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

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

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Abstract

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

**Keywords:** Phenolic compounds; carotenoids; antioxidant capacity; reactive oxygen species; reactive nitrogen species.
1. Introduction

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

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

The *Couepia bracteosa* Benth species (Brazilian name: “pajurá”) belongs to the Chrysobalanaceae R. Br. family. It is native to the Tropical Amazon and naturally found in the following Brazilian States: Amazonas, Amapá, Pará and Rondônia. The *C. bracteosa* tree has medium size (up to 25 m high); the fruits are globose drupes with 8 to 12 cm long by 8 to 15 cm in diameter and 80-200 g of weight. The peel (exocarp) is dark-brown with rough surface, covered with numerous white dots (lenticels). The pulp (mesocarp) is thick, fleshy and oily, yellow-brown colour, with grainy consistency, sweetness flavour vaguely reminiscent of nuts and a thick endocarp with rough surface, dark brown colour, abundant endosperm and just one large seed. The pulp is traditionally consumed *in natura*, as well as used to prepare different kind of sweets, such as jams.⁷
To the best of our knowledge, no data related to the bioactive compounds or biological potential of *C. bracteosa* fruits have been published in the literature. However, tocopherols, flavonoids and derivatives and also triterpenes were reported for other species from *Couepia* genus, such as *C. edulis* and *C. paraensis*. Furthermore, there are other reports in the literature that support the presence of interesting bioactive compounds in *Couepia* genus. For example, the chemopreventive activity (induction of quinone reductase activity) of *C. ulei* compounds, the antibacterial, antioxidant and cytotoxicity activity against *Artemia salina* of *C. grandiflora* extracts, and the anticancer activity (lyase inhibitors of DNA β-polymerase activity) of *C. polyandra*.

In this paper, we are reporting, for the first time, the tentative identification and quantification (HPLC-DAD-MS) of bioactive compounds (phenolic compounds and carotenoids) of seed, shell and pulp extracts obtained from *C. bracteosa* fruits, and also the antioxidant potential of each extract against some ROS and RNS with high relevance in biological systems: superoxide radical (O$_2^\cdot$), hydrogen peroxide (H$_2$O$_2$), hypochlorous acid (HOCl), nitric oxide (\textsuperscript{\textbullet}NO) and peroxynitrite (ONOO\textsuperscript{-}). These results may help to stimulate the rational exploitation of natural resources from the Amazonian biome due to the little number of scientific studies about native fruits from that region, including the potential benefits not only for the local people, but also for the food, pharmaceutical and cosmetic industries.

2. Experimental

2.1. Chemicals

Nitroblue tetrazolium chloride (NBT), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), lucigenin, 30% hydrogen peroxide, sodium hypochlorite solution (4% available chlorine), dihydrorhodamine 123 (DHR), 4,5-diaminofluorescein (DAF-2), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), quercetin, acacetin, apigenin, all-trans-lutein, all-trans-zeaxanthin, all-trans-β-cryptoxanthin, all-trans-β-carotene, dimethyl sulfoxide (DMSO), ethanol, methanol, methyl tert-butyl ether (MTBE), acetonitrile
and all other chemical salts and solvents of analytical grade were obtained from Sigma-Aldrich (St. Louis, USA). Ultrapure water was obtained from the arium® pro system (Sartorius, Germany). All phenolic compounds and carotenoids standards showed at least 95% of purity, as determined by HPLC-DAD.

