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Profisetinidin type tannins responsible for antioxidant activity in *Copaifera reticulata*

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The *in vitro* antioxidant and free radical scavenging properties in bark extracts of South American tree *Copaifera reticulata* Ducke. (Caesalpinaceae) were studied using different bioassays. Lipid peroxidation was assessed by means of the production of thiobarbituric acid reactive substances (TBARS) in rat liver homogenate. All the extracts tested were effective in this method. The highest activity was observed in the aqueous extract, showing an IC_{50} of 30 μg/ml. DNA sugar damage induced by Fe (II) salts was also used to determine the capacity of the samples to suppress hydroxyl radical-mediated degradation of DNA. Although all the extracts tested were effective in reducing oxidation of DNA, the highest activity was observed in the methanol extract, showing an IC_{50} of 2 μg/ml. Bioassay-guided fractionation of a total methanol extract monitored by luminol-enhanced chemiluminescence, together with structural elucidation using ^{13}C NMR and FABMS, led to the identification of profisetinidin type tannins in a semi-pure fraction. The fraction containing the active compounds also reduced the production of TBARS in rat liver homogenates ($IC_{50} = 530$ μg/ml) and DNA damage ($IC_{50} = 1$ μg/ml), suggesting that profisetinidins could be responsible for the free radical scavenging and antioxidant activities observed in the extracts.

1. Introduction

Several plant derived natural products such as Vitamins E and C, β-carotene, flavonoids, phenolic acids and nitrogen compounds including alkaloids and chlorophyll derivatives, have proved to be effective as oxygen radical scavengers and in reducing oxidative stress related processes such as lipid peroxidation and DNA damage [1–3]. One successful methodology for the investigation of plants as a source of new pharmacologically active compounds such as antioxidants includes the screening of plant extracts followed by a bioassay-guided fractionation leading to isolation of pure active plant constituents. This methodology usually entails the testing of a drug by means of *in vitro* methods, which can then be used for monitoring the activity during purification of the active plant constituents [4, 5].

The bark of South American tree *Copaifera reticulata* Ducke. (Caesalpinaceae) is widely used among local inhabitants in the Amazon river basin for the treatment of arthritis and other inflammatory diseases. Copalic acid and many sesquiterpenes have been identified in the oleoresin of *C. reticulata*, which could be responsible for the anti-inflammatory and analgesic activities observed in rats [6, 7]. Furthermore, recent studies have demonstrated that different extracts of *C. reticulata* contain compounds that act as peroxyl radical ($ROO^{\cdot}$) scavengers [8]. Since peroxyl radicals are important agents that mediate lipid peroxidation [9] the aim of this study was to elucidate the antioxidant properties present in different extracts of *C. reticulata*, and to subject the active lead(s) to bioassay-directed procedures and structure elucidation techniques, namely ^{13}C NMR and FABMS analysis, for the procurement of the active principle(s).

2. Investigations and results

2.1. Inhibition of lipid peroxidation in rat liver homogenate

Lipid peroxidation was assessed by means of an assay that determines the production of malondialdehyde and related compounds in rat liver homogenates. Thiobarbituric-acid-reactive substances (TBARS) are produced as by-products of lipid peroxidation that occurs in the hydrophobic core of biomembranes [10]. Previous control experiments (data not shown) indicated that addition of *C. reticulata* extracts to liver homogenate that was either intact or already oxidatively modified did not interfere with the absorption at 515–555 nm. Moreover, omission of the homogenate from the reaction mixture abolished chromogen formation, suggesting that the extracts did not affect TBARS measurement. However, all the extracts tested were effective in reducing the production of TBARS in a dose-dependent manner, thus allowing to calculate the concentration that would inhibit the TBARS production by 50%, i.e the IC_{50} values. The results obtained are shown in Table 1. The highest activity was observed in the aqueous extract, showing an IC_{50} lower to that observed for Trolox, used as a standard. Catechin was also effective in reducing the production of TBARS.

