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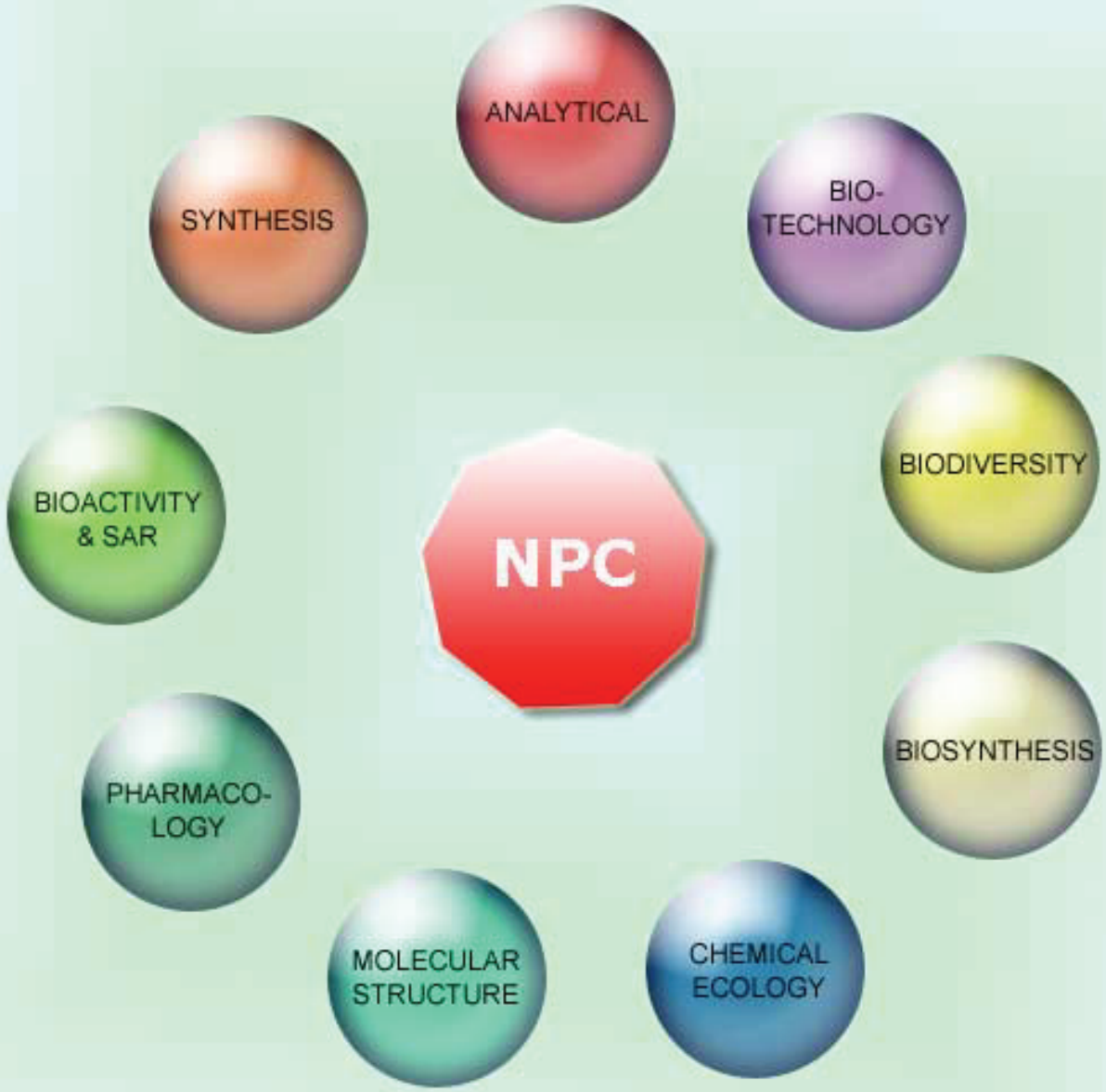

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Argentinean *Larrea* Dry Extracts with Potential Use in Vaginal Candidiasis

