Antioxidant Activity of Mediterranean Plant Leaves: Occurrence and Antioxidative Importance of α-Tocopherol

S. Chevolleau, J.F. Mallet, A. Debal and **E. Ucciani***

Laboratoire de Chimie Organique Appliquée, URA CNRS 1409, Faculté des Sciences et Techniques, 13397 Marseille Cedex 20, France

α-Tocopherol was identified as the main antioxidant in hexane extracts of leaves of sixteen Mediterranean plant species. The α-tocopherol content was determined by a two-step procedure involving column and gas chromatography with α-tocopherol acetate as internal standard. The tocopherol content of the extracts was in the range of 0.0–4.7%, and that of the dry leaves was 0–846 ppm. The highest α-tocopherol content was found in the leaves of a Mediterranean oak, *Quercus ilex*. The antioxidative activity, which was previously investigated, was correlated with the α-tocopherol content. Correlation coefficients were 0.947 and 0.904 for extracts and leaves, respectively.

KEY WORDS: Antioxidant activity, hexane extracts, Mediterranean plant leaves, α-tocopherol.

Antioxidative properties of common spices and herbs were systematically investigated in the 1950s by Chipault *et al.* (1). Since then, numerous studies have been devoted to vegetable sources, not only for their possible utilization as antioxidative extracts but also to look for new, naturally efficient antioxidants. Several antioxidants have been characterized from plant leaves (2–7), and some products or extracts proved to be as good as the synthetic butylated hydroxyanisole and butylated hydroxy toluene.

In a recent communication (8) we presented a simple and rapid method for obtaining plant leaf extracts, as well as comparative data on the antioxidant properties of sixteen Mediterranean plant species. Research in our laboratory is in progress to continue the screening of the local flora. We present here the identification of the major antioxidant, α-tocopherol, and its content in leaf extracts and dry leaves by gas chromatographic determination. The α-tocopherol content and antioxidant activity were correlated.

EXPERIMENTAL PROCEDURES

Air-dried leaves were extracted with hexane by a percolation method that has been previously described (8). Crude extracts were stored under argon at −20°C.

Antioxidant activity was determined in emulsion by the carotene bleaching method of Taga *et al.* (9), involving a coupled oxidation of linoleic acid and β-carotene at 50°C. The reaction was monitored by absorbance decrease at 470 nm in a Sequoia Turner 690 spectrophotometer (Sequoia Turner Corporation, Mountain View, CA). Chemical reagents were purchased from Fluka (Mulhouse, France).

Hexane extracts were analyzed by thin-layer chromatography (TLC) on silica gel 60 (0.2 mm) pre-coated aluminum sheets (Merck, Darmstadt, Germany). The developing solvent was hexane/diethyl ether (4:1, vol/vol).

*To whom correspondence should be addressed at LCOA, boite 412, Faculté des Sciences et Techniques, 13397 Marseille Cedex 20, France.

The spots were visualized with an ultraviolet lamp (254 and 365 nm) and by soaking the plate in a 3% sulfuric acid diethyl ether solution and heating at 200–250°C. After development, the plates were sprayed with mixed β-carotene–chloroform and linoleic acid–ethanol solutions (10). The plates were then exposed to daylight until disappearance of background color. Yellow spots indicated an antioxidant activity.

Hexane extracts (120 mg), added to 5 mg of α-tocopherol acetate (Fluka) as internal standard, were fractionated on a chromatographic column (1.5 × 40 cm) filled with 12 g of 70–230 mesh silica gel 60 (Merck). Elution with hexane/diethyl ether (4:1, vol/vol) was monitored by TLC, and samples of α-tocopherol and its acetate were used as reference standards. Fractions containing α-tocopherol and its acetate yielded a purified tocopherol extract.

Gas chromatographic (GC) analysis of tocopherol extracts was carried out on a Girdel 300 chromatograph (Girdel, Suresnes, France) equipped with a flame-ionization detector and an Enica 10 integrator (Delsi Instruments, Suresnes, France). The column was an OV-17 fused-silica capillary column (Rescom, Kortrijk, Belgium) of 25 m length, 0.25 mm i.d. and 0.2 μm film thickness. Programmed temperature was from 230 to 300°C at 10°C/min. Split-injector and detector temperatures were 250 and 300°C, respectively. Helium (80 KPa) was used as carrier gas.

GC-mass spectrometric (MS) analysis was performed on a Delsi-Nermag Automass (Argenteuil, France) under the aforementioned GC conditions. Spectrometer source temperature was held at 120°C. An emission current of 0.329 mA and an ionization energy (EI mode) of 70 eV were used for mass fragmentation.