Table 1: Antioxidant activity of *C. reticulata* bark extracts: inhibition of the production of thiobarbituric acid-reactive substances (TBARS) in rat liver homogenates (n = 2), IC_{50} and 95% confidence interval (n = 3)

Sample	Inhibition (%)			IC_{50} and 95% confidence interval (μg/ml)
	1000 μg/ml	100 μg/ml	110 μg/ml	
Trolox	61	49	17	232 (480–131)
Catechin	87	74	56	5 (12–1)
H_2O extract	71	68	37	30 (65–9)
CH_2Cl_2 extract	41	25	14	>1000
MeOH extract	45	31	24	>1000
CRAE-S2–8	53	42	30	530 (9518–169)

Rat liver homogenates were pre-incubated with DMSO (1%, control) or various concentrations of the extracts at 37 °C for 15 min (n = 3). Inhibition percentage calculated by (1-Ptm/Pcm) × 100; Ptm: production of TBARS of test samples, Pcm: production of TBARS in control samples. Trolox and catechin were used as reference compounds.

2.2. Free radical mediated DNA damage

The Fenton reaction generates hydroxyl radicals (OH·) which degrade DNA deoxyribose, using Fe^{2+} salts as an important catalytic component [11]. Addition of low concentrations of transition metal ions such as iron to DNA causes degradation of the sugar into malondialdehyde and other related compounds which form a chromogen with thiobarbituric acid (TBARS). As in the case of lipid peroxidation, previous control experiments (data not shown) indicated that neither the addition of plant extracts to calf thymus DNA which was either intact or already oxidatively modified, nor the omission of DNA from the reaction mixture, interfered with the absorption at 515–555 nm and TBARS measurement. The addition of different extracts of *C. reticulata* into a reaction medium containing DNA and Fe^{2+} salts decreased the production of TBARS, measured as a reduction in fluorescence, indicating the presence of antioxidant activity (Table 2). The methanol and aqueous extracts inhibited the DNA-sugar damage in a dose dependent manner, the former showing an IC_{50} of 3 μg/ml. Trolox and catechin were also effective in reducing the production of TBARS. By contrast, the dichloromethane extract showed a decreased antioxidant activity at higher concentrations.

Table 2: Effect of different bark extracts of *C. reticulata* on free radical-mediated DNA-sugar damage (n = 3)

Sample	Inhibition (%)			IC_{50} and 95% confidence interval (μg/ml)
	1000 μg/ml	100 μg/ml	10 μg/ml	
Trolox	65	44	23	199 (420–108)
Catechin	93	74	59	5 (12–1)
H_2O extract	66	31	–8	376 (560–267)
CH_2Cl_2 extract	12	21	48	N.D.
MetOH extract	72	70	54	2 (15–0.001)
CRAE-S2-8	72	58	45	1 (10–0.1)

Calf thymus DNA was pre-incubated with DMSO (1%, control) or various concentrations of the extracts and ferrous ammonium sulphate at 37 °C for 1 h. Inhibition percentage of iron (II)-mediated DNA sugar-damage was calculated by (1-Dtm/Dcm) × 100; Dtm: fluorescence in test samples, Dcm: fluorescence in controls. Trolox and catechin were used as standards. ND: not determined.

2.3. Bioassay guided fractionation and phytochemical analysis

Bioassay-guided fractionation of a total methanol extract of *C. reticulata* was performed using luminol-enhanced chemiluminescence [12] as a monitor of biological activity. A previous screening for antioxidant activity in medicinal plant extracts using this method revealed that *C. reticulata* was the most active [6]. An initial fractionation of the extract with solvents of increasing polarity (hexane, chloroform, ethyl acetate, *n*-butanol and water) revealed that the ethyl acetate fraction was the most active peroxyl radical scavenger (TRAP = 3047 μM Trolox equivalents). A preliminary ^{13}C NMR spectrum of the fraction was performed, showing a typical profile of condensed tannins [13] (data not shown). Further purification by gel filtration on Sephadex LH-20 afforded an active fraction (Fraction 4) (TRAP = 6300 μM Trolox equivalents) that was studied by ^{13}C NMR. The spectrum obtained by this method did not differ from that of the total extract (Fig. a). The lack of signals between 95 and 100 ppm ruled out the presence of procyanidins (A-ring: 5,7-diOH, B-ring: 3′,4′-diOH) or prodelfinidins (A-ring: 5,7-diOH, B-ring: 3′,4′,5′-triOH) type tannins since free C-6 and C-8 present in these compounds resonate in that region, thus suggesting the presence of profisetinidins and/or prorobinetidin type tannins [13, 14].
The chemical shift of carbon signals of prorobinetidins (C-2′ and C-6′) were coincident with those of C-6 of all A-rings (Fig. a, f), hence, the presence of this type of oligomer could not be discarded. Even though, due to the low intensity of this signal, it was presumed that if it was presented, it might be in a relatively low concentration, leading to the interpretation of the spectra on the basis of the presence of profisetinidin type oligomers.