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Larrea divaricata (Ld), *Larrea cuneifolia (Lc)* and *Larrea nitida (Ln)* are shrubs occurring in Northwestern Argentina used in traditional medicine to treat fungal and bacterial infections and as anti-inflammatory. Antibacterial and antifungal activity of several *Larrea* species has been reported. However, their potential use in vaginal infections has been so far not assessed. The aim of this work was to determine the botanical difference between *Larrea* species, the chemical composition and the activity of *Larrea* dry extracts (DE) on *Candida* species isolated from vaginal infections and to assess their potential as antioxidant agents because infections are usually associated with oxidative processes. The main botanical difference between *Larrea* species resides in the morphology and shape of leaf, leaflets and stipules, presence or absence of mucron and rachis, percentage of coalescence of the leaflets. The position and abundance of the sclerenchymatic tissue at the mind vein and petiole transection allows the differentiation of the three species. The profile of phenolic compounds in the *Larrea* DE was determined. HPLC-ESI-MS/MS analysis of DE allowed the identification of 2 flavonoids and 10 lignans. Trihydroxy-6,7′cyclolignan was found only in *L. divaricata* and dihydroxy-methoxy-epoxylignan in *L. cuneifolia* and *L. nitida*, nordihydroguaiaretic acid (NDGA) was found in the three species. All extracts showed antioxidant capacity. The DE showed to be effective against *Candida albicans* and non-albicans strains. According to our results, the local vaginal use of *Larrea* DE in the concentration range of MIC values for *Candida* species does not affect the *Lactobacillus* normal vaginal microbiota. This work adds evidence to the potential use of *Larrea* DE as phytomedicine in vulvovaginal candidiasis with multiple effects, including antifungal and antioxidant activity.

Keywords: *Larrea* species, Dry extracts, Antioxidant, Anti-*Candida*.

Vulvovaginal candidiasis (VVC) is estimated to be the second most common cause of vaginitis. Up to 75% of women suffer this infection during their lifetime, and 5–8% of adult women have recurrent candidiasis [1a]. In this sense, it is important to find products without resistance to the treatment of *Candida* infection. Plant extracts (a single extract or an extract combination) could be considered as an alternative therapy because they are multi-component drugs with a binding ability for one or several targets by different action mechanisms [1b]. Therefore, microorganisms are less likely to develop mechanisms of resistance to phyto-extracts.

Several shrubs belonging to genus *Larrea* (Zygophyllaceae) occur in arid region from Argentina and are used in traditional medicine to treat fungal and bacterial infections [1c]. The most common species includes *Larrea divaricata* Cav. (common names: "jarilla", "jarilla hembra", "chamanilla", "jarilla del cerro", "yarilla"), *Larrea cunefolia* Cav. (common names: "jarilla", "jarilla macho", "jarilla crespa", "jarilla norte-sur", "jarilla del campo") and *Larrea nitida* Cav. (common names: "jarilla", "jarilla de la montaña", "crespa", "pispa o pispita", "jarilla fina"). A wide range of pharmacological activities was previously described indicating the potential use as alternative or complementary medicine.

The aqueous and/or alcoholic extracts from *L. divaricata* exert antibacterial [2a,b] and inmunomodulatory [2c] effect. Organic solvent extracts were active against phytopathogenic fungi [2d, e]. The aqueous and ethyl acetate extracts of *L. divaricata* decreased cell proliferation and induced apoptosis [2f-2i]. Preliminary phytochemical studies reported the presence of NDGA, essential oils and flavonoids in some *Larrea* species [2i-k, 3a]. Antioxidant properties [3b] and the synergistic antifungal effect of *L. nitida* and *Zuccagnia punctata* were reported [3c]. In *Larrea cuneifolia* organic extracts, several flavonoids including quercetin, apigenin and kaempferol derivatives were identified [3d]. The antimicrobial activity of the species against Gram-negative bacteria [2b], the antifungal activity against phytopathogenic filamentous fungi and yeast [2d, 3e] and the larvicidal activity [3f] has been reported. The aim of this study was to evaluate the antifungal and antioxidant activity and to compare the chemical composition and botanical

difference between of three *Larrea* species for their potential use as functional ingredients to treat vaginal candidiasis.

