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abeo-Abietanes from *Teucrium polium* roots as protective factors against oxidative stress

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ABSTRACT

Six new 17(15→16)-*abeo*-abietanes, along with seven known compounds, were isolated and characterized from ethyl acetate root extract of *Teucrium polium* L., a medicinal plant belonging to the Labiatae family reported to have hypolipidemic, hypoglycaemic, anti-nociceptive and anti-inflammatory effects. Their structures were elucidated by 1D (^{1}H, ^{13}C and DEPT) and 2D (COSY, TOCSY, HSQC, HMBC, and NOESY) NMR and mass spectral data. The antioxidative properties of pure metabolites were analyzed on the basis of their DPPH radical scavenging capability. The antioxidant capacity in cell-free systems of the isolated metabolites was carried out by measuring their capabilities to inhibit the synthesis of thiobarbituric acid reactive species in assay media using as oxidable substrates a vegetable fat and the pentose sugar 2-deoxyribose and to prevent oxidative damage of the bovine serum albumin (BSA) hydrosoluble protein. All of the compounds showed a significant and dose–response efficacy although weaker than that exercised by the standard Trolox®.

1. Introduction

Free radical production occurs continuously in cells as a consequence of both enzymatic and non-enzymatic processes. Some free radicals arise normally during metabolism: the function of reactive oxygen species (ROS) in host defense is well known, and their cell signaling role in biological systems by inducing apoptosis or necrosis, by suppressing the expression of many genes or by activating cell signaling cascades is now clear.[1]

These highly reactive organic species, when produced in high quantities, can interact with biomolecules of subcellular structures. To cope with potentially damaging ROS, aerobic tissues contain endogenous antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalase, as well as several exogenously acquired radical scavenging substances such as vitamins E and C and carotenoids.[2] Other scavengers, like free amino acids, peptides,[3,4] and polyphenols in vegetables and other plants,[5] can influence ROS concentration in the cell.[6]

Environmental factors, such as pollution, radiations, cigarette smoke and herbicides, can break down the balance between free radicals and endogenous antioxidants leading to pathogenesis of several diseases.[7]

Many functional foods contain antioxidant components[8] that could increase the endogen protection against free radicals, preventing lipid oxidation in alimentary fats and oils, retarding the formation of toxic oxidation products, maintaining nutritional quality and prolonging the products shelf life.[9] Synthetic antioxidants have been widely used to retard lipid oxidation in foods,[10] but nowadays there is growing interest in finding naturally occurring antioxidants[11] to be used as alimentary integrators. Plant extracts, which minimize or retard lipid oxidation in lipid-based food products and several natural molecules are continually proposed as food additives.[12]

In order to discover new antioxidant compounds, we undertook the phytochemical study of Mediterranean wild plants species.[13–15] Among them, *Teucrium polium* is a medicinal plant belonging to the Labiatae family. This plant has been reported to have hypolipidemic,[16] hypoglycaemic,[17] anti-nociceptive[18] and anti-inflammatory[19] effects, but few adverse effects of *T. polium* have been described indicating the relatively safe nature of this medicinal herb.[20]

From the ethyl acetate root extract we isolated 13 *abeo*-abietane diterpenes, including six new analogues. Antioxidant and radical scavenging properties of all the isolated diterpenes have been assessed in cell-free systems.

2. Results and discussion

Six new 17(15→16)-*abeo*-abietanes **1**–**6** (Fig. 1), along with seven known compounds **7**–**13** (Fig. 2) were isolated and characterized from *T. polium* EtOAc root extract.

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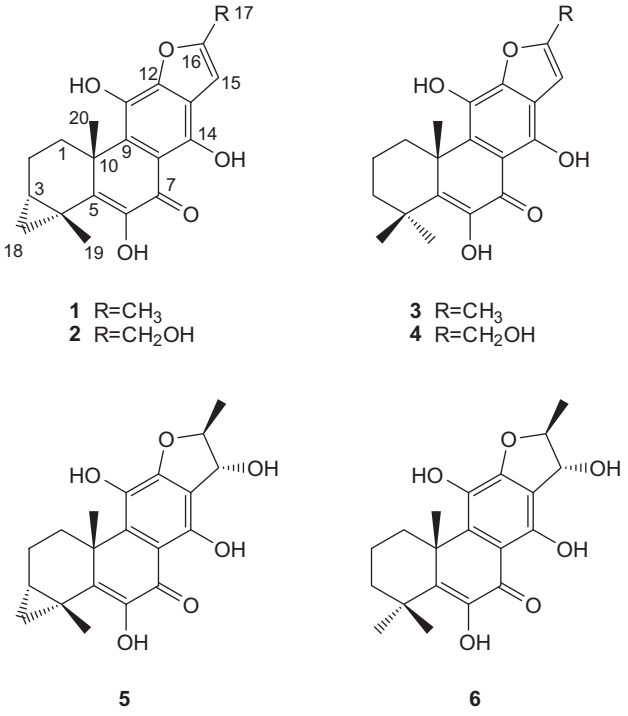

Figure 1. New *abeo*-Abietanes **1**–**6** from *T. polium*.

Compound **1** had a molecular formula $C_{20}H_{20}O_5$ as suggested by the 20 signals in ^{13}C NMR spectrum, by EIMS and elemental analysis data, according to the presence of 11 unsaturations in the molecule.