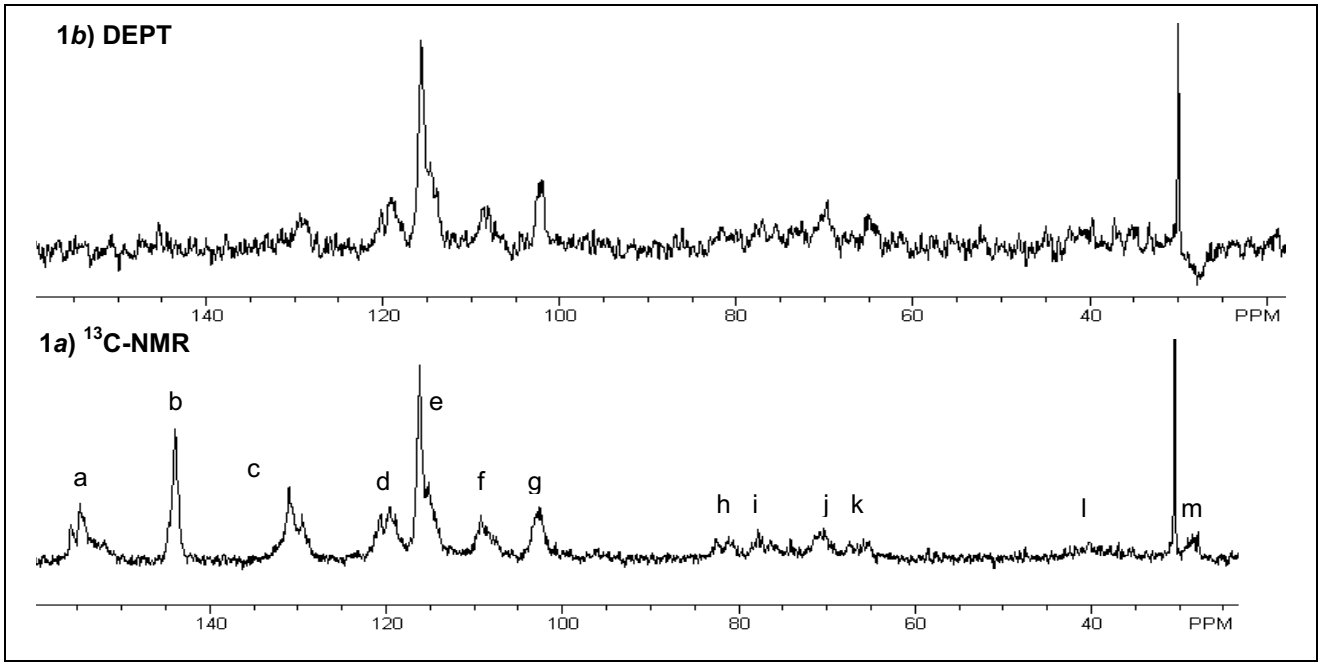
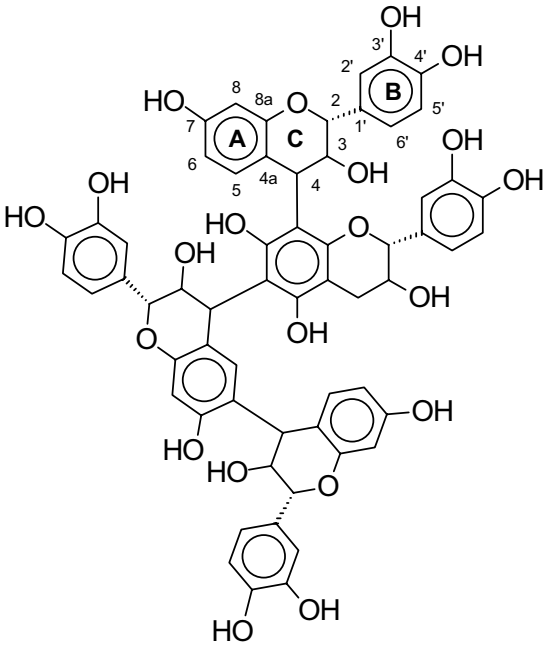