Larrea species are evergreen xerophytic, erect aromatic shrubs 1-4 m with opposite leaves pubescent, sub-sessile and stipulate leaves which show a resinous yellowish appearance. The main botanical difference between *Larrea* species resides in the morphology and shape of leaf, leaflets and stipules, presence or absence of mucron and rachis, and percentage of coalescence of the leaflets (SM Figures.1A-F). Anatomically characters such as non-glandular trichomes, stomata types, thick striated cuticle, resinous deposits, mesophyll type are common to all three species, however, we found that the position and abundance of the sclerenchymatic tissue at the mid vein and petiole transection allows the differentiation of the three species (SM Table 1, SM Figure 2). The plant anatomical and histological information provided in this report allow users to identify the botanical source for potential use in phytotherapy products. The information updates the previous study by Ragonese [3g], published in 1960 in an Argentinian journal (in Spanish). Phytochemical studies were performed with the lyophilized hydroalcoholic extracts from the *Larrea* aerial parts as used in traditional medicine. Extracts contained a high level of total phenolic compounds (TPC between 370.6 to 397.9 mg GAE/g dry extract) and flavonoid phenolic compounds (FPC between 201.6 to 240.4 mg GAE/g dry extract). Condensed tannin with values between 25.06 and 39.52 mg/g dry extract was detected. Hydrolyzed tannin was not detected in any of the analyzed samples. Soluble sugars were also quantified (89.7 to 139 mg GE/g DW). The results are summarized in SM Table 2.

The HPLC-ESI-MS/MS analysis of the dry extracts (SM Figure 3) allowed the tentative identification of 12 compounds including 2 flavonoids and 10 lignans. Compounds **1** and **2** with $[M-H]^-$ ion at *m/z* 463 and 477 amu, showed the neutral loss of 162 amu, leading to the base peak at m/z 301 and 315 amu, respectively, in agreement with quercetin (Q) and quercetin methyl ether. The compounds were assigned as Q-hexoside (**1**) and Q-methyl ether hexoside (**2**). The main compound with $[M-H]^-$ ion at *m/z* 301 was identified as nordihydroguaiaretic acid **7** by the characteristic fragments at 177, 122 and 109 amu, in agreement with the data reported by Agüero *et al*. (2011) [3a] as well as by comparison with a reference sample of the compound. Compound **10** with $[M-H]^-$ion at *m/z* 285 differs from **7** by 16 amu, indicating the presence of three hydroxyl functions in the aromatic rings. The compound was identified as 4-[4-(4-hydroxy-phenyl)-2,3-dimethyl-butyl]-benzene-1,2-diol in agreement with Agüero *et al*. (2011) [3a]. Compounds **11** and **12** differ from compound **7** in 14 amu, supporting the presence of a methoxy group in the molecules. The compounds have longer retention time as **7**, in agreement with the substitution of a free OH function by a OCH_3 group. Compounds **11** and **12** were assigned as methyl nordihydroguaiaretic acid isomers, in agreement with the data reported by Agüero *et al.* (2011) [3a].

Compounds **3** and **4** differ from **7** in 14 amu, presenting fragments supporting additional oxygen and an unsaturation (cycle), as required for epoxylignans. The mass spectra are in agreement with 3,4,3′,4′-tetrahydroxy-7,7′-epoxylignan and 3,4,3′,4′-tetrahydroxy-7,7′-epoxylignan isomer, as reported by Agüero *et al*. (2011) [3a] for propolis from *Larrea nitida*. The mass spectrum of the related compound **5** with $[M-H]^-$ion at *m/z* 329 amu, suggest*s* the presence of a methyl ether function. The compound was tentatively identified as 3,3′,4′-trihydroxy-4-methoxy-7,7′-epoxylignan [3a]. Compound **9** differs from compound **5** by 16 amu and was assigned as dihydroxy-methoxy-epoxylignan, the fragmentation pattern is in agreement with the structure proposed.

Table 1: Identification of *Larrea* extract constituents by HPLC-MS-MS in the negative ion mode.