The absorption bands at 392, 330 and 270 nm, in the UV–vis spectrum, together with the presence of bands at 1734, 1581 and 907 cm^{-1} in the IR spectrum, suggested the presence of a phenolic aryl ketone in the molecule.[21] The ^1H NMR spectrum showed a singlet at δ 6.59 and three broad singlets at δ 5.10, 6.58 and 12.46 which disappeared in the ^1H NMR spectrum after addition of D_2O. In the upfield region of the spectrum, signals of a doublet methyl at δ 2.46, two singlet methyls at δ 1.45 and 1.69, as well as those of three diastereotopic methylene protons, at δ 2.88/0.96, 2.35/1.90, 0.87/0.48 and a multiplet methine a δ 1.01 were also present.

The 20 carbons, evidenced in the ^{13}C NMR spectrum, were identified, on the basis of a DEPT experiment, as 3 methyl, 3 methylene, 2 methine, and 12 tetrasubstituted carbons. The existence of an *abeo*-abietane skeleton with a 2-methylbenzo[*b*]furan moiety was established by one-bond and long range correlations evidenced in the 2D NMR experiments. The DQ-COSY and TOCSY experiments showed correlations of the following spin systems: both the methylene protons at δ 2.88/0.96 showed cross peaks with the methylene protons at δ 2.35/1.90, which correlated to the methine at δ 1.01. This latter was, in turn, correlated with a doublet of doublet at δ 0.87/0.48, whose values are characteristics of a cyclopropane ring formed by a bond between the C-3 carbon and the C-18 methyl.

The HSQC experiment allowed the assignment of each proton of the molecule to the corresponding carbon, while the HMBC experiment allowed us to assemble the partial structures as follows. The doublet methyl at δ 2.46, which, in the HSQC experiment, correlated with the carbon at δ 14.0, showed cross peaks with the carbons at δ 155.0 and 117.0, and with the signal at δ 101.4, correlated with the aromatic proton at δ 6.59. This proton, in turn, showed cross peaks with C-12, C-13, and C-16 carbons at δ 148.8, 117.0, and 155.0, respectively. The carbon at δ 148.8 also showed correlations with the phenolic hydroxyl proton at δ 5.10, assigned to the hydroxyl group C-11, which correlated with the C-11 and C-9 tetrasubstituted carbons at δ 131.7 and 129.6, respectively. Furthermore, this latter carbon showed a cross peak with the H-20 methyl protons at δ 1.69 which, in turn, correlated with the tetrasubstituted C-5 and C-10 carbons at δ 142.2 and 39.3 and with the signal at δ 26.4 attributed to the C-1 methylene carbon. Finally, the proton at δ 2.88, bonded to this latter carbon, correlated with the signals at δ 18.1 and 20.2 assigned to the C-3 and C-2,

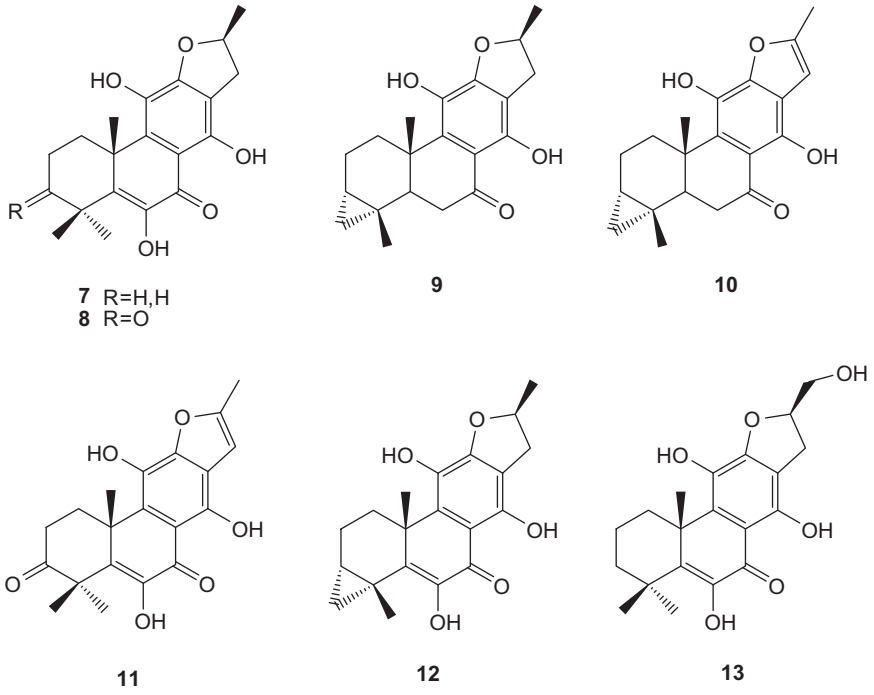

Figure 2. *abeo*-Abietanes **7**–**13** from *T. polium*.

respectively. These data were in good accordance with the structure of 12,16-epoxy-6,11,14-trihydroxy-17(15→16)-*abeo*-3α,18-*cyclo*-5,8,11,13,15-abietapentaen-7-one.

Compound **2** showed a molecular formula $C_{20}H_{20}O_6$, as deduced from spectroscopic and elemental analysis data. The spectral data were very similar to those of the previous metabolite, indicating the presence of a cyclopropane ring formed by C-3, C-4, and C-18 carbons, a phenolic system (C-ring) conjugated with the diosphenol moiety in the B ring and a trisubstituted furane moiety. The main difference was the lack of the C-17 methyl signals, in the NMR spectra, and the presence of a methylene resonating at δ 4.77 and 58.0 in the ^{1}H and ^{13}C NMR spectra, respectively. The correlations, in the HMBC experiment, between these protons and the C-15 and C-16 carbons at δ 103.6 and 158.4 agreed with the structure of 12,16-epoxy-6,11,14,17-tetrahydroxy-17(15→16)-*abeo*-3α,18-*cyclo*-5,8,11,13,15-abietapentaen-7-one.