Fig.: ^{13}C NMR and DEPT-135 spectra of fraction 4. Profisetinidin type tannins from *Copaifera reticulata* bark. Key: a, C-7 and C-8a; b, C-3′ and C-4′; c, C-1′ and C-5; d, C-6′; e, C-2′, C-5′ and C-4a; f, C-6; g, C-8; h, C-2 of the 2,3-*trans* systems; i, C-2 of the 2,3-*cis* systems; j, no terminal C-3; k, terminal C-3; l, C-4 linked to a phenolic ring; m, free C-4 methylene

The carbon signal at ~103 ppm (Fig. a, g) was assigned to the free C-8 signal of the fisetinidol units, since it appeared as a positive signal in the DEPT-135 (Fig. b) experiment, suggesting that it was a methine carbon. This evidence, together with the fact that the C-8 signal appeared at ~103 ppm, only for A-rings of the resorcinol type (7-OH substituted), reinforced the idea that the active fraction (as well as the total ethyl acetate extract) was mainly composed by profisetinidin type tannins. The modification on the profile of this peak in the DEPT-135 spectrum was due to the absence of signals of the quaternary C-8's of the interflavan linkages. The intense signal at ~115 ppm (Fig. a, e) accounted for the C-2′ and C-5′ signals of a 3′,4′-dihydroxy substituted B-ring as for fisetinidol units. Signals of the quaternary carbons C-7/C-8a (Fig. a, a) and C-3′/C4′ (Fig. a, b) are not present in Fig. b, as expected. The carbon signals in Fig. a, c distinguished the methine C-5 of all units and C-1′ of fisetinidinol B-rings. The change in the profile of this signal (Fig. b) was due to the absence of the quaternary carbon C-1′. Signals at ~120 ppm proved to be due to methine carbons, and were assigned to C-6′of the fisetinidol units. The remaining signals were coincident with literature data as follows: h, C-2 (2,3-*trans*); i, C-2 (2,3-*cis*); j, C-3 (middle units); k, C-3 (terminal units); l, C-4 (middle units) and m, C-4 (methylene of terminal units) that appeared as a negative signal.

Assuming that the main components of the stem bark extract of *C. reticulata* are profisetinidins, the most active fractions were combined and purified by gel filtration. Subfraction CRAE-S2-8, having displayed the highest activity in this group of sub-fractions, was analyzed by FABMS. A molecular ion at m/z = 1106 (8%) was consistent with a molecular formula of $C_{60}H_{50}O_{21}$, as for a profisetinidin tetramer. The fragments at 833 (100%) and 561 (38%) indicated stepwise losses of 273 ($C_{15}H_{12}O_5$) amu and 272 ($C_{15}H_{13}O_5$) amu, corresponding to a terminal and middle fisetinidol unit loss from the tetramer, respectively. It is known that tri- and tetraprofisetinidins occur in nature as "angular" [4,6 : 4,8]-oligomers with the fisetinidol monomers condensed on a catechin unit (or gallocatechin) whose C-6 at the A-ring (5,7-diOH) is more nucleophilic that the C-6 in the fisetinidol unit [13, 15–17].



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Further biological studies were performed in order to determine if the profisetinidin tannins present in fraction CRAE-S2-8 are responsible for the capacity of the extracts to reduce the *in vitro* lipid peroxidation and DNA damage. As expected, the presence of CRAE-S2-8 in the reaction medium reduced the production of TBARS in both rat liver homogenates (Table 1) and calf thymus DNA (Table 2), when subjected to oxidative stress. Based on the evidence presented, we suggest structure **1** as a representative tannin present in the *C. reticulata* ethyl acetate extract, which would be responsible for the antioxidant and free radical scavenging activities described in different extracts of this species. Stereochemistry at all the C-3 and C-4 asymmetric carbons were considered to be either *R* or *S*.