Compound	Rt (min)	$[M-H]^-$	MS/MS	Compounds
1	9.0	463	301	Q-hexoside
2	12.2	477	315(100)	Q-methyl ether hexoside
3	23.5-26.1	315	301(50), 137(19)	3,4,3′,4′-tetrahydroxy-7,7′-epoxylignan*
4	34.6.	315	300(100)	3,4,3′,4′-tetrahydroxy-7,7′-epoxylignan isomer*
5	37.6-39.3	329	314(100)	3,3′,4′-trihydroxy-4-methoxy-7,7′-epoxylignan*
6	37.6-39.3	299	299(100), 243(10), 109(27)	3,4,3',4'-tetrahydroxy 6,7'-cyclolignan
7	43.1-47.0	301	273(19),177(9), 122(25), 109(13)	Nordihydroguaiaretic acid (NDGA)*
8	51.4-52.2	283	227(36),209(25), 189(21),173(6), 92.3(9)	Trihydroxy-6,7′cyclolignan
9	52.1-53.9	313	298(100), 109(5)	Dihydroxy-methoxy-7,7′-epoxylignan
10	58.2-59.9	285	122	4-[4-(4-hydroxy-phenyl)-2,3-dimethyl-butyl]-benzene-1,2-diol*
11	60.2-62.8	315	300(100),149(40)	Methyl-nordihydroguaiaretic acid*
12	61.8-62.4	315	300(100), 149(35)	Methyl-nordihydroguaiaretic acid isomer*

Cyclolignans have been previously isolated from *Larrea divaricata* [2e]. In the HPLC-ESI-MS/MS of our samples, compounds **6** and **8** are compatible with cyclolignans differing in the number of free hydroxyl and methoxy groups in the aromatic rings. The compounds were tentatively identified as hydroxyl methoxy derivatives of 6,7'cyclolignan on the basis of $[M-H]^-$ ions and fragmentation. Compounds include tetrahydroxy- and trihydroxy 6,7'-cyclolignan (compounds **6** and **8**, respectively). The lignans occurring in the extracts can be assigned either to NDGA and its derivatives, cyclolignans and epoxylignans according to the molecular mass and fragmentation patterns. Several biological activities (anti-lipoxygenase, antiproliferative, antitumor, antifungal and antioxidant) were attributed to lignans [2f, 2j, 3h-j]. The tentative identification of *Larrea* extract constituents is summarized in Table 1 and the chemical structures in SM Figure 4.

The antifungal activity of DE was assayed *in vitro* against 10 yeast strains obtained from vaginal exudates of patients with vaginal yeast infection. They included three strains of *Saccharomyces cerevisiae*, three strains of *C. albicans*, three strains of *C. glabrata* and one strain of *C. tropicalis*. Some *S. cerevisiae* and *C. albicans* and non-albicans strains are azole-susceptible as fluconazole, voriconazole, itraconazole (85%) or resistant (R) (15%), SM Table 3. The *Candida* and *Saccharomyces* species were also susceptible to nystatin and amphotericin B (SM Table 3).

Dry extracts were effective against *C. albicans* and non-*albicans*, in microdilutions assays. The MIC_{50} values were around 25 µg GAE/mL in all cases but MIC_{90} values (25 to 100 µg GAE/mL) showed that *Ld* and *Ln* extracts were more active than Lc extract (SM Table 3). The activity of the extracts was further examined in term of minimum fungicidal concentration (MFC), to ascertain whether the antifungal susceptibility results correlated with their killing capacity. For all isolates, MFC values of extract were two to four times higher than MIC_{90} values (SM Table 3). Our results are consistent with powerful candidacidal activity. Tangarife-Castaño *et al*. (2011) [4a] suggested a classification system for antifungal activity in plant derivatives based on MIC values as strong inhibitors (MIC of < 0.5 mg/mL); moderate inhibitors (MIC of 0.6-1.5 mg/mL); and weak inhibitors (MIC of > 1.6 mg/mL). Therefore, *Ln* and *Ld* extracts could be considered as strong natural antifungals. Lactic acid-producing bacteria, mainly *Lactobacillus* spp. are normal vaginal microbiome in women and maintain the acidic pH of vaginal fluids (pH 3.5-4.5). Therefore, the evaluation of the effect of *Larrea* extracts on vaginal lactic bacteria is essential in order to avoid an imbalance in the vaginal microbiome and the