The mass and NMR spectra of new metabolite **3** indicated a molecular formula $C_{20}H_{22}O_5$, in accordance with the presence of 10 unsaturations in the molecule. The ^{1}H NMR showed the signal of the furan ring as a singlet at δ 6.58. In the upfield region of the same spectrum, signals of a doublet methyl at δ 2.46 and three singlet methyls at δ 1.46, 1.47 and 1.70 as well as those of three diastereotopic methylenes at δ 3.16/1.75, 1.69/1.90, 1.42/2.05 were evident.

The DQ-COSY and TOCSY experiments showed correlations of the following spin systems: both the methylene protons at δ 1.75/3.16 showed cross peaks with the methylene protons at δ 1.69/1.90, which were both correlated to the protons at δ 1.42/2.05. The ^{13}C NMR experiment revealed 20 carbons identified, on the basis of a DEPT experiment, as 4 methyl, 3 methylene, 1 methine, and 12 tetrasubstituted carbons. The HMBC heterocorrelations and NOE effects allowed us to identify this compound as 12,16-epoxy-6,11,14-trihydroxy-17(15→16)-*abeo*-5,8,11,13,15-abietapentaen-7-one.

The molecular formula of metabolite **4** was $C_{20}H_{22}O_6$. Its spectral data were very similar to those of compound **3**. The differences, evidenced in the ^{1}H and ^{13}C NMR spectra, indicated the presence of a hydroxyl at the C-17 carbon. 2D NMR data confirmed the structure of 12,16-epoxy-6,11,14,17-tetrahydroxy-17(15→16)-*abeo*-5,8,11,13,15-abietapentaen-7-one.

Compound **5** showed a molecular formula $C_{20}H_{22}O_6$ according to the EIMS data and elemental analysis. Its ^{1}H NMR spectrum showed the C-6 diosphenol proton at δ 6.52, two further oxygen-bonded protons at δ 12.40 (OH-14) and 5.12 (OH-11), two carbinol methine protons as a doublet at δ 5.27 and a double quartet at δ 4.89, two singlet methyls at δ 1.64 and 1.43, and a doublet methyl at δ 1.46. The COSY and TOCSY experiments allowed the identification of a spin system constituted by a methine at δ 1.00 and three diastereotopic methylenes at δ 0.94/2.79, 1.89/2.32, and 0.46/0.86. This latter, bonded to the carbon at δ 17.7, agreed with the H-18 proton of a cyclopropane ring. In fact, in the HMBC experiment (Fig. 3) the carbon at δ 17.7 correlated with the methyl at δ 1.43 (H-19) and the H-3 methine at δ 1.00. The 2D homocorrelation experiments evidenced a second spin system constituted by the doublet methyl and both the carbinol protons. The ^{13}C NMR and DEPT experiments confirmed the reported observations indicating the presence of two carbinol carbons at δ 90.1 and 76.5. The first carbon, bonded to the proton at δ 4.89, correlated with the methyl and with the proton at δ 5.27, which correlated with the carbon at δ 76.5 in the HSQC experiment.

These data suggested the presence of an *abeo*-abietane with a cyclopropane formed by the C-3, C-4 and C-18 carbons, and a further hydroxyl at the C-15 carbon. As the absolute configuration to the C-16 carbon in *abeo*-abietanes reported from *Teucrium* spp. has been established as S,[21] the C-15 absolute configuration has been assigned, in respect to this latter carbon, on the basis of a NOESY experiment: the β-oriented H-17 methyl showed NOE interactions with both the C-15 and C-16 carbinol methines indicating its *trans* orientation respect to the hydroxyl group. This observation was in accordance with the *R* configuration for the C-15 carbon.

NMR data of compound **6** were very similar to those of the previous metabolite. In fact, it showed the H-15 doublet at δ 5.21 and the H-16 double quartet at δ 4.88, indicating the presence of a hydroxyl group at the C-15 carbon. The differences with compound **5** were the lack of H-18 methylene of the cyclopropane moiety and the presence of a further singlet methyl (H-18) at δ 1.45. The homo- and heterocorrelations arisen by the 2D NMR experiments confirmed the hypothesized structure and the NOE effects, evidenced in the NOESY experiment, indicated a *R* configuration for the C-15 carbon.

Physical data of the known metabolites **7**–**12** were identical to literature values; compounds **7**–**9** and **12**, named, respectively teuvincenones B, A, D and C, have been previously isolated from root of *T. polium* L. subsp. *vincentium* (Rouy) D. Wood.[22] The abietane **10** was identified from acetone extract of the roots of *Teucrium lanigerum*,[23] while compound **9** was reported as constituent of *Teucrium* species.[24] Finally, compound **13**, the last abietane isolated from *T. polium*, was identified as villosin C, already reported from the plant *Teucrium divaricatum* subsp. *villosum*.[25]

abeo-Abietanes from *T. polium* were investigated for their radical scavenging and antioxidant capacities using four different methods. Each compound was tested at four concentrations (37.5 μM, 75 μM, 125 μM, and 250 μM) in triplicate analysis (n = 3). Detected