3. Discussion

In this study, different bark extracts of medicinal tree *Copaifera reticulata* were studied to determine their effects on *in vitro* lipid peroxidation and DNA damage. In the case of lipid peroxidation, liver homogenate is useful for investigation of oxidative stress and its effects on cell membranes. Regarding DNA damage, besides generation of hydroxyl radicals, ferrous ion may also stimulate the formation of perferryl and ferryl species [18, 19]. Although all extracts studied were active as free radical scavengers, both aqueous and methanol extracts showed a high capacity of reducing lipid peroxidation and DNA sugar damage, respectively. These activities were higher than that observed for the reference drug Trolox, suggesting the presence of compound(s) with high antioxidant activity, taking into account that the materials under study were crude extracts. On the other hand, the dichloromethane extract showed a decreased antioxidant activity at higher concentrations, when tested in the DNA damage assay. This behaviour could be due to the presence of antioxidants that lose their activity at high concentrations, such as desferrioxiamine [20].

The lack of phytochemical information available on *C. reticulata* led to further studies involving bioassay guided fractionation and structural elucidation of a total methanol extract, in order to identify the compound(s) responsible for the biological activity described. Studies by ^{13}C NMR and FABMS on purified fractions showed the presence of profisetinidin tannins as the active principles. These compounds demonstrated to be responsible not only for the peroxyl radicals scavenging activity previously described for *C. reticulata*, but also for the capacity of the extracts to reduce lipid peroxidation and DNA damage. Tannins and flavonoids possess phenolic hydrogens which confer them with free radical-scavenging activity, and the *O*-dihydroxyl (catechol) structure in the B ring is the main radical target site for these compounds [21, 22]. The additional presence of both 3- and 5- hydroxyl groups is responsible for maximal radical scavenging potentials and strongest radical absorption. Trolox, on the other hand, acts to block the chain reaction of lipid peroxidation by scavenging the intermediate peroxyl radicals which are generated in the hydrophilic domains of membranes during this process [3], mainly due to the long lipophilic chain present in its structure [23]. The differences in the mechanisms of action of Trolox and profisetinidin tannins

could explain the relatively higher efficiency of the reference compound in reducing lipid peroxidation (Table 1). In contrast, profisetinidins were more effective in scavenging hydroxyl radicals that lead to degradation of DNA deoxyribose (Table 2).

Tannins are widely distributed in nature and are, in many cases, the active compounds of medicinal plants. However, unlike proanthocyanidins, reports on profisetinidins as biologically active plant-derived compounds are not common in literature. Several *in vitro* assays demonstrated potentially significant interactions of tannins with biological systems, such as antiviral, antibacterial and molluscicidal activity; inhibition of human immunodeficiency viral replication, inhibition of human simplex virus, host mediated antitumour activity, anti-inflammatory, antioxidant and free radical-scavenging properties [3]. ^{13}C NMR spectroscopy proved to be the most useful technique to study free phenolic tannins, although their spectra are quite complex. These provide broad signals which are due to the multiplicity produced by the conformational isomerism arose from the restricted rotation at the C-4 (sp^3-sp^2) interflavan-linkage [17].

Although medicinal plants are rarely used as "antioxidants" in traditional medicine, their claimed therapeutic properties could be due, in part, to their capacity of scavenging oxygen free radicals which may be involved in many diseases, such as in the case of plants used to treat inflammatory diseases and gastric ulcers, which could act by reducing oxidative stress [24, 25]. Further investigations will be necessary to determine the effects of *C. reticulata* on the oxidative stress process *in vivo*, together with the possible involvement of profisetinidin tannins in the anti-inflammatory activity previously described for this species.

4. Experimental

4.1. Plant material

The bark of *C. reticulata* was collected by one of the authors (C. Desmarchelier) in the District of Totaizal, province of Beni, Bolivia, during November 1995. Botanical identification was done by botanists of the Museum of Botany, School of Pharmacy and Biochemistry, University of Buenos Aires, where a voucher specimen (N° 540) is deposited.

