Supporting Information

***Rec. Nat. Prod*. 7:2 (2013) 147-151**

Acetylcholinesterase Inhibitory and Antioxidant Properties of *Euphorbia characias* Latex

Francesca Pintus[a], Delia Spanò[a], Claudia Mascia[a], Alberto Macone[b], Giovanni Floris[a] and Rosaria Medda[a,*]

[a]*Department of Sciences of life and environment*, University of Cagliari

[b]*Department of Biochemical Sciences "A. Rossi Fanelli", University of Rome "La Sapienza" and CNR Institute of Molecular Biology and Pathology, Rome, Italy*

*To whom correspondence should be addressed: Prof. Rosaria Medda, Dipartimento di Scienze della vita e dell'ambiente, Cittadella Universitaria, I–09042 Monserrato (CA) (Italy)

Tel: +39 070 6754517; Fax: +39 070 6754523; e-mail address: rmedda@unica.it

Table of Contents **Page**

MATERIALS AND METHODS

All chemicals were obtained as pure commercial products and used without further purification. Acetylcholinesterase (AChE) from *Electrophorus electricus*, acetylthiocholine iodide, 4-aminoantipyrine, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), aluminum nitrate, benzoic acid, caffeic acid, cinnamic acid, *p*-coumaric acid, 3,5-dimethoxy-4-hydroxycinnamic acid, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), 5,5′-dithiobis(2-nitrobenzoic acid), ferulic acid, Folin-Ciocalteu phenol reagent, galanthamine hydrobromide, gallic acid, 4-hydroxybenzylalchol, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), horseradish peroxidase (HRP), myricetin, quercetin, sinapic acid, tyrosol, and vanillic acid were from Sigma Chemical Co (St. Louis, USA).

Spectrophotometry

Data from all activity assays were obtained with an Ultrospec 2100 spectrophotometer (Biochrom Ltd, Cambridge, England) using cells with a 1 cm path length.

Plant material

Euphorbia characias latex, drawn from cut branches of several plants, was collected in the four seasons at several locations in southern Sardinia (Italy) and immediately used.

The water content in the latex is different in every season, ranging from 68-70% in spring and autumn to 55-58% in winter and summer. Thus, all data reported were referred to the dry weight after liophylization and normalized to 60% in water.

Analysis of crude latex extracts does not show significant differences in antioxidant and AChE inhibitor content over the four seasons, indicating that the oxidative stress does not change during the various vegetative phases of the shrub.

Preparation of crude extracts

Euphorbia latex (5 mL) was treated with:

(i) Trichloroacetic acid (TCA). This method appears particularly suitable for obtaining a clear extract from the milky latex. Briefly, 0.5 mL TCA (100%) was added to 5 mL latex and immediately centrifuged at 9000 rpm for 30 min. The precipitate was separated and the pH of the supernatant was corrected to 7.0 with NaOH 1 M.

(ii) Methanol (MeOH; 50 mL)

(iii) Ethanol (EtOH; 50 mL)

(iv) Petroleum ether (PE; 5 mL) followed by MeOH (5 mL) (PE/MeOH).

All the samples were kept end-over-end from 1 to 12 h in sealed vials. Afterwards they were centrifuged at 9000 rpm for 30 min. The precipitate from all the three extracts was separated,

treated again with the respective solvent and centrifuged at 9000 rpm for 30 min. All supernatants were used for antioxidant activity assays.

For each extraction method, all the antioxidant activities and the inhibitory effects on AChE activity, were checked in the first and in the successive treatment. We reported the results obtained with the first treatment only because very few antioxidant activities and few AChE inhibitors (≈ 0.2-1.0%) were detected in the successive treatment.

All determinations were performed in triplicate and at least three times for each of the four seasons.

Free radical-scavenger methods

In every extract total free radical-scavenging molecules were determined by $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ methods using Trolox as antioxidant standard. Trolox (2.5 mM) was prepared in EtOH as a stock standard solution and it was used with a final concentration ranging from 0 to 20 μM.