The infrared spectra of CCl_4 solutions were obtained on an Acculab 4 spectrophotometer (Beckman Instruments, Fullerton, CA). Nuclear magnetic resonance (NMR) spectra were obtained on an AC 200 spectrometer (Brücker, Wissembourg, France) and an XL 200 spectrometer (Varian, Palo Alto, CA) in $CDCl_3$ as solvent with Me_4Si as internal standard.

RESULTS AND DISCUSSION

In our previous paper (8) we examined the antioxidant activity of leaf extracts of sixteen Mediterranean plant species. We found that hexane extracts were much more efficient than the corresponding methanol extracts. Subsequently, we tried to determine the compounds responsible for the high antioxidant activity of the hexane extracts.

The TLC test based on the decoloration of β-carotene was used to detect any antioxidant present in these hexane extracts (10). Several types of compounds were thus visualized, but only one of them showed high activity based on the intensity of the spot. The R_f value of this compound was the same as that of an α-tocopherol sample (R_f 0.45). Purified extracts obtained from silica

gel column chromatography were then analyzed. By GC analysis, we found identical retention times for both α-tocopherol and the unknown compound. Structural elucidation was finally achieved by GC–MS analysis, which gave the following fragment ions: *m/z* 430 (59%) corresponding to $C_{29}H_{50}O_2$, *m/z* 205 (10%), *m/z* 165 (100%), corresponding to an α-tocopherol structure (11).

The ^{13}C NMR spectrum clearly indicated the presence of a 2,5,7,8-tetramethyl chroman-6-ol ring with a 2-phytyl chain, as previously reported (12,13). The ^1H NMR and infrared spectrum matched those of authentic α-tocopherol. All the analytical and spectroscopic data obtained are in agreement with the identification of α-tocopherol as the main antioxidant in the hexane extracts of the evaluated leaves.

Tocopherols are natural phenolic substances synthetized only in the plastids of higher plants (14), so it is not surprising to find them in leaf extract. Their occurrence in leaves has been already reported in different plants (15–17).

Methods for quantitative determination of α-tocopherol in natural products are abundantly described in the literature, especially in fats and oils analysis. However, direct analysis of leaf tocopherols in the crude extracts is practically impossible. Tocopherol contents in leaves have been determined previously by colorimetry (15,17) and by high-performance liquid chromatography (16). We selected a two-step method to determine the α-tocopherol content of the leaves—a chromatographic purification followed by a GC analysis. Extracts in which α-tocopherol acetate was added as internal standard were first purified on silica gel. Fractionation gave a tocopherol extract consisting mainly of α-tocopherol and its acetate. These extracts were then directly analyzed by GC.

α-Tocopherol acetate is not a natural plant product and is not present before its introduction in the samples. It represented a good reference standard for our method because the retention ratio acetate/α-tocopherol was 1.03.

The α-tocopherol content of the leaf extracts was calculated from the following equation:

$$M_T = K\,(A_T/A_S) \cdot M_s \qquad [1]$$

where M_T and A_T are, respectively, mass and peak area of α-tocopherol, M_S and A_S are, respectively, mass and peak area of the internal standard (acetate) S, and K is the response factor of the ratio acetate/α-tocopherol. K was evaluated from a calibration curve after GC analysis of different synthetic mixtures, and a value of 1.09 was found. Table 1 gives the α-tocopherol content calculated as percent of hexane extracts and as ppm of dry leaves.

Except for *Lavandula angustifolia*, all the species contained α-tocopherol. Levels varied from 18 ppm in leaves of *Coronilla juncea* to 846 ppm for *Quercus ilex*. These values are in accordance with those obtained by Booth (15), who used a colorimetric method for the determination of α-tocopherol in some hundred other species. This author observed a maximum content of 1000 ppm in leaves of *Urtica dioica*.

In comparison with some common edible seeds, well known for their high tocopherol content, leaves could be a valuable source of α-tocopherol. Amounts found in leaves of *Q. ilex* (846 ppm), *Globularia alypum* (663 ppm) and *Myrtus communis* (627 ppm) are greater than those of total seed tocopherols in rapeseed (190–528 ppm), soybean (144–484 ppm), sunflower (130–415 ppm) and corn (27–312) (18,19).