restoration of the ecological equilibrium of the tract after their administration. All DE of *Larrea* spp. produce inhibition of the growth of *L. casei* CRL1267, *L. paracasei* CRL1291 and *L. johnsonii* CRL1292 isolated from human vagina, with MIC values higher than those against *Candida* strains (>400 µg/mL). According to these results, the local vaginal use of *Larrea* DE in the concentration range of MIC values for *Candida* species does not affect the *Lactobacillus* normal vaginal microbiota. Furthermore, our results would stimulate further research on the use of joint therapies in VVC of *Larrea* extracts and beneficial *Lactobacillus* for vaginal applications [4b]. The VVC is associated with signals following *Candida*-vaginal epithelial cell interactions that promote the release of free radicals and inflammatory response that results in mucosal damage [4c]. Products released due to the activation of proinflammatory enzymes are the major physiological sources of free radicals or reactive oxygen species (ROS) [4c].

Larrea extracts showed antioxidant capacity. The *Ld* extract was more active as $ABTS^{\bullet+}$ scavenger than *Lc* and *Ln* with SC_{50} values of 2.68; 4.10 and 4.50 µg/mL, respectively (SM Table 4). SC_{50} values did not show significant differences between *L. divaricata* DE and the major lignan identified in the three species, NGDA. In all cases the antioxidant effect of *Larrea* extracts was higher than BHT and quercetin, two commercial antioxidants. Similar results were reported to *Zuccagnia punctata* flower extract, a jarilla that grows in association with *L. divaricata* and *L. cuneifolia* in the same arid region of Argentina (SC_{50} values of 3.8 µg GAE/mL for ABTS) [4d]. In the assay of the oxidative hemolysis inhibition, *Lc* exhibited a stronger inhibitory effect on lipoperoxidation of red blood cells, with IC_{50} values of 0.12 µg GAE/mL. The main compounds in all extracts were NDGA and its derivatives, with known antifungal and antioxidant properties [3h-j] .

Experimental

Plant material: The plant parts used were leaves and stems (aerial parts), according to the traditional use. *Larrea cuneifolia* Cav. *(Lc)* and *L. divaricata* Cav. *(Ld)* were collected in April 2015 at Amaicha del Valle, Tucumán, Argentina at 2000 m.a.s.l.. The sample of *Larrea nitida (Ln)* was collected in April 2015 at Vinchina, La Rioja, Argentina at 3485 m.a.s.l. The plants investigated are shown in Supplementary material (SM Figure 1). The plants were identified by Dra Soledad Cuello (INBIOFIV-CONICET). Voucher specimens (*L. cuneifolia*: LIL 614829; *L. divaricata*: LIL 614299; *L. nitida*: LIL 615845) were deposited at the Herbarium of Fundación Miguel Lillo (Tucumán, Argentina). The samples were dried in a forced air oven at 40°C.

Histological analysis: Samples of leaves and stems of each species were fixed in FAA (formalin, acetic acid, 50% ethanol, 5:5:90 v/v/v), and then, were embedded in 3% agarose and sectioned (10-25 µm) with a rotation microtome. Sections were stained astra blue-safranin and mounted in 50% glycerol [5a]. Sections were visualized with a Zeiss Axiolab optic microscope equipped with a Zeiss Axiocam ERc 5s digital camera. For scanning electron microscopy (SEM) samples were fixed in glutaraldehyde phosphate 5% buffered with 0.1 M sodium cocadylate at pH 7, and postfixed in 1.5% osmium tetroxide buffered with 0.1 M sodium cocadylate at pH 7.2. Leaflets were dehydrated in acetone, dried by CO_2 critical point drying method and covered with a thin gold layer (200Å) by using an ion-sputter. Observations were carried out on a field emission scanning electron microscope (FESEM-ZEISS SUPRA-55 VP).

Dry extract preparation: The powdered air-dried plant material (10 g) was macerated in 200 mL of 60° ethanol for 1 h with ultrasonic application five times for 10 minutes. Combined extracts were filtered, taken to dryness under reduced pressure and then lyophilized to afford the extracts. The w/w extraction yield was determined. Dry extracts were placed in oxygen barrier bags and vacuum-packed (Multivac, D-8941, Germany). The bags were divided into two batches that were stored under two different conditions, at room temperature and at 4°C. The dry extract was dissolved in ethanol 60°C to carry out the phytochemicals and biological assays.