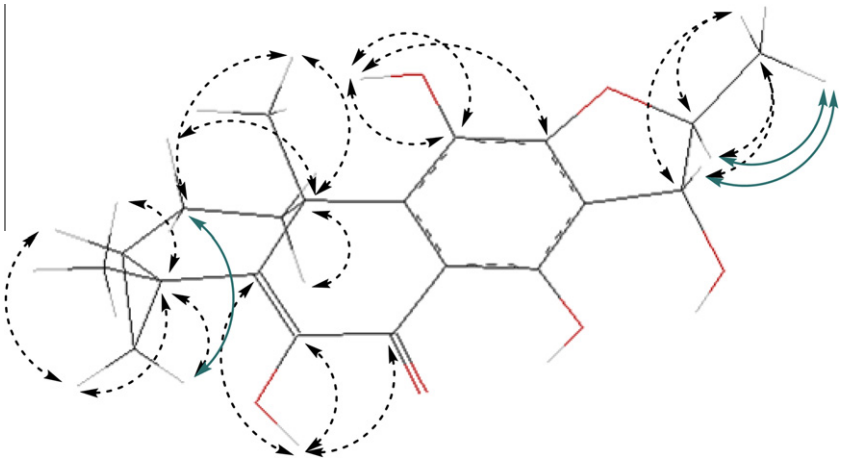

Figure 3. Selected HMBC (dashed black arrows) and NOESY (plain gray arrows) correlations of the new *abeo*-abietane **5**.

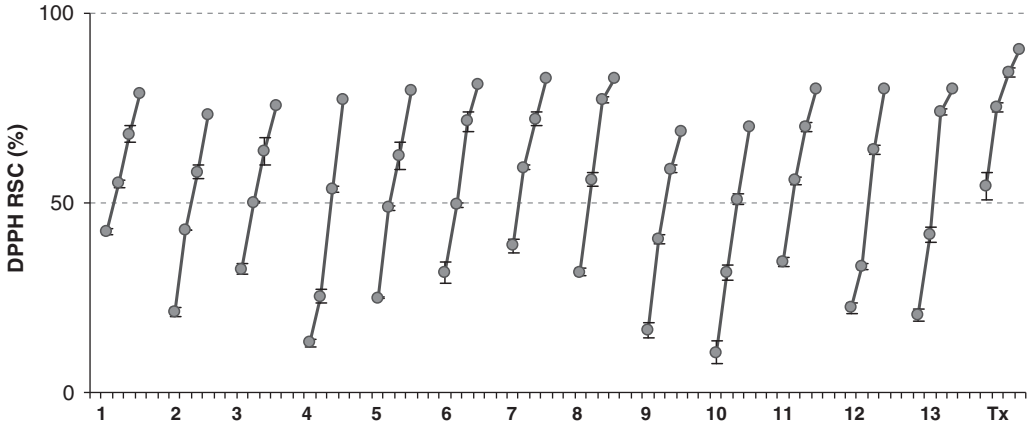

Figure 4. DPPH radical scavenging capacity (RSC, %) of *T. polium* metabolites **1**–**13**. Values are reported as percentage versus a blank ± SD.

activities were compared to those exercised from Trolox®, a vitamin E water-soluble derivative.

The antiradical efficacy of pure isolated compounds was tested by the DPPH assay. The change in coloration from violet to yellow and subsequent fall in absorbance of the stable radical DPPH· was measured at 515 nm, and the results were plotted (Fig. 4). The IC_{50} value for each compound, defined as the concentration of metabolite causing 50% inhibition of absorbance, was determined from the curves plotted and tabulated (Table 3). Since IC_{50} is a measure of inhibitory concentration, a lower IC_{50} value would reflect greater antioxidant activity of the sample.

Considering the graph of the radical scavenging capacity versus tested concentrations, metabolites seem to exert a similar antiradical behaviour; the different response of the molecules is highlighted by their IC_{50} values that emphasize how structural variants of the basic molecular skeleton can modulate the activity. Comparing the antiradical effects of metabolites **1**–**2** and **3**–**4**, the weak decrease of the activity in metabolites **2** and **4** could be ascribed to the presence of a hydroxymethyl function at C-16 carbon. This is probably due to the capacity of the primary hydroxyl to form a hydrogen bond with the oxygen of the 2,3-dihydrofuranic unit. Analogously metabolite **13** did not show a remarkable activity although it presents a further hydroxyl function on tetrahydrofuranic ring. The loss of the α-hydroxy dienonic function in metabolites **9** and **10** defined a decrease of the scavenging efficacy. The metabolites exerted a moderate antiradical effect with IC_{50} values equal to 111 μM and 130 μM, respectively.

Antioxidant properties of all pure metabolites were examined through analysis of the thiobarbituric acid reactive species (TBARS) synthesis under oxidative stress conditions, using as oxidisable substrate rape seed oil (RSO) and 2-deoxyribose (2-DR), and by determination of protein carbonyl groups. Results are reported in Figure 5. As regards the response to TBARS test, all compounds showed a significant and dose–response efficacy although weaker than that exercised by Trolox®. Peculiar antilipoperoxidative activity was exercised by compound **1** which was able to reduce the synthesis of lipoperoxidation secondary products by 76.9% at the highest tested concentration. The same molecule also exerted a good hydroxyl radicals scavenging capability. The inhibiting effects of the pentose 2-deoxyribose oxidation were unclear for the other investigated substances. It is noteworthy that except for the metabolites **1**–**3**, which achieved an inhibition of the oxidative process by approximately 50% at the highest tested concentration, the increase in dose reflected a slight increase in response. When the free radical-mediated oxidation of protein was tested, a similar effect was recorded. The presence of the *abeo*-abietanes ineffectively inhibited the synthesis of protein carbonyl group in a concentration-dependent manner. Again the most active compound was *abeo*-abietane **1** capable of inhibiting protein oxidation by 31% already at the lowest concentration.