4.2. Plant extracts

Extracts were prepared following recommendations of CYTED [26]. The infusions were made by pouring 100 ml of boiling H_2O on 5 g of powdered bark placed in a stoppered flask. The mixture was left standing for 20 min an then filtered. The resulting infusion was freeze-dried in a Gamma A lyophiliser (Chriss, Germany). The resulting powder was considered as the aqueous extract. The CH_2Cl_2 extract was prepared by extracting 5 g of dry powdered plant material during 24 h with 50 ml of CH_2Cl_2 (Dorwill, Argentina). The extract was filtered and concentrated under reduced pressure at 43 °C in a Savant Speed Vac Plus SC210A concentrator. The marc was extracted with MeOH (Mallinckrodt, USA), under the same conditions as described for the CH_2Cl_2 extract. The total MeOH extract was prepared by extracting 5 g of dry powdered plant material for 24 h with 50 ml of MeOH. The extract was filtered and concentrated as described for the other extracts. DMSO (Merck, Argentina) was used to pre-solubilize CH_2Cl_2 and MeOH extracts (final concentration = 1% V/V).

4.3. Preparation of rat liver homogenate and lipid peroxidation (TBARS) assay

Adult Wistar rats of 180–200 g on a standard laboratory diet and water ad libitum were used. The livers were excised, perfused and homogenised with 120 mM KCl, 50 mM phosphate buffer, pH 7.4, (1 : 10 w/v). The samples were centrifuged at 700 × g for 10 min at 0–4 °C. The supernatant fraction was kept at −20 °C until use. Protein concentration was measured as described previously [27], using bovine serum albumin (Dorwill, Argentina) as a standard. Thiobarbituric acid-reactive substances (TBARS) were determined as described [10]. Rat liver homogenates, adjusted to 10 mg protein/ml in 120 mM KCl, 50 mM phosphate buffer, pH 7.4, were incubated with 1000, 100 and 10 μg dry weight/ml of plant extract at 37 °C for 15 min. Sodium dodecyl sulphate (Sigma, USA) (0.2 ml of 3% (w/v)) was included in the reaction mixture, and after mixing, 2 ml of 0.1 N HCl, 0.3 ml of 10% (w/v) phosphotungstic acid (Sigma) and 1 ml of 0.7% (w/v) TBA (Sigma) were added. The mixture was heated for 60 min in boiling H_2O, and TBARS were extracted into 5 ml n-butanol (Mallinckrodt). After centrifugation, the fluorescence of the butanol layer was measured at 515 nm excitation and 555 nm emission using an Hitachi F-3010 fluorescence spectrophotometer. The values were expressed as the ratio of the amount of TBARS formed in the presence of plant extracts compared to control.

4.4. Free radical mediated DNA damage

The DNA-sugar damage was assayed as described by Halliwell and Gutteridge [11], with modifications. The reaction mixture in a total volume of 1.2 ml contained 0.5 ml calf thymus DNA (Calbiochem, USA) (1 mg/ml of 0.15 M NaCl), 0.5 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml ferrous ammonium sulphate (Mallinckrodt) (4.8 mM) and 1000, 100 and 10 μg/ml extracts. The reaction mixture was incubated for 1 h at 37 °C in a water bath shaker. After the incubation was over, 1 ml TBA 1% (w/v) plus 1 ml 2.8% (w/v) TCA (Sigma) were added to the reaction mixture and kept in a boiling water bath for 15 min. The TBA reacting species so generated were extracted into 2 ml n-butanol. After centrifugation, the fluorescence of the butanol layer was measured as described for the lipid peroxidation (TBARS) assay. The iron (II)-dependent DNA-sugar damage inhibition values are expressed as the ratio of TBA reacting species in the presence of plant extracts to that in their absence (control).

4.5. Standards and statistical analysis

Finney's statistical programme of Probit analysis [28] was used to calculate the concentration of the extract that would inhibit the thiobarbituric acid-reactive substances production (TBARS) and DNA damage in 50%, i.e the IC_{50} values. α-tocopherol synthetic analog Trolox (Sigma) was used as a positive control or standard. Antioxidant activity was also compared to that observed for catechin (Sigma), due to its high antioxidant activity [29].