$ABTS^{\bullet+}$ assay

This method [1] is based on the capacity of an antioxidant to scavenge the free radical $ABTS^{\bullet+}$. $ABTS^{\bullet+}$ reagent was produced by reacting 7 mM ABTS with 2.45 mM potassium persulfate (final concentration) in aqueous solution, kept in the dark at room temperature for 24 h before use. The $ABTS^{\bullet+}$ solution, which has an absorption maximum at 734 nm, was diluted in the appropriate solvent to obtain an A_{734} of about 0.70. Ten μL of the extracted sample was added to 1 mL of diluted $ABTS^{\bullet+}$ solution and incubated in the dark at room temperature for 1 min. Afterwards the A_{734} was recorded. The decrease in A_{734} was calculated and referred to the Trolox standard and expressed as Trolox equivalent antioxidant capacity (TEAC; mM).

$DPPH^{\bullet}$ assay

$DPPH^{\bullet}$ radical shows a strong absorption maximum at 515 nm. $DPPH^{\bullet}$ radical goes through a change of color after the transfer of a hydrogen atom from an antioxidant. Thus, the antioxidant effect can be monitored by evaluating the decrease in A_{515} [2]. An aliquot of extracts (25 μL) was added to 975 μL of 6×10^{-5} M $DPPH^{\bullet}$ solution. The reaction mixture was incubated in the dark for 15 min and, thereafter, the A_{515} was recorded against $DPPH^{\bullet}$ solution alone as a control. The decrease in absorbance of $DPPH^{\bullet}$, after addition of test samples compared to the control, was used to calculate the antioxidant activity which was expressed as TEAC (mM).

Estimation of the total polyphenol content

Total polyphenol content in the extracts was determined by the Folin-Ciocalteu reagent and by an enzymatic method, using a calibration curve obtained with gallic acid as polyphenolic reference standard.

Folin-Ciocalteau method

Ten μL of the extracts was mixed with 50 μL of the Folin-Ciocalteu reagent and 790 μL of distilled water. After 1 min, 150 μL of saturated sodium carbonate (20% aqueous solution) was added to the mixture. The mixture was kept in the dark for 45 min, and then the A_{750} was measured [2].

Enzymatic method

The enzymatic method was used as previously reported [3]. In a typical determination 50 μL of the extracts was added to 1 mL of 0.1 M potassium phosphate buffer pH 8.0, containing 3 mM 4-aminoantipyrine, 2 mM H_2O_2 and 0.33 μM horseradish peroxidase (HRP). The phenoxyl radicals, formed from the phenolic groups of polyphenols in the presence of HRP and hydrogen peroxide, reacted with 4-aminoantipyrine, giving a colored quinone-imine. The absorbance at 500 nm was determined at the end of the reaction (e.g. after 5 min incubation time).

In both of the methods, the polyphenol content was expressed as gallic acid equivalents (GAE; mM).

Determination of total flavonoid content

Total flavonoid content was determined as reported [4]. The extracts (0.5 mL) were added to 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1 M sodium acetate and 4.3 mL of 80% EtOH. After 40 min at room temperature, the absorbance was determined at 415 nm. Flavonoid concentration was calculated by a calibration curve obtained using quercetin as reference standard and expressed as quercetin equivalent molarity (QE, mM).

Acetylcholinesterase activity inhibition

AChE activity was measured spectrophotometrically using Ellman's reagent according to the method previously reported [5]. The test is based on the hydrolysis of acetylthiocholine by AChE to acetate e thiocholine that reacts with 5,5'-dithiobis-2-nitrobenzoate yielding a yellow coloured anion having an ε_{405} = 1.36 $M^{-1}cm^{-1}$. The velocity of the reactions was measured and AChE activity was calculated as a percentage of the velocity in the presence (100 μL) and in the absence of latex extracts and comparing the rate for the sample to the appropriate blank. The amount of AChE inhibitor was calculated by a calibration curve obtained using galanthamine as reference standard and expressed as galanthamine equivalent molarity (GE, μM). Standard galanthamine solutions were prepared in 10% MeOH (v/v) with

a final concentration ranging from 0.1 to 100 μM. AChE inhibition of 50% (IC_{50}) was achieved with 15 μM galanthamine.