Phytochemical analysis of dry extracts: Total phenolic compound (TPC), flavone and flavanone, condensed and hydrolyzable tannins and sugar content were determined according to Costamagna *et al*., [5b]. Non-flavonoid phenols (NF-P) were determined by the Folin–Ciocalteau method after precipitation of the flavonoids phenols with acidic formaldehyde [5c]. Flavonoid phenolic (FP) content was calculated by difference between TPC and NF-P.

Identification of phenolics by HPLC-ESI-MS/MS: Extracts were analyzed by HPLC-ESI-MS/MS to compare the composition of the samples and to identify principal constituents. Mass spectra were recorded using an Agilent 1100 (Agilent Technologies Inc., CA, USA) liquid chromatography system connected through a split to an Esquire 4000 Ion Trap LC/MS(n) system (Bruker Daltoniks, Germany). Ionization was performed at 3000 V assisted by nitrogen as a nebulizing gas at 50 psi and as a drying gas at 365ºC and a flow rate of 10 L/min. Negative ions were detected using full scan (m/z 20-2200) and normal resolution (scan speed 10,300 m/z/s; peak with 0.6 FWHM/m/z). Trap parameters were set in ion charge control (ICC) using manufacturer default parameters, and maximum accumulation time of 200 ms. Mass spectrometric conditions for analysis were as follows: electrospray needle, 4000 V; end plate offset, -500 V; skimmer 1, 56.0 V; skimmer 2, 6.0 V; capillary exit offset, 84.6 V; capillary exit, 140.6 V. Collision induced dissociation (CID) spectra were obtained with a fragmentation amplitude of 1.00 V (MS/MS) using helium as the collision gas and was automatically controlled through Smart Frag option. Extracts were analyzed using a MultoHigh 100 RP 18-5µ (250 x 4.6 mm) column (CS-Chromatographie Service GmbH, Langerwehe, Germany) maintained at 25 °C. The HPLC-MS analyses were performed using a linear gradient solvent system consisting of 1% formic acid in water (A) and acetonitrile (B) as follows: 30% to 40% B over 35 min, increasing to 45% B at 50 min, changing to 70% B at 70 min, 70 to 100% B from 70 to 80 min, 100% to 30% B from 80 to 85 min, and kept to 30% to 95 min. Flow rate was 0.5 mL/min and the volume injected was 20 µL. Compounds were monitored at 254 nm.

Antimicrobial assays: *Candida* strains were provided by Instituto Nacional de Enfermedades Infecciosas- Administración Nacional de Laboratorios e Institutos de Salud (INEI-ANLIS) 'Dr. Carlos G. Malbrán', Buenos Aires, Argentina, 2015. The strains used were *Candida albicans* (144783; 134333; 2089), *C. glabrata* (031646; 042030; 031982), *C. tropicalis* (1841), *S. cerevisiae* (134528; 134544; 124263), *C. parapsilosis* DMic 134410 and *C. krusei* DMic 134409. MIC and MFC values of dry extracts against *Candida* and *Saccharomyces* were determined by the broth microdilution method [5d]. The inoculum (200µL) containing $0.5\text{-}2.5\times10^3$ CFU/mL and DE (6.25–400 µg/mL) were added to each well. *Lactobacillus casei* CRL 1267, *L. paracasei* CRL 1291 and *L. johnsonii* CRL 1292 strains were provided by CERELA (Centro de Referencia de Lactobacilos, Tucumán, Argentina). MIC values of extracts against *Lactobacillus* were performed by the agar macrodilution method [5e]. Two-fold serial dilutions of the original extract (6.25 – 400 µg/mL) were used.

Antioxidant activity: The antioxidant capacity of the dry extracts (concentration range between 0.1 and 11 µg GAE/mL) was carried out by the improved ABTS radical cation ($ABTS^{\bullet+}$) method as described by Costamagna *et al*. (2013) [5b] . Butylated hydroxytoluene (BHT), quercetin and NDGA were used as reference compounds.The protection of oxidative hemolysis of RBC by the DE (0.1 and 2.3 µg GAE/mL) was determined according to Mendes *et al*. (2011) [5f]. BHT, quercetin and NDGA were used as reference compounds.

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