2.2. *C. bracteosa* samples and extract preparation

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

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

2.3.1. Equipments

The identification and quantification of phenolic compounds in all extracts was performed in an Accela HPLC system (Thermo Fisher Scientific, San Jose, CA) equipped with a quaternary pump (Accela 600), a DAD detector and an auto-sampler cooled to 5 °C. The equipment was also connected in series to a LTQ Orbitrap™ XL mass spectrometer (MS) (Thermo Fisher Scientific, San Jose, CA) with electrospray ionization source (ESI), and a hybrid system combining a linear ion-trap and the Orbitrap as the m/z analyzer. The identification of carotenoids was performed in a Shimadzu HPLC (Kyoto, Japan) equipped with a quaternary pump (LC-20AD), a degasser unit (DGU-20A5), a Rheodyne injection valve with
a 20 μL loop, a DAD detector (SPD-M20A), and connected in series to a MS from Bruker Daltonics (AmaZon speed ETD, Bremen, Germany) with atmospheric pressure chemical ionization (APCI) and an ion-trap as the m/z analyzer. The quantification of carotenoids was carried out in a LaChrom HPLC system (D-700, Merck Hitachi Ltd., Tokyo, Japan) equipped with a quaternary pump (L-7100) and DAD detector (L-7455). For all the chromatographic analysis, samples and solvents were filtered using, respectively, membranes of 0.22 and 0.45 μm, both from Millipore (Billerica, MA, USA).

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

The phenolic compounds were analysed after solubilising 50 mg of the freeze-dried extract from each fruit part in methanol/water (80:20, v/v) and the compounds were separated on a C18 Synergi Hydro column (4 μm, 250 x 4.6 mm, Phenomenex), at 0.9 mL/min, column temperature at 29 °C and mobile phase consisting of water/formic acid (99.5:0.5, v/v) acetonitrile/formic acid (99.5:0.5, v/v) in a linear gradient. The column eluate was split to allow only 0.3 mL/min to enter the ESI interface. The UV-Vis spectra were obtained between 200 and 600 nm, and the chromatograms were processed at 280, 320 and 360 nm. Mass spectra were obtained after ionization in an ESI source in the negative ion mode, with a scan range from m/z 100 to 1000, and the MS parameters were set at the same conditions as described in our previous work. Phenolic compounds were tentatively identified based on the following data: elution order, retention time of peaks and characteristics of the UV-visible and mass spectra in comparison with authentic standards (data not shown) analysed under the same conditions and data available in the literature. The quantification was carried out by comparison to external analytical curves (1 to 100 μg/mL, in duplicate) using five-point for the standards apigenin (at 339 nm, r² ≥ 0.99) and acacetin (at 327 nm, r² ≥ 0.99).

For carotenoid analysis, 50 mg of each freeze-dried extract of C. bracteosa were solubilised in acetone and directed to the steps of exhaustive extraction, liquid-liquid partition, saponification and drying under N₂ flow, following the same procedures described in details by
Ribeiro et al. The dried saponified carotenoid extracts were re-suspended in methanol/MTBE (70:30, v/v) and injected into the chromatographic systems. The carotenoids were separated on a C30 YMC column (5 µm, 250 mm x 4.6 mm) with a linear gradient of methanol and MTBE at 0.9 mL/min and temperature column set at 29 °C. The UV-Vis spectra were recorded between 200 and 600 nm and the chromatograms were processed at 450 nm. The column eluate was directed to the APCI interface and the mass spectra were obtained after ionization in the positive ion mode, with a scan range from m/z 100 to 800 and MS parameters set as described by Chisté and Mercadante. The carotenoids were tentatively identified according to the following combined information: elution order, retention time, co-chromatography with authentic standards, UV-visible spectrum (λmax, spectral fine structure (%III/II), peak cis intensity (%AII/AII)) compared with data available in the literature. The characterization of each cis-isomers of carotenoids was based on the observed decrease in the %III/II values and increase %AII/AII values (≈ 7-11% = 9-cis; ≈ 45% = 13-cis e ≈ 56% = 15-cis carotenoid) as the cis double bond moves from the end to the centre of the molecule. The carotenoids were quantified by HPLC-DAD by comparison to standards using five-point external analytical curves (0.5-30 µg/mL, in duplicate) for all-trans-lutein, all-trans-zeaxanthin, all-trans-β-cryptoxanthin and all-trans-β-carotene. All other carotenoids (including epoxy and cis isomers) were estimated using the curve of the corresponding all-trans-carotenoid.