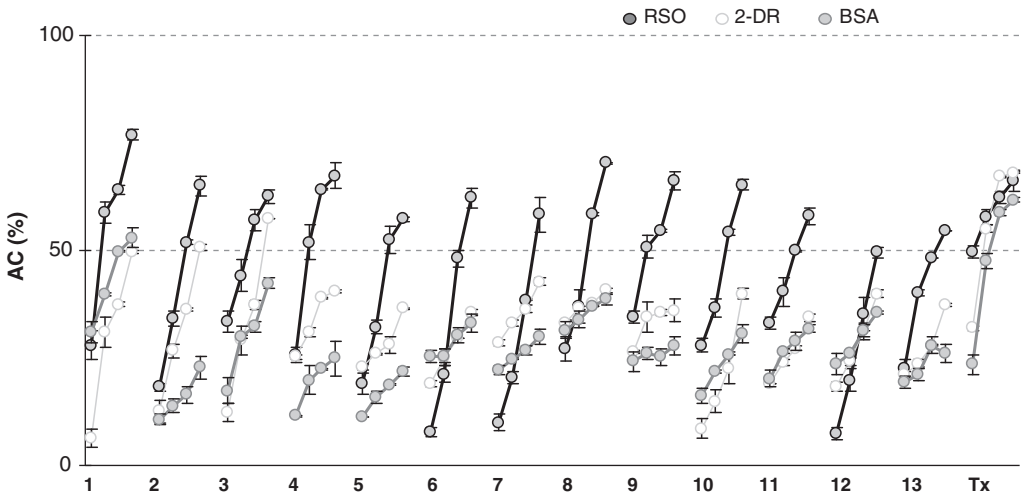

Figure 5. Antioxidant capability of *T. polium* metabolites **1**–**13** on (A) rape seed oil (RSO), (B) 2-deoxyribose (2-DR), and (C) bovine serum albumin (BSA). Values are reported as percentage versus a blank ± SD.

Although there are no reports on the antioxidant activity of *abeo*-abietanes, several abietanes have been screened for their antioxidative capacity. Carnosic acid and carnosol were reported to be responsible for 90% of antioxidant activity of rosemary.[26] Abietanes from *Salvia barrelieri* were indicated as promising antioxidants which could be used as food additives.[27] When Wang et al.[28] evaluated the antioxidant activity of the specific diterpenes from *Taiwania cryptomerioides* heartwood, employing the DPPH radical scavenging activity assay, observed that the presence of the phenol structure in the abietane-type diterpene is essential to its radical scavenging activity. Furthermore, as an electron-withdrawing group nears the aromatic ring, the observed antioxidant activity decreased.

In conclusion, in this paper we report the isolation and structural elucidation of six new 17(15→16)-*abeo*-abietanes, along with seven known compounds, from *T. polium* roots. Their structures were elucidated by using 1D and 2D NMR experiments and mass spectral data. The isolated metabolites were screened for their antioxidant capacity in cell-free system showing a significant and dose–response efficacy although the detected activity was not very pronounced. Further studies will be addressed to the evaluation of cytotoxic properties of these metabolites. It is known that molecules having similar hydroquinoid substructures showed a strong antiproliferative capability due to their capacity to be oxidized in quinoid derivatives.[29] Slameňová et al. investigating the cytotoxic effects of several cyclic diterpenoid quinones observed that all diterpenoid quinones tested acted cytotoxically and damaged DNA in human colon and hepatoma cells.[30]

3. Experimental

3.1. General experiment procedures

NMR spectra were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C on a Varian 300 spectrometer Fourier transform NMR in $CDCl_3$ at 25 °C. The spectrum width was 2300 Hz. The initial matrix of 2 k × 2 k data points was zero-filled to give a final matrix of 4 k × 4 k points. The TOCSY experiments were performed in the phase-sensitive mode with a mixing time of 90 ms. The spectral width was 3000 Hz. The NOESY experiments were performed in the phase-sensitive mode. The mixing time was 500 ms and the spectral width was 3000 Hz. For both the homonuclear experiments, the initial matrix of 512 × 512 data points was zero-filled to give a final matrix of 1 k × 1 k points. Proton-detected heteronuclear correlations were measured using a gradient heteronuclear single-quantum coherence (HSQC), optimised for $^1J_{HC}$ = 140 Hz, a gradient heteronuclear multiple bond coherence (HMBC), optimised for $^nJ_{HC}$ = 8 Hz. The HSQC experiment was performed in the phase-sensitive mode with field gradient. The spectral width was 22,000 Hz in f_1 (^{13}C) and 3200 Hz in f_2 (^{1}H) and the matrix of 1 k × 1 k data points was zero-filled to give a final matrix of 2 k × 2 k points. The HMBC experiment was performed in the absolute value mode with field gradient. The spectral width was 20,000 Hz in f_1 (^{13}C) and 1100 Hz in f_2 (^{1}H) and the matrix of 1 k × 1 k data points was zero-filled to give a final matrix of 4 k × 4 k points. UV spectra were obtained on UV-1700 Shimadzu spectrophotometer in MeOH solution. Optical rotations were measured on a Perkin–Elmer 141 (Perkin–Elmer Co., Norwalk, CT) in MeOH solution. IR spectra were determined in the KBr pellet using a FT-IR Perkin–Elmer 'Spectrum GX' spectrometer.