GC/MS analysis

GC-MS analyses were performed with an Agilent 6850A gas chromatograph coupled to a 5973N quadrupole mass selective detector (Agilent Technologies, Palo Alto, CA, USA). Chromatographic separations were carried out with an Agilent HP5ms fused-silica capillary column (30 m × 0.25 mm i.d.) coated with 5%- phenyl-95%-dimethylpolysiloxane (film thickness 0.25 μm) as stationary phase.

GC/MS analysis of phenolic compounds as TBDMS derivatives

TCA latex extract (300 μL) was extracted three times with 1 mL ethyl acetate by vortexing for 2 min in a 10 mL tube after the pH was adjusted to 2.0 by adding 0.5 M HCl. The mixture was then centrifuged and the top layer (ethyl acetate) was removed. Once pooled, the ethyl acetate extracts were passed through anhydrous $MgSO_4$ packed in a Pasteur pipette and evaporated to dryness under nitrogen. The extracts were resuspended in 30 μL dichloromethane, derivatized by the addition of 50 μL of N-tert-butyldimethylsilyl-N-methyltrifluoro-acetamide (TDMS) (70 °C, 1 h), evaporated to dryness under nitrogen and resuspended in 20 μL ethyl acetate for GS-MS analysis. Injection mode: splitless at a temperature of 260 °C. Column temperature program: 70 °C (1 min) then to 300 °C (10 min) at a rate of 10 °C/min. The carrier gas was helium at a constant flow of 1.0 mL/min. The spectra were obtained in the electron impact mode at 70 eV ionization energy; ion source 280 °C; ion source vacuum 10^{-5} Torr. Mass spectra were obtained both in total ion current increment mode (TIC) (mass range m/z 30- m/z 600) and in selected ion monitoring mode (SIM) checking two characteristic ions for each phenolic compound.

GC/MS analysis of quercetin and myricetin as TMSi derivatives

Acidified methanol (0.5 mL) containing 1% (v/v) HCl and 0.5 mg mL^{-1} TBHQ was added to the TCA latex extract (0.5 mL). HCl (1.2 M, 0.1 mL) was added and the mixture was stirred at 90°C under reflux for 2 h to obtain the aglycons by hydrolysis of the flavonol glycosides. The extract was cooled to room temperature and extracted three times with ethyl acetate (1:1, v/v). Ethyl acetate extracts were pooled, passed through anhydrous $MgSO_4$ packed in a Pasteur pipette and evaporated to dryness under nitrogen. The extracts were resuspended in 30 μL dichloromethane and derivatized by the addition of 50 μL of the trimethylsilyl chloride (TMS) (70 °C, 3 h), evaporated to dryness under nitrogen and resuspended in 20 μL ethyl acetate for GS-MS analysis. Injection mode: splitless at a temperature of 280 °C. Column temperature program: 120 °C (1 min) then to 320 °C (10 min) at a rate of 10 °C/min. The

carrier gas was helium at a constant flow of 1.0 mL/min. The spectra were obtained in the electron impact mode at 70 eV ionization energy; ion source 280 °C; ion source vacuum 10^{-5} Torr. Mass spectra were obtained both in TIC mode (mass range m/z 50- m/z 800) and in SIM mode monitoring three characteristic ions for both quercetin (m/z 647, m/z 575, m/z 487) and myricetin (m/z 735, m/z 663, m/z 575).

REFERENCES

[1] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **26**, 1231–1237.