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

2.4. ROS- and RNS-scavenging assays

The scavenging assays against all ROS and RNS were carried out in a microplate reader (Synergy HT, Biotek, Vermont, USA) equipped with thermostat and detection systems for the measurement of fluorescence, UV-Vis and chemiluminescence. Quercetin was used as positive control in all assays and its IC50 values were similar to those already reported by our research.
DMSO was used to solubilise all extracts of *C. bracteosa* fruit in all assays, except for the HOCl-scavenging assay (ethanol). Additional experiments were performed with all extracts to ensure the results are not flawed by any interference of solvents or fluorescence/chemiluminescence/absorbance response of extracts (data not shown). The IC<sub>50</sub> values (*in vitro* inhibitory concentration of the extract which is able to reduce, by 50%, the effect of ROS or RNS) were calculated from the curves of percentage of inhibition versus antioxidant concentration using GraphPad Prism 6 software. For each assay, four independent experiments were performed, in duplicate, using six different concentrations.

2.4.1. O<sub>2</sub><sup>•</sup>-scavenging assay

The non-enzymatic system NADH/PMS/O<sub>2</sub> was used to generate O<sub>2</sub><sup>•</sup>−, which promotes the reduction of NBT into a purple coloured diformazan compound. This reaction was followed by spectrophotometry, at 560 nm, for 2 minutes, by monitoring the effect of each *C. bracteosa* extract and the positive control against the O<sub>2</sub><sup>•</sup>−-induced reduction of NBT. The scavenging capacities were expressed as the percentage of inhibition of the NBT reduction to diformazan.

2.4.2. H<sub>2</sub>O<sub>2</sub>-scavenging assay

The effect of each *C. bracteosa* extract and the positive control against the H<sub>2</sub>O<sub>2</sub>-induced oxidation of lucigenin was monitored by chemiluminescence, at 37 °C and the signal was detected immediately after the introduction of the plate in the reader. The scavenging capacities were expressed as percentage of inhibition of H<sub>2</sub>O<sub>2</sub>-induced oxidation of lucigenin.

2.4.3. HOCl-scavenging assay

HOCl was immediately prepared before the assay using a NaOCl solution 1% (w/v) and adjusting to pH 6.2 with a diluted solution of H<sub>2</sub>SO<sub>4</sub>. The concentration of HOCl obtained after
the reaction was determined by spectrophotometry at 235 nm using a molar absorption coefficient of $100 \text{ M}^{-1}\text{cm}^{-1}$. The capacity of each extract and the positive control to scavenge HOCl was determined by monitoring the HOCl-induced oxidation of DHR (non-fluorescent) to rhodamine 123 (fluorescent). The scavenging capacities were expressed as percentage of inhibition of HOCl-induced oxidation of DHR.

2.4.4. *NO-scavenging assay

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

2.4.5. ONOO−-scavenging assay

The ONOO− was synthesized as previously described by Fernandes, Gomes, Costa & Lima. The capacity of each extract and the positive control in scavenging ONOO− was determined by monitoring the ONOO−-induced oxidation of non-fluorescent DHR to the fluorescent rhodamine 123. Parallel experiments simulating physiological concentrations of CO$_2$ were performed using 25 mM NaHCO$_3$. The scavenging capacities were expressed as percentage of inhibition of ONOO−-induced oxidation of DHR.