The preparative HPLC apparatus consisted of Knauer Smartline 31/40 module equipped with Knauer Smartline 1000 pump, UV Knauer Smartline 2500 detector and RI Knauer Smartline 2300 detector and PC CromGate® *software*. Preparative HPLC was performed using Luna RP-8 (10 μm, 250 × 10.0 mm i.d., Phenomenex). Analytical TLC was performed on Merck Kieselgel 60 F_{254} or RP-8 F_{254} plates with 0.2 mm layer thickness. Spots were visualized by UV light or by spraying with H_2SO_4/AcOH/H_2O (1:20:4). The plates were then heated for 5 min at 110 °C. Preparative TLC was performed on Merck Kieselgel 60 F_{254} plates, with 0.5 or 1.0 mm film thickness. Column chromatography (CC) was performed on Merck Kieselgel 60 (70–240 mesh), and Merck Kieselgel 60 (40–63 μm).

Electronic ionization mass spectra (EIMS) were obtained with a HP 6890 instrument equipped with a MS 5973 N detector.

4. Plant material

Plants of *T. polium* L. were collected at vegetative state, in July 2006 at 'Castel Volturno' Nature Reserve, a flat coastal area in the north of Naples (Southern Italy) and identified by Dr. Assunta Esposito of the Second University of Naples. A voucher specimen (CE034) has been deposited with the Herbarium of the Dipartimento di Scienze della Vita of Second University of Naples.

5. Extraction and isolation

Roots of *T. polium* were dried in a ventilated thermostat at 45 °C for 48 h obtaining a root dried material (761.3 g) which was powdered and extracted by maceration in ethyl acetate (EtOAc, 7.0 L) at 4 °C in a dark refrigerated room for five days. After the removal of the solvent by a rotary evaporator, we obtained a crude extract (63.0 g) which was stored at −80 °C until purification.

The organic extract was chromatographed on SiO_2 to obtain five fractions A–E of 600 mL volume each.

Fraction A, eluted with PE/$CHCl_3$ (3:2), was chromatographed on Sephadex LH-20, eluting with hexane/$CHCl_3$/MeOH (4:1:1) collecting fractions of 10 mL; fraction 25–28 contained pure compound **9** (7.0 mg), fraction 32–36, chromatographed by SiO_2 TLC (0.5 mm) eluting with hexane/CH_2Cl_2 (3:7), gave two spots. The first spot was identified as compound **10** (3.0 mg), the second one as **7** (8.0 mg). Fraction 40–47, purified by SiO_2 TLC (1.0 mm) using as eluent hexane/CH_2Cl_2 (3:7), furnished pure compound **3** (9.0 mg), finally fraction 53–63 was rechromatographed by SiO_2 TLC (1.0 mm) eluting with hexane/EtOAc (4:1) and gave pure metabolites **11** (6.0 mg) and **1** (5.0 mg).

Fraction B, eluted with PE/$CHCl_3$ (11:9), was chromatographed on Sephadex LH-20, eluting with hexane/$CHCl_3$/MeOH (4:1:1) collecting fractions of 10 mL. Fraction 30–32 was rechromatographed by SiO_2 TLC (1.0 mm), eluting with hexane/CH_2Cl_2 (3:7), to give pure metabolite **12** (6.0 mg). Fraction 33–36, chromatographed by SiO_2 TLC (1.0 mm) eluting with hexane/CH_2Cl_2 (3:7), gave a pure metabolite which was identified as **7** (11.0 mg). Finally, fraction 38–42, purified by SiO_2 TLC (1.0 mm) with hexane/EtOAc (4:1), furnished compound **1** (3.0 mg).

Fraction C, eluted with PE/$CHCl_3$ (1:1), was chromatographed on Sephadex LH-20, eluting with hexane/$CHCl_3$/MeOH (4:1:1) collecting fractions of 10 mL. Fraction 33–40 was chromatographed by SiO_2 TLC (1.0 mm), eluting with hexane/EtOAc (4:1), to give pure **8** (15.0 mg).

Fraction D, eluted with $CHCl_3$, was chromatographed on Sephadex LH-20, eluting with hexane/$CHCl_3$/MeOH (3:1:1) and collecting fractions of 10 mL. Fraction 36–47, rechromatographed by RP-8 HPLC eluting with MeCN/MeOH/H_2O (1:6:3), gave pure compounds **5** (7.0 mg), and **6** (3.0 mg).

Fraction E, eluted with EtOAc, was chromatographed on Sephadex LH-20, eluting with hexane/$CHCl_3$/MeOH (3:1:1) and collecting fractions of 10 mL. Fraction 41–47, rechromatographed by SiO_2 TLC (0.5 mm) eluting with hexane/$CHCl_3$/*i*PrOH (2:7:1), gave pure compound **13** (8.0 mg); fraction 58–64, purified by SiO_2 preparative TLC using as eluent $CHCl_3$/MeOH (10:1), furnished pure metabolite

Table 1
Selected ^{1}H NMR data of the new *abeo*-abietanes **1**-**6** from *T. polium* in CD_3OD