[2] A. Mansouri, G. Embarek, E. Kokkalou, P. Kefalas (2005). Phenolic profile and antioxidant activity of the Algerian ripe date palm fruit (*Phoenix dactylifera*). *Food Chem*. **89**, 411–420.

[3] R. Stevanato, S. Fabris, F. Momo (2004). New Enzymatic method for the determination of total phenolic content in tea and wine. *J. Agr. Food Chem.* **52**, 6287–6293.

[4] M.I.N. Moreno, M.I. Isla, A.R. Sampietro, M.A. Vattuone. (2000). Comparison of the free radical-scavenging activity of propolis from several regions of Argentina. *J. Ethnopharmacol.* 7**1**, 109–114.

[5] I.K. Rhee, M. van de Meent, K. Ingkaninan, R. Verpoorte (2001). Screening for acetylcholinesterase inhibitors from Amaryllidaceae using silica gel thin-layer chromatography in combination with bioactivity staining. *J. Chromatogr. A* **915**, 217–233.

Table 1. Antioxidant and AchE inhibitor content in *Euphorbia characias* latex**.**

Extraction method	Free radical-scavenging compounds[a]		Polyphenols[b]		Flavonoids[c]	AchE inhibitors[d]
	ABTS	DPPH	Folin-Ciocalteau	HRP		
MeOH	11 ± 1.4	3.6 ± 0.7	24 ± 2.3	96 ± 12	26.63 ± 2.3	8.6 ± 1.4
EtOH	6.4 ±0.8	4.2 ± 0.8	28 ± 3.2	104 ± 15	29 ± 4	21.5 ± 2.3
PE/MeOH	2.8 ± 0.5	1.9 ± 0.2	25 ± 2.8	14.4 ± 1.6	13.7 ± 1.8	15.6 ± 1.6
TCA	18 ± 0.8	5.9 ± 0.4	28.3 ± 1.2	30 ± 1	23.6 ± 0.8	170 ± 15

[a]expressed as Trolox equivalent antioxidant capacity (TEAC; mM)
[b]expressed as gallic acid equivalents (GAE; mM)
[c]expressed as quercetin equivalent molarity (QE, mM)
[d]expressed as galanthamine equivalent (GE, μM)

Table 2. Mass spectral data for TBDMS and TMSi derivatives of phenolic and benzoic acids identified in TCA latex extract.

COMPOUND	Retention time (min)	TBDMS groups[a]	M^+	Fragment ions[b,c]
benzoic acid	11.53	1	236(0)	221(4), **179**(100), 77(29), **135**(26), 105(40)
cinnamic acid	15.06	1	262(0)	**205**(100), 161(28), 145(9), **131**(37), 103(31), 75(22)
4-hydroxybenzyl-alcohol	16.80	2	352(2)	**295**(32), **221**(100), 179(9), 147(9),73(29)
tyrosol	17.51	2	366(0)	**309**(100), 235(37), **219**(55), 193(16), 177(6), 126(8)
vanillic acid	19.42	2	396(0)	**339**(100), 295(12), **267**(39), 265(11), 223(10), 193(12), 126(12), 73(18)
p-coumaric acid	21.19	2	392(1)	377(2), **335**(100), 291(13), **261**(26), 73(33), 57(5)
ferulic acid	22.35	2	422(1)	407(4), **365**(100), **293**(53), 249(7), 219(16), 191(7), 146(14), 73(23)
sinapic acid	23.42	2	452(1)	437(4), **395**(100), **323**(64), 279(6), 249(18), 218(5), 161(20),73(23)
caffeic acid	24.14	3	522(2)	507(4), **465**(100), 293(7), 249(5), **219**(22), 191(7), 73(87)
		TMSi groups[a]		
quercetin	21.38	5	662(0)	**647**(16), **575**(100), 545(1), 517(1), **487**(16), 415(4), 73(51)
myricetin	21.79	6	750(0)	**735**(4), **663**(100), 647(5), **575**(22), 503(3), 73(50)

[a] TBDMS groups add 114 to the original molecular weight for each hydroxyl proton it displaces, whereas TMSi groups add 72.
[b]m/z values, with relative abundances (%) in parentheses.
[c]Ions monitored during SIM acquisition are written in bold.