3. Results and Discussion

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

The phenolic compounds (Fig. 1) and carotenoids (Fig. 2) of all *C. bracteosa* extracts in this study were separated, identified and quantified by HPLC-DAD-MS. In relation to the
phenolic compounds identification, according to Table 1, peak 1 and 4 were assigned as acacetin sulphate, probably positional isomers, since both peaks showed [M-H]⁻ at m/z 363 and exhibited neutral loss of 80 u (m/z 283) in the MS² spectra, which indicated loss of a sulphate moiety esterified to acacetin [M-H-SO₃]⁻, and fragments at m/z 268 [M-H-SO₃-CH₃]⁻, indicating loss of a methyl group after losing the sulphate moiety. The identity was confirmed by the same MS characteristics observed after analyzing acacetin standard (data not shown). Peak 2 showed [M-H]⁻ at m/z 349 with a high intense loss of a sulphate moiety [M-H-SO₃]⁻ (m/z 269) and was tentatively identified as apigenin sulphate, since the fragmentation of m/z 269 (MS³), presented the same fragmentation pattern of the authentic standard of apigenin [neutral losses of 28u (CO) and 44u (CO₂)] (data not shown). Peak 3 presented [M-H]⁻ at m/z 377 and was tentatively identified as oleuropein derivative after comparing the MS² and MS³ features with those data already well described in the literature.¹⁶,¹⁹

Sulphate esters of flavonoids are relatively rare compounds and their functional significance in plant tissues is not clear. They are found mainly in species occurring in coastal and swampy areas rich in mineral salts, as well as in plants occurring in arid habitats.²⁰,²¹ In such plants, bind reaction of inorganic sulphate to flavonoids is probably one of the mechanisms connected with biochemical adaptation of species to environment.²¹ Although the phenolic profile of C. bracteosa fruits was reported for the first time in this study, there is another report available, in which two active compounds were identified in the ethyl acetate extracts of C. ulei stems: erythro-2,3-bis(4-hydroxy-3-methoxyphenyl)-3-ethoxypropan-1-ol and a known compound, evofolin-B, along with five inactive compounds (betulinic acid, oleanolic acid, pomolic acid, (+)-syringaresinol and ursolic acid).¹²

Regarding the carotenoid profile, 18 compounds were separated and tentatively identified and quantified (Table 2). The MS² experiments confirmed the assignment of the protonated molecule ([M+H]⁺) of all identified peaks through the fragments expected for the carotenoid polyene chain and functional groups, along with the UV-Vis spectra features.¹⁵,¹⁷ The carotenoid composition was slightly different for each extract, with predominance of xanthophylls with one to three hydroxyl groups (OH), mostly with one or two epoxide groups.
The identification of all-trans-lutein (peak 9), all-trans-zeaxanthin (peak 10), all-trans-β-cryptoxanthin (peak 13) and all-trans-β-carotene (peak 17) was positively confirmed through co-elution with authentic standards, as well as by comparison of their UV-vis and MS spectra features with standards. Peaks 2, 4 and 5 (Table 2) presented the same MS and MS² spectra characteristics: [M+H]+ at m/z 601 and three consecutive neutral losses of water from the protonated molecule were observed at m/z 583 [M+H-18]+, m/z 565 [M+H-18-18]+ and m/z 547 [M+H-18-18-18]+, as well as a fragment at m/z 491 [M+H-18-92]+ resulting from an additional loss of toluene moiety (92 u) from the polyene chain. Peak 6 also presented [M+H]+ at m/z 601, but only two consecutive losses of water were observed in the MS² spectrum (m/z 583 and m/z 565). Peaks 7, 8, and 11 showed [M+H]+ at m/z 585 and the indication of two OH attached to the carotenoid molecules was demonstrated by the consecutive losses of two water moieties in its MS² spectra (m/z 567 and m/z 549). In addition, the fragment at m/z 221 was observed in all these peaks that correspond to an epoxy substituent in a β-ring with a OH group. Moreover, all these previous peaks showed a hypsochromic shift of 10 nm (peak 11) and 25-30 nm (peaks 7 and 8) in relation to β-carotene (450 nm, peak 17), which indicates the presence of a 5,6-epoxy or 5,8-furanoid groups in the carotenoid structures. Although 5,8-epoxides were already found in other Amazonian fruits, such as buriti, marimari, palm oil, peach palm, physalis and tucuma, it is not possible to assure that 5,6-epoxy to 5,8-furanoid rearrangement did not occur during preparation and storage of the extracts obtained from C. bracteosa. However, peaks 7, 8 and 11 were assigned as “not identified” due to the lack of visible fragments at m/z 205 (β-ring with an epoxy group) in their MS² spectra. In the same sense, other minor peaks (peaks 3, 12 and 14) were also assigned as “not identified” due to the lack of consistent data between its UV-visible and MS spectra features as compared with data available in the literature to ensure its tentative identification.

