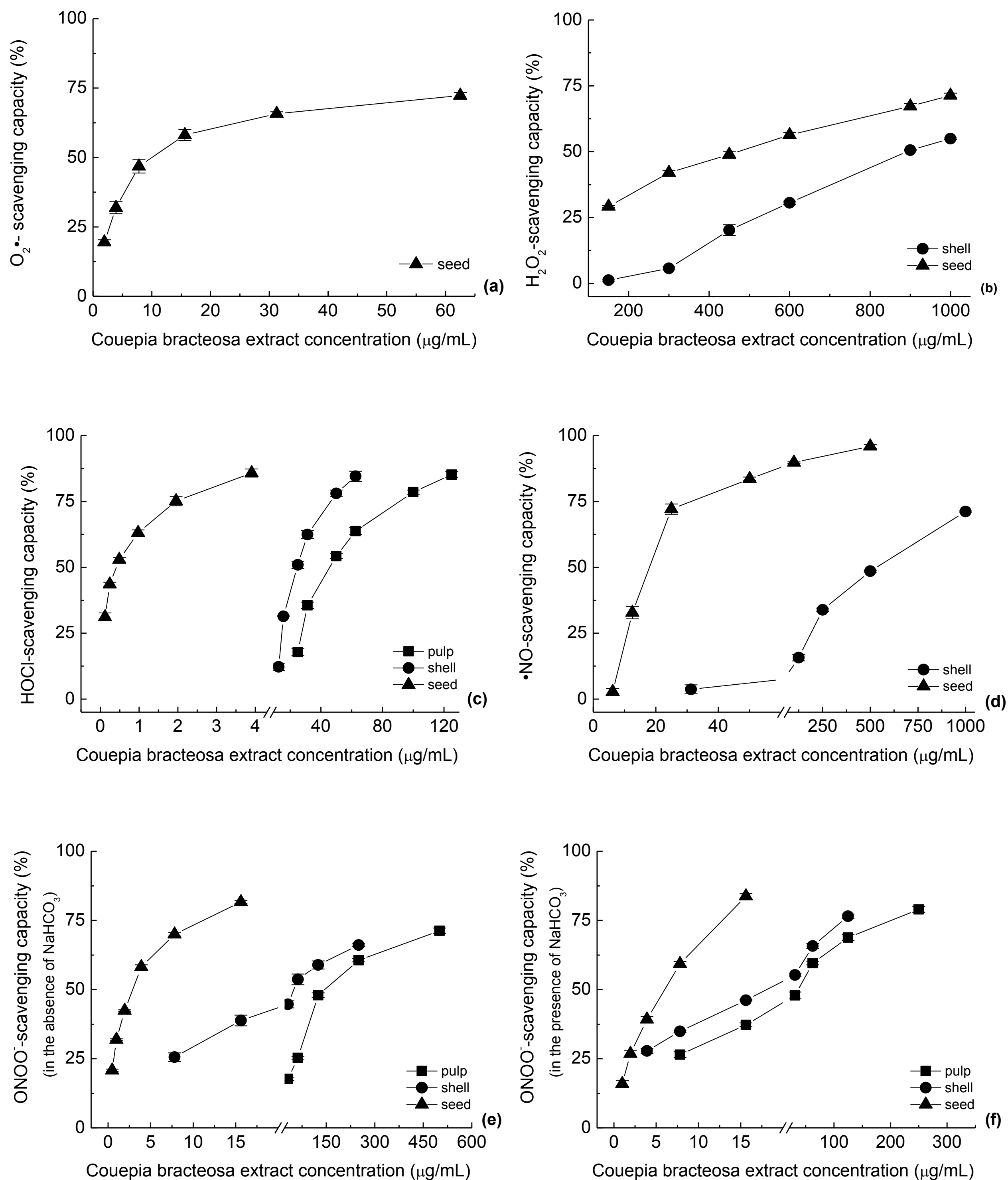

Figure 3

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