Figure 1

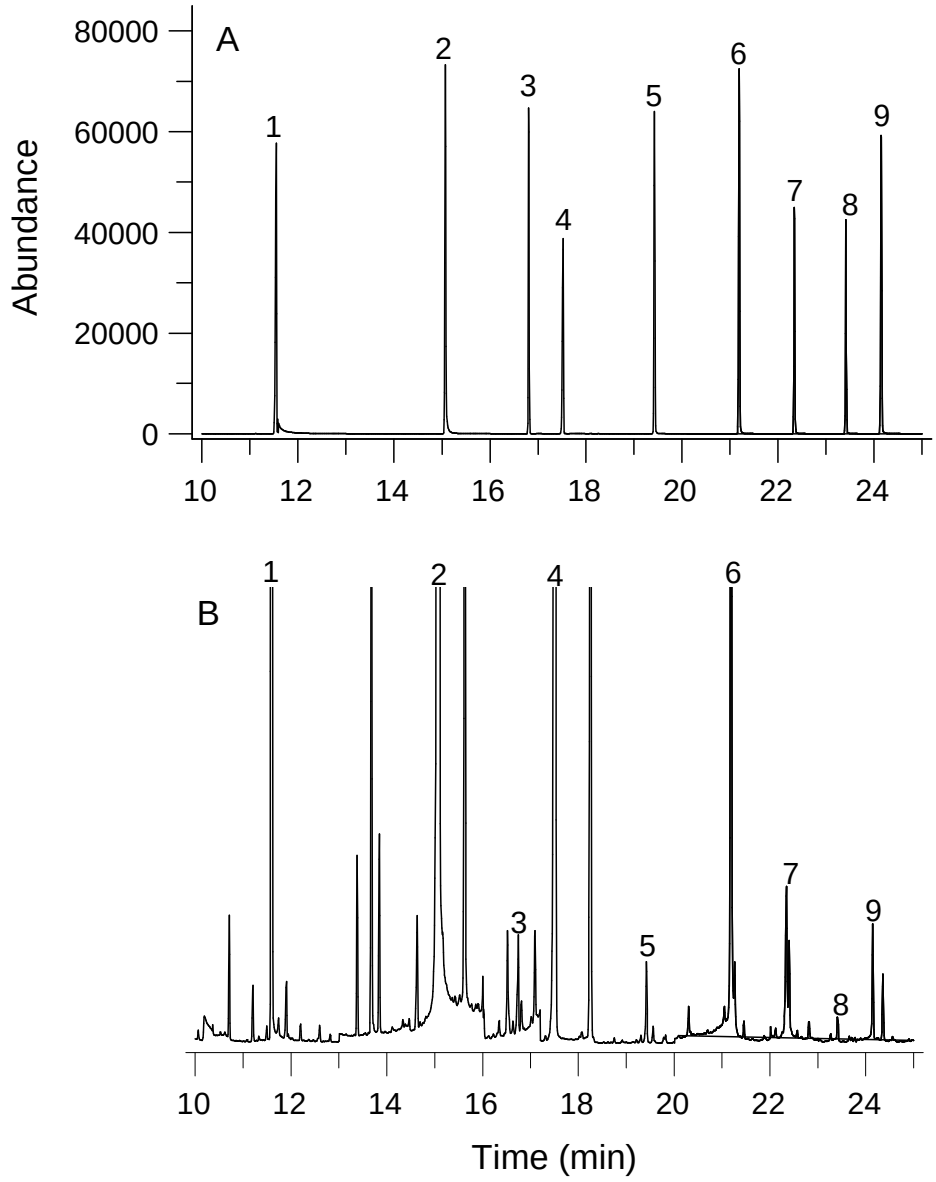

Figure 1. **GC/MS chromatograms of phenolic compounds**.
Panel A: GC/MS chromatogram of TBDMS derivatives of nine different phenolic compounds used as standard: 1. benzoic acid; 2. cinnamic acid; 3. 4-hydroxybenzyl-alcohol; 4. tyrosol; 5. vanillic acid; 6. *p*-coumaric acid; 7. ferulic acid; 8. sinapic acid; 9 caffeic acid. Panel B: GC/MS chromatogram of TCA latex extract.