Peaks 15 to 18 belong to the carotene group since all presented [M+H]+ at m/z 537 with a characteristic neutral loss of toluene at m/z 444 [M+H-92]+. The presence of fragments at m/z 481 and m/z 444 (peaks 15 and 16) corresponds to the respective losses of ε-ring and toluene as
in α-carotene and its isomers. Additionally, the assignment of all cis-isomers considered that
the spectral fine structure (%III/II) decreases and the intensity of cis-peak (%AII/AII) increases
as the cis-double bond is getting closer to the centre of the molecule.

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

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

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

The seed extract was the most efficient one against all tested ROS and RNS
notwithstanding its lower contents of phenolic compounds (763 µg/g) and carotenoids (34 µg/g)
compared to the amounts found in the shell and pulp extracts (Tables 1 and 2). The high
scavenging capacity of the seed extract of *C. bracteosa* fruits may be probably attributed to the
presence of apigenin sulphate, even at low concentration (Fig. 1, Table 1), since this compounds
was only detected in the extracts obtained from the seeds. Some studies have already reported
the high antioxidant properties of apigenin.\textsuperscript{23-25} Additionally, the other identified phenolic compounds (acacetin and oleuropein) are also promising bioactive compounds and they have also been studied due to its beneficial effects to human health.\textsuperscript{26-28}

The seed extracts showed high scavenging capacity against $\text{O}_2^{\bullet-}$ (Fig. 3a) with an IC$_{50}$ of 11.5 µg/mL, while the pulp and shell extracts showed no activity against this ROS, at the highest tested concentration (1000 µg/mL). Although $\text{O}_2^{\bullet-}$ is not considered as a potent pro-oxidant species \textit{per se}, it represents a key point in the oxidative stress as a primary generated ROS. The $\text{O}_2^{\bullet-}$ production plays an important role in cellular signalling and in the development of pathophysiological conditions, as hypertension, ischemia-reperfusion, inflammation, and atherosclerosis.\textsuperscript{6} In our study, the scavenging capacity of \textit{C. bracteosa} seed extract against $\text{O}_2^{\bullet-}$ was higher than that found for quercetin (positive control) (IC$_{50}$ = 14.2 µg/mL) and also higher than those reported for water and ethanol/water extracts of \textit{Caryocar vilosum} fruit pulp\textsuperscript{3}, another Amazonian fruit, pulp and peel extracts of \textit{Psidium cattleianum} fruits\textsuperscript{4}, as well as than infusion and decoction extracts obtained from artichoke leaves\textsuperscript{29}.