Position	1	2	3	4	5	6
H-1$_\alpha$	0.96 td (13.5, 13.5, 5.4)	0.96 td (14.1, 13.5, 5.4)	1.75 dd (13.5, 8.4)	1.68 ob	0.94 dt (14.1, 13.5, 5.4)	1.69 ob
H-1$_\beta$	2.88 ddd (13.5, 6.0, 1.8)	2.88 ddd (14.1, 6.6, 1.7)	3.16 ddd (13.5, 6.0, 1.5)	3.16 ddd (13.8, 5.7, 1.7)	2.79 ddd (13.5, 6.3, 1.7)	3.10 m
H-2$_\alpha$	1.90 dd (13.8, 5.4)	1.91 dd (14.1, 4.8)	1.69 ob	1.64 ob	1.89 dd (14.1, 5.4)	1.41 ob
H-2$_\beta$	2.35 tt (13.8, 13.5, 6.0)	2.36 tt (14.1, 13.5, 6.6)	1.90 m	1.92 m	2.32 tt (14.1, 13.5, 6.3)	2.02 m
H-3$_\alpha$	—	—	1.42 ov	1.43 ob	—	1.60 ob
H-3$_\beta$	1.01 m	1.06 m	2.05 dt (13.2, 5.4)	2.02 dd (12.0, 5.1)	1.00 m	1.87 m
H-15	6.59 s	6.89 s	6.58 s	6.88 s	5.27 d (2.4)	5.21 d (2.4)
H-16	—	—	—	—	4.89 dq (6.9, 2.4)	4.88 dq (6.3, 2.4)
H-17	2.46 d (0.9)	4.77 s	2.46 d (0.9)	4.78 s	1.46 d (6.9)	1.46 d (6.3)
H-18$_{endo}$	0.48 dd (5.4, 4.8)	0.49 dd (5.4, 4.5)	—	—	0.46 dd (5.1, 4.9)	—
H-18$_{exo}$	0.87 dd (8.7, 4.8)	0.87 dd (7.2, 4.5)			0.86 dd (8.4, 5.1)	
18 Me	—	—	1.47 s	1.47 s	—	1.45 s
19 Me	1.45 s	1.46 s	1.46 s	1.46 s	1.43 s	1.43 s
20 Me	1.69 s	1.70 s	1.70 s	1.71 s	1.64 s	1.66 s
H–O(6)	6.58 s	6.58 s	6.57 s	6.95 s	6.52 s	6.89 s
H–O(11)	5.10 s	5.30 s	5.28 s	5.25 s	5.12 s	5.14 s
H–O(14)	12.46 s	12.44 s	12.42 s	12.40 s	12.40 s	12.38 s

d = Doublet; dd = doublet of doublets; ddd = doublet of doublet of doublets; dq = doublet of quartets; m = multiplet; ob = obscured; ov = overlapped; s = singlet; t = triplet; td = triplet of doublets; tt = triplet of triplets. The *J* values, in the brackets, are reported in Hertz.

4 (8.3 mg); finally, fraction 65–72 was rechromatographed by SiO_2 TLC (0.5 mm) eluting with hexane/$CHCl_3$/*i*PrOH (2:7:1), to have pure metabolite **2** (5.0 mg).

12,16-Epoxy-6,11,14-trihydroxy-17(15→16)-*abeo*-3α,18-*cyclo*-5,8,11,13,15-abietapentaen-7-one (**1**). Colorless oil. $[\alpha]_D^{25} = -14.4$ (c = 0.21, MeOH). UV (MeOH) λ_{max} (log ε): 392 (3.51), 330 (3.61), 270 (3.97). IR ν_{max} cm^{-1} 3573, 2928, 2856, 1734, 1581, 1346, 998, 907. ^{1}H NMR ($CDCl_3$, 300 MHz) data, see Table 1. ^{13}C NMR ($CDCl_3$, 75 MHz) data, see Table 2. EIMS *m/z*: 340 $[M]^+$, 325 $[M-CH_3]^+$. Anal. Calcd for $C_{20}H_{20}O_5$: C, 70.57; H, 5.92. Found: C, 70.53; H, 5.95.

12,16-Epoxy-6,11,14,17-tetrahydroxy-17(15→16)-*abeo*-3α,18-*cyclo*-5,8,11,13,15-abietapentaen-7-one (**2**). Colorless oil. $[\alpha]_D^{25} = -7.8$ (c = 0.18, MeOH). UV (MeOH) λ_{max} (log ε): 388 (3.17), 326 (3.36), 270 (3.71). IR ν_{max} cm^{-1} 3570, 2922, 2860, 1575, 1340, 990. ^{1}H NMR ($CDCl_3$, 300 MHz) data, see Table 1. ^{13}C NMR ($CDCl_3$, 75 MHz) data, see Table 2. EIMS *m/z*: 356 $[M]^+$, 341 $[M-CH_3]^+$. Anal. Calcd for $C_{20}H_{20}O_6$: C, 67.41; H, 5.66. Found: C, 67.44; H, 5.70.

12,16-Epoxy-6,11,14-trihydroxy-17(15→16)-*abeo*-5,8,11,13,15-abietapentaen-7-one (**3**). Colorless oil. $[\alpha]_D^{25} = +13.1$ (c = 0.29, MeOH). UV (MeOH) λ_{max} (log ε): 394 (3.49), 330 (3.60), 272 (3.90).

IR ν_{max} cm^{-1} 3574, 2927, 1734, 1641, 1569, 1472, 1344, 986, 907. ^{1}H NMR ($CDCl_3$, 300 MHz) data, see Table 1. ^{13}C NMR ($CDCl_3$, 75 MHz) data, see Table 2. EIMS *m/z*: 342 $[M]^+$, 327 $[M-CH_3]^+$. Anal. Calcd for $C_{20}H_{22}O_5$: C, 70.16; H, 6.48. Found: C, 70.12; H, 6.45.