Figure 2

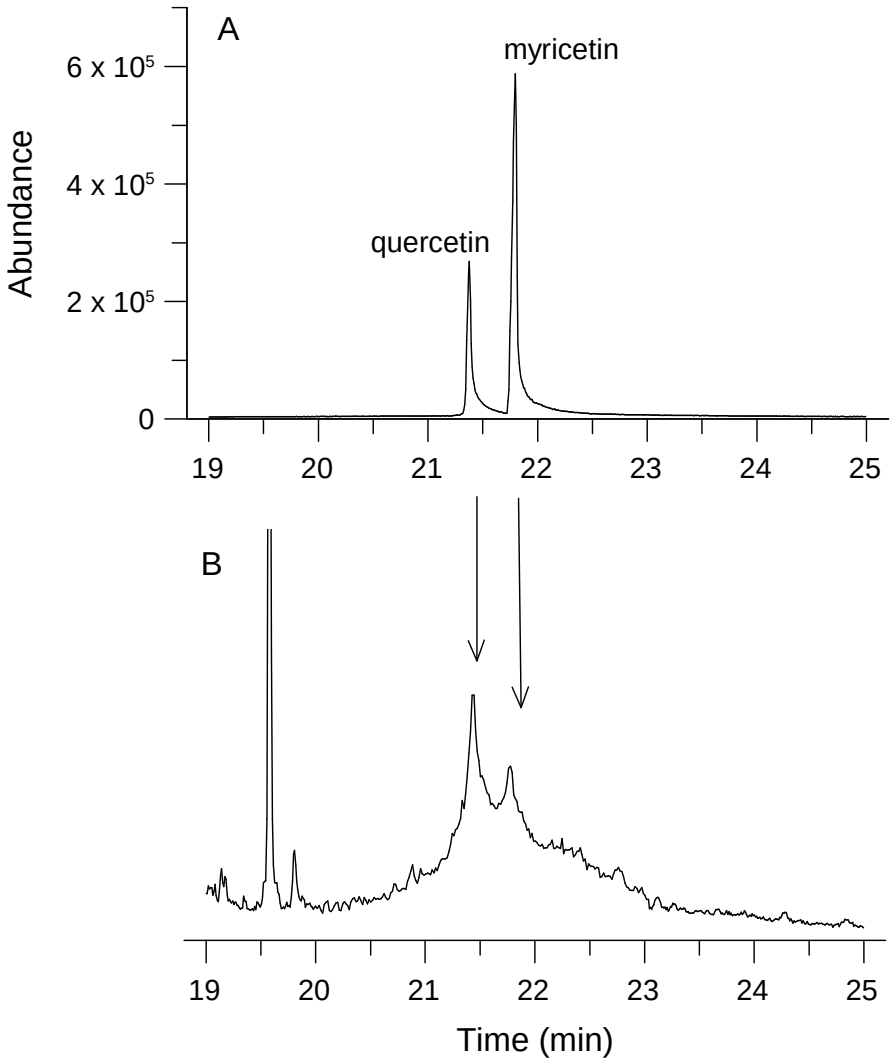

Figure 2. **GC/MS chromatogram of flavonoid compounds.**
Panel A: GC/MS chromatogram of TMSi derivatives of quercetin and myricetin as standard compounds.
Panel B: GC/MS chromatogram of TCA latex extract.