Once formed, $\text{O}_2^{\bullet-}$ can be physiologically dismutated to H$_2$O$_2$ by the action of the superoxide dismutase (SOD) enzyme, or spontaneously at acid conditions. H$_2$O$_2$, although not a free radical, presents high reactive potential, since it has a long lifetime, it is able to cross cell membranes and therefore may be potentially cytotoxic, mainly due to its participation in \textasteriskcentered{OH} generation by the reactions catalysed by iron and/or copper ions (Fenton and Haber-Weiss reactions).\textsuperscript{30} Again, the seed extract of \textit{C. bracteosa} was the most efficient extract against H$_2$O$_2$ (Fig. 3b), with an IC$_{50}$ at high µg/mL level (426 µg/mL), followed by the shell extract (894 µg/mL), while the pulp extract could decrease the oxidizing effect of H$_2$O$_2$ only by 29%, at the highest tested concentration (1000 µg/mL) (Table 3). The seed extract also exhibited higher scavenging efficiency against H$_2$O$_2$ than quercetin (509 µg/mL) and peel extract of \textit{P. cattleianum} fruit\textsuperscript{4}, but lower than hydrophilic extracts of murici (228 µg/mL)\textsuperscript{5} and \textit{V. cauliflora} plant (medicinal plant from Amazonia) (IC$_{50}$ from 106 to 401 µg/mL)\textsuperscript{31}. 
Most part of $\text{H}_2\text{O}_2$ produced by phagocytes (neutrophils and monocytes) is used by the myeloperoxidase (MPO) enzyme to catalyse the oxidation of $\text{Cl}^-$, yielding HOCl. HOCl has been considered as a strong pro-inflammatory agent, and consequently it has been implicated in several diseases associated to chronic inflammation, such as atherosclerosis, ischemia-reperfusion renal injury, multiple sclerosis, disease Alzheimer's and some cancers.\textsuperscript{32, 33} This reactive specie presents a very fast reaction rate with various compounds in biological systems, such as sulfhydryl, polyunsaturated fatty acids, DNA pyridine nucleotides and aminoacids, and its toxicity has been referred between 100 to 1000 times higher than $\text{O}_2^-$ and $\text{H}_2\text{O}_2$.\textsuperscript{34} Our results suggest that all $C. \text{bracteosa}$ extracts have high potential to scavenge HOCl (Table 3 and Figure 3c) with the seed extract as the most efficient one ($\text{IC}_{50} = 0.39 \mu\text{g/mL}$), followed by the shell and pulp extracts. All the extracts presented higher scavenging capacity against HOCl than the freeze-dried extracts of $Cytisus \text{scoparius}$ (56 to 60 $\mu\text{g/mL}$)\textsuperscript{35} and the ethanol or ethyl acetate/ethanol extracts of $C. \text{villosum}$ pulp (199 and 299 $\mu\text{g/mL}$, respectively)\textsuperscript{3}, but lower activity than quercetin (0.10 $\mu\text{g/mL}$).

Not only ROS are involved in the oxidative stress, but RNS are also known to interfere with the biological activity of several molecules, which may affect the shelf-life and the quality of food\textsuperscript{36}, as well as being implicated in several human diseases.\textsuperscript{37} Therefore, research strategies directed for searching isolated compounds or plant extracts that act as natural antioxidants against RNS has been assumed an important role in the modern science. Regarding this issue, $\text{NO}$ is produced by the nitric oxide synthase (NOS) enzyme, throughout by the conversion of $\text{L}$-$\text{arginine}$ to $\text{L}$-$\text{citrulline}$\textsuperscript{37}, and at low concentrations $\text{NO}$ exhibit important activity in physiological conditions. However, if the production exceeds normal levels, it can cause harmful effects in the tissues leading to serious inflammatory conditions, as well as being involved in endotoxin shock.\textsuperscript{37} As can be seen in Table 3, among all $C. \text{bracteosa}$ extracts, the seeds extract was, by far, the most active against $\text{NO}$ ($\text{IC}_{50} = 18 \mu\text{g/mL}$) (Fig. 3d), with higher scavenging capacity than seed extracts of sesame ($Sesamum \text{indicum}$) (98-238 $\mu\text{g/mL}$), $\alpha$-tocopherol (57 $\mu\text{g/mL}$)\textsuperscript{38}, extracts of some fruits used in traditional Indian medicine ($Terminalia$
Chebula, Terminalia belerica and Emblica officinalis) (33-41 µg/mL) and curcumin (91 µg/mL)\(^{39}\). However, the \(^*\)NO scavenging capacity of C. bracteosa seed extracts was lower than that found for quercetin (0.15 µg/mL), and also less efficient than V. cauliflora extracts (0.9 to 3.6 µg/mL)\(^{31}\), extracts of P. cattleianum fruit (2.7 µg/mL)\(^4\) and infusion, decoction and hydroalcoholic extracts of artichoke leaves (5.5-11 µg/mL)\(^{29}\).