TABLE 1

α-Tocopherol Content of Hexane Leaf Extract of Various Plant Species

Species	α-Tocopherol content	
	Hexane extract (%)	Dry leaves (ppm)
Centranthus ruber DC. (Valerianaceae)	0.4	100
Cistus albidus L. (Cistaceae)	0.1	33
Conium maculatum L. (Apiaceae)	0.6	210
Coronilla juncea L. (Fabaceae)	0.1	18
Eucalyptus globulus Labil. (Myrtaceae)	0.3	333
Ferula communis L. (Apiaceae)	0.3	75
Globularia alypum L. (Globulariaceae)	3.9	663
Hedera helix L. (Araliaceae)	1.3	75
Lavandula angustifolia L. (Lamiaceae)	0.0	0
Myrtus communis L. (Myrtaceae)	3.3	627
Phillyrea angustifolia L. (Oleaceae)	2.4	480
Pinus halepensis Mill. (Pinaceae)	0.2	210
Quercus ilex L. (Fagaceae)	4.7	846
Rhamnus alaternus L. (Rhamnaceae)	3.4	442
Smilax aspera L. (Liliaceae)	2.1	357
Staehelina dubia L. (Asteraceae)	0.3	138

α-Tocopherol is an efficient liposoluble antioxidant, so it could be responsible for most of the activity observed in the hexane extracts. To confirm this hypothesis, we searched for a correlation between the α-tocopherol content and the antioxidant activity. Figures 1 and 2 show the antioxidant activity coefficient (8) in relation to the α-tocopherol content of the hexane extract and in the dry leaves, respectively. A positive and proportionate influence of the α-tocopherol content appeared from these two graphs. The experimental data can be fitted by a straight line. The values of the correlation coefficient were 0.95 for

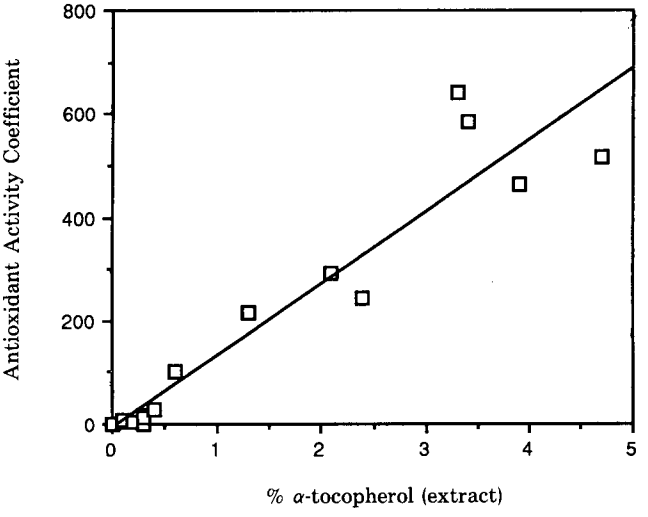

FIG. 1. Correlation between the antioxidant activity coefficient and the α-tocopherol content (%) in hexane extracts of leaves from 16 Mediterranean plant species.

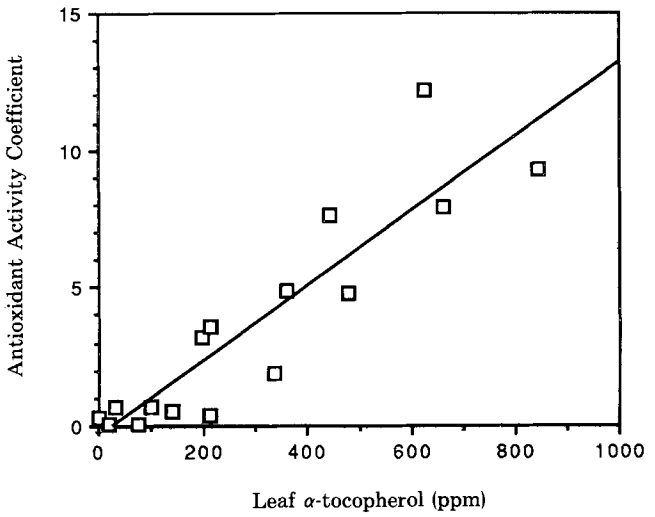

FIG. 2. Correlation between the antioxidant activity coefficient and the α-tocopherol content (ppm) of leaves from 16 Mediterranean plant species.

extracts and 0.91 for dry leaves. These values are given as square terms by the computer or calculated from the usual equation of linear regression analysis (20). Because the correlation coefficients are close to unity, α-tocopherol could be considered as responsible for the main part of the antioxidant activity of the leaf extracts.

Plant tissues are rich in phenolic compounds, some of which have powerful antioxidant properties, but they are not extracted by hexane, neither as glycosides nor as aglycones. α-Tocopherol could be the main liposoluble antioxidant. Screening of more species is still in progress to characterize those possessing high α-tocopherol content. Work is also in progress to identify the other lipids in the hexane extracts.

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