12,16-Epoxy-6,11,14,17-tetrahydroxy-17(15→16)-*abeo*-5,8,11,13,15-abietapentaen-7-one (**4**). Colorless oil. $[\alpha]_D^{25} = +3.6$ (c = 0.22, MeOH). UV (MeOH) λ_{max} (log ε): 390 (2.51), 328 (2.58), 270 (3.01). IR ν_{max} cm^{-1} 3392, 2935, 1704, 1515, 874. ^{1}H NMR ($CDCl_3$, 300 MHz) data, see Table 1. ^{13}C NMR ($CDCl_3$, 75 MHz) data,

Table 2
Selected ^{13}C NMR data of the new *abeo*-abietanes **1**-**6** from *T. polium* in CD_3OD

Position	1	2	3	4	5	6
1	26.4	26.7	30.4	30.3	25.9	29.7
2	20.2	20.5	17.7	17.7	20.1	17.6
3	18.1	18.0	36.3	36.2	18.0	36.2
4	17.6	17.7	36.6	36.7	17.9	36.6
5	142.2	143.1	145.2	145.6	142.1	144.6
6	142.2	142.8	141.6	141.6	141.8	141.5
7	184.1	184.3	184.1	184.1	182.9	182.9
8	108.1	108.7	107.0	107.4	108.0	106.9
9	129.6	131.0	132.8	131.2	131.7	132.0
10	39.3	40.0	41.7	42.0	39.7	42.0
11	131.7	131.2	130.9	135.8	138.4	138.2
12	148.8	149.4	149.2	149.5	154.6	154.6
13	117.0	116.4	116.6	115.7	112.7	112.4
14	150.9	150.7	150.8	151.6	154.2	154.4
15	101.4	103.6	101.4	103.4	76.5	76.6
16	155.0	158.4	155.0	158.4	90.1	90.2
17	14.0	58.0	13.9	57.8	19.2	19.2
18	18.0	18.6	27.1	27.0	17.7	27.2
19	23.2	23.4	28.0	28.0	23.1	27.9
20	19.4	19.6	27.9	27.8	18.8	27.0

Table 3
DPPH radical scavenging capacity of *T. polium* metabolites **1**–**13**

Compound	IC_{50}
1	55
2	101
3	76
4	120
5	84
6	71
7	55
8	63
9	111
10	130
11	64
12	97
13	86
Trolox®	24

Values are reported as IC_{50} (μM).

see Table 2. EIMS *m/z*: 358 $[M]^+$, 343 $[M-CH_3]^+$. Anal. Calcd for $C_{20}H_{22}O_6$: C, 67.03; H, 6.19. Found: C, 67.0; H, 6.16.

12,16-Epoxy-6,11,14,15-tetrahydroxy-17(15→16)-*abeo*-3α,18-*cyclo*-5,8,11,13-abietatetraen-7-one (**5**). Colorless oil. $[\alpha]_D^{25} = -7.5$ ($c = 0.20$, MeOH). UV (MeOH) λ_{max} (log ε): 388 (3.73), 323 (3.53), 260 (3.90). IR ν_{max} cm^{-1} 3440, 3388, 2987, 2929, 1722, 1575. ^1H NMR ($CDCl_3$, 300 MHz) data, see Table 1. ^{13}C NMR ($CDCl_3$, 75 MHz) data, see Table 2. EIMS *m/z*: 358 $[M]^+$, 343 $[M-CH_3]^+$, 340 $[M-H_2O]^+$. Anal. Calcd for $C_{20}H_{22}O_6$: C, 67.03; H, 6.19. Found: C, 67.10; H, 6.22.

12,16-Epoxy-6,11,14,15-tetrahydroxy-17(15→16)-*abeo*-5,8,11,13-abietatetraen-7-one (**6**). Colorless oil. $[\alpha]_D^{25} = -1.97$ ($c = 0.30$, MeOH). UV (MeOH) λ_{max} (log ε): 382 (3.66), 325 (3.44), 267 (3.92). IR ν_{max} cm^{-1} 3690, 3414, 2929, 1684, 1569, 984. ^1H NMR ($CDCl_3$, 300 MHz) data, see Table 1. ^{13}C NMR ($CDCl_3$, 75 MHz) data, see Table 2. EIMS *m/z*: 360 $[M]^+$, 345 $[M-CH_3]^+$, 342 $[M-H_2O]^+$. Anal. Calcd for $C_{20}H_{24}O_6$: C, 66.65; H, 6.71. Found: C, 66.60; H, 6.74.

6. Antioxidant capability determination

6.1. DPPH radical scavenging capacity

The DPPH radical scavenging capacity of metabolites was measured according to the method of Brand-Williams et al.[31] Methanolic DPPH· solution (9.4×10^{-5} M; 1.5 mL) was added at room temperature to rates of pure isolated metabolites in order to obtain samples with final concentration equal to 37.5, 75.0, 125.0, 250.0 μM. The absorbance at 515 nm was measured at 30 min against a blank using a UV-1700 Shimadzu spectrophotometer. Analyses were carried out in triplicate ($n = 3$). Results are expressed in terms of the percentage reduction of the initial DPPH radical adsorption by test samples. The DPPH radical scavenging activity was compared to that exercised by Trolox® used as standard. The IC_{50} value was also estimated.

6.2. Determination of TBARS

Rape seed oil (62.0 μL) was emulsified with 188.0 mg of Tween-40 initially dissolved in 24.0 mL of Tris–HCl buffer (0.2 M, pH 7.4). The emulsion (0.5 mL) was added to rates of pure isolated metabolites in order to obtain samples with final concentration equal to 37.5, 75.0, 125.0, 250.0 μM. Samples were irradiated with UV light at 254 nm for 60 min. TBA–TCA reagent (2.0 mL) was added to test tubes. The TBA–TCA reagent was prepared mixing TBA and TCA solutions. TBA solution was prepared dissolving 375.0 mg of thiobarbituric acid and 30.0 mg of tannic acid in 30.0 mL of hot water. TCA solution was prepared dissolving 15.0 g of trichloroacetic acid in 68.0 mL of hydrogen chloride aqueous solution (70.0 mL, 2 N). Test tubes were placed into a boiling water bath for 30 min. The absorbance of samples was measured at 532 nm by Shimadzu UV-1700 spectrophotometer. Lipid peroxidation inhibition