4.6. Peroxyl radical scavenging activity: total reactive antioxidant potential (TRAP)

Free radical scavenging activity during bioassay guided fractionation was monitored by the luminol-enhanced chemiluminescence method [12], using the radical cation 2,2′-azo-bis(2-amidinopropane) (ABAP) as a source of peroxyl radicals. The relative ability of antioxidants to scavenge free radicals, as compared to standard amounts of the synthetic antioxidant Trolox, allows to measure the antioxidant activity of mixtures of substances such as plant extracts [30]. The reaction medium consisted of phosphate buffer 100 mM (pH: 7.4), ABAP (Acros Organics, USA), 20 mM, 10 μM luminol (Acros Organics), and increasing volumes (5–10 μl) of tested plant extract fractions (0.1 mg/ml). Light intensity was measured at room temperature in a WinSpectral (1414) liquid scintillation counter with the circuit coincidence out of mode. ABAP is a source of free radicals which react with luminol yielding chemiluminescence. The system was calibrated using Trolox (Sigma) (150 μM). A comparison of the induction times after addition of known concentrations of Trolox and antioxidants allows to obtain TRAP values as equivalents of Trolox concentration necessary to suppress the emitted luminescence by employing the following equation:

$$\text{TRAP}\ (\mu\text{M Trolox}) = (\mu l_{total}/\mu l_{sample}) \times (\delta_{i\ sample}/\delta_{i\ Trolox}) \times \delta_{i\ Trolox\ (1\,\mu M)}$$

where μl_{total} is the final volume (3000 μl), μl_{sample} is the volume of the sample added to the reaction (5–10 μl of a 0.1 mg/ml solution), $\delta_{i\ sample}$ is the induction time observed for the different volumes of the sample, $\delta_{i\ Trolox}$ is the induction time observed for the reference compound (Trolox), and $\delta_{i\ Trolox\ (1\,\mu M)}$ is the induction time observed for the reference compound at a 1 μM final concentration.

4.7. Bioassay-guided fractionation and phytochemical analysis

The ^{13}C NMR and DEPT spectra were recorded in acetone-d_6 (ethyl acetate extract) and D_2O (Fraction 4) solutions on a Bruker AC-200 spectrometer at 50.32 MHz. Chemical shifts are given in parts per million (δ). The spectra obtained in acetone-d_6 and D_2O were referenced using the solvent peak and dioxane as external standard, respectively. Temperature was maintained at 303 K. FABMS were recorded on a ZAB-CQ(V6) Hybrid BeqQ mass spectrometer. Column chromatography of a total MeOH extract was carried out on Sephadex LH-20 (Pharmacia, Sweden) using MeOH or Me_2CO as eluents. The extract was previously dissolved in 600 ml MeOH–H_2O (5 : 1). The MeOH–H_2O mixture was further extracted with hexane, and the remaining MeOH–H_2O extract was concentrated to eliminate MeOH and extracted, successively, with $CHCl_3$, EtOAc and *n*-BuOH. Each partitioned fraction was evaporated to dryness *in vacuo* to give hexane, $CHCl_3$, EtOAc, *n*-BuOH and water extracts. Free radical scavenging activity of the resulting fractions was monitored by luminol-enhanced chemilumines-

cence, showing TRAP values of 139, 563, 3047, 1547 and < 100 μM Trolox equivalents, respectively. The ^{13}C NMR of the ethyl acetate extract, being the most active, showed the typical signals for condensed tannins [14, 17]. This fraction (20.8 g) represented 64% of the total extract; 1 g was purified by gel filtration on Sephadex LH-20 using MeOH as an eluent, and the biological activity in the resulting fractions were monitored using luminol-enhanced chemiluminescence. Fraction 4 showed the highest activity and was analyzed by ^{13}C NMR revealing a similar profile to that observed in the total extract (Fig.). Fractions 3–12 (487.3 mg) were purified by gel filtration on Sephadex LH-20 using MeOH and $(CH_3)_2CO$ as eluents. Luminol enhanced chemiluminescence revealed that peroxyl radicals scavenging activity was concentrated in sub-fractions 2/6–2/10 (188.5 mg). The FABMS of fraction 2/8 designed as CRAE-S2-8 (the most active of the group), showed ions at m/z = 1106 (M^+, 2%), 833 (100%), 561 (36%) was consistent with the proposed kind of compound. Further purification of sub-fractions 2/6–2/10 using similar procedures did not reveal a considerable increase in biological activity.

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