The toxicity of \(^*\)NO is related to the high concentration in the biological systems and in the presence of O\(_2^\cdot\) a highly oxidant species is formed: ONOO\(^-\). This RNS has been shown to oxidize a variety of biomolecules including thiols, lipids, proteins, carbohydrates, DNA and has been implicated in the development of some diseases, including arteriosclerosis, cardiovascular diseases, inflammation, ischemia-reperfusion, cancer, diabetes and neurodegenerative disorders, such as Alzheimer's or Parkinson's diseases.\(^{37}\) The ONOO\(^-\)-scavenging capacity of C. bracteosa seed extract, in absence (2.64 µg/mL) or in presence of NaHCO\(_3\) (4.9 µg/mL), was superior to that found for the shell and pulp extracts (Figures 3 e and f). The evaluation of the scavenging capacity of ONOO\(^-\) in the presence of NaHCO\(_3\) is important because, under physiological conditions, the reaction between ONOO\(^-\) and CO\(_2\) is predominant\(^{40}\) and may lead the formation of further reactive species that are also responsible for the nitration and oxidation reactions observed in vivo. These reactive species have the ability to oxidize a variety of biomolecules (thiols, lipids, proteins, carbohydrates, DNA, among others) via complex mechanisms of oxidation reaction and which are strongly pH dependent.\(^{37}\) An interesting effect was observed in the ONOO\(^-\)-scavenging effect of both the shell and pulp extracts of C. bracteosa, where they were more efficient in scavenging ONOO\(^-\) in the presence of NaHCO\(_3\) (20.6 and 35 µg/mL, respectively) than in its absence (53 µg/mL and 167 µg/mL, respectively). The efficiency of seed extracts, in absence and in presence of NaHCO\(_3\), was higher than extracts of P. cattleianum fruit\(^4\), V. cauliflora fruit\(^{11}\), artichoke laves\(^{29}\) and a hydrophilic extract of B. crassifolia (Mariutti et al., 2014). In contrast, quercetin (Table 3) showed higher ONOO\(^-\)-scavenging capacity than all C. bracteosa extracts, in absence or in presence of NaHCO\(_3\). Therefore, as the C. bracteosa
extracts could scavenge ONOO$^-$, both in absence and in presence of NaHCO$_3$, they are also supposed to exhibit scavenging capacity against other reactive species, such as *NO$_2$* and CO$_3$.

4. Conclusion

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

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The authors have declared no conflicts of interest.


Figure captions

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

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

Figure 3. Scavenging capacities of pulp, shell and seed extracts of *Couepia bracteosa* fruits against (a) superoxide radical (O$_2^-$), (b) hydrogen peroxide (H$_2$O$_2$), (c) hypochlorous acid (HOCl), (d) nitric oxide (**NO**), and (e) peroxynitrite (ONOO$^-$) in the absence and (f) presence of NaHCO$_3$. Each point shows the standard error of the mean (SEM) bars and represents the values from four experiments, performed in duplicate.
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).

<table>
<thead>
<tr>
<th>Peaks</th>
<th>t&lt;sub&gt;R&lt;/sub&gt; range (min)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>[M-H]&lt;sup&gt;c&lt;/sup&gt; (m/z)</th>
<th>Fragments (m/z)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Compounds</th>
<th>Concentration (µg/g extract)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pulp</td>
</tr>
<tr>
<td>1</td>
<td>23.4-23.6</td>
<td>270, 330, 338</td>
<td>363.01749</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt;[363]: 348, 320, <strong>283</strong>, 268</td>
<td>Acacetin sulphate&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4461 ± 195</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MS&lt;sup&gt;3&lt;/sup&gt;[363→283]: <strong>268</strong>, 255, 239, 165</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MS&lt;sup&gt;2&lt;/sup&gt;[349]: 331, 283, <strong>269</strong>, 239, 211</td>
<td>Apigenin sulphate&lt;sup&gt;e&lt;/sup&gt;</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MS&lt;sup&gt;3&lt;/sup&gt;[349→269]: 241, <strong>225</strong>, 197, 149</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>30.6-30.8</td>
<td>268, 320(sh), 344</td>
<td>349.00427</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt;[377]: <strong>362</strong>, 334, 297, 282, 252</td>
<td>Oleuropein aglycon&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2037 ± 45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MS&lt;sup&gt;3&lt;/sup&gt;[377→362]: 333, 298, 281, <strong>252</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>31.7-31.9</td>
<td>270, 330</td>
<td>377.03336</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt;[363]: <strong>348</strong>, 283, 268, 253, 225</td>
<td>Acacetin sulphate&lt;sup&gt;e&lt;/sup&gt;</td>
<td>654 ± 16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MS&lt;sup&gt;3&lt;/sup&gt;[363→348]: 330, 320, <strong>268</strong>, 238</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>39.7-39.9</td>
<td>270, 330, 342</td>
<td>363.01874</td>
<td>MS&lt;sup&gt;2&lt;/sup&gt;[363]: <strong>348</strong>, 283, 268, 253, 225</td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td>MS&lt;sup&gt;3&lt;/sup&gt;[363→348]: 330, 320, <strong>268</strong>, 238</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sum of phenolic compounds 7152 ± 1385</td>
<td></td>
<td>16621 ± 1612</td>
</tr>
</tbody>
</table>

<sup>a</sup>Retention time on the C<sub>18</sub> Synergi Hydro (4µm) column. <sup>b</sup>Solvent: gradient of 0.5% formic acid in water and acetonitrile with 0.5% formic acid. <sup>c</sup>In the MS<sup>2</sup> and MS<sup>3</sup>, the most abundant ions are shown in boldface. <sup>d</sup>Mean ± standard deviation (n = 3, dry basis). The peaks were quantified as equivalent of acacetin<sup>e</sup> and apigenin<sup>f</sup>. nd = not detected.
Table 2. Chromatographic, UV-Vis, mass spectroscopy characteristics (HPLC-DAD-MS<sup>a</sup>) and contents of carotenoids of pulp, peel and seed extracts obtained from *Couepia bracteosa* fruit.

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

Total carotenoids (µg/g) | 130 ± 7 | 328 ± 41 | 34 ± 4

<sup>a</sup> Retention time on the C<sub>18</sub> column. <sup>b</sup> Linear gradient of methanol/MTBE. <sup>c</sup> Mean ± standard deviation (n = 3, dry basis). nc = not calculated. nd = not detected. The peaks were quantified as equivalent to all-trans-β-carotene<sup>d</sup>, all-trans-lutein<sup>d</sup>, all-trans-zeaxanthin<sup>d</sup> and all-trans-β-cryptoxanthin<sup>d</sup>. RAE = retinol activity equivalent
Table 3. Scavenging capacities of pulp, shell and seed extracts obtained from *Couepia bracteosa* fruits against superoxide radical (O$_2^*$), hydrogen peroxide (H$_2$O$_2$), hypochlorous acid (HOCl), nitric oxide (*NO) and peroxynitrite (ONOO$^-$).

<table>
<thead>
<tr>
<th>Reactive species</th>
<th>IC$_{50}$ (µg/mL) (n = 4)</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Couepia bracteosa</em> extracts</td>
<td>quercetin</td>
</tr>
<tr>
<td></td>
<td>pulp</td>
<td>shell</td>
</tr>
<tr>
<td>ROS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O$_2^*$</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>29.4 ± 0.2%*</td>
<td>894 ± 3</td>
</tr>
<tr>
<td>HOCl</td>
<td>47.1 ± 0.6</td>
<td>25.3 ± 0.4</td>
</tr>
<tr>
<td>RNS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*NO</td>
<td>36.1 ± 0.3%*</td>
<td>485 ± 2</td>
</tr>
<tr>
<td>ONOO$^-$</td>
<td>167 ± 5</td>
<td>53 ± 1</td>
</tr>
<tr>
<td>ONOO$^-$ **</td>
<td>35 ± 1</td>
<td>20.6 ± 0.3</td>
</tr>
</tbody>
</table>

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$_2$. 
Figure 1
Figure 2

Detector response at 450 nm (AU)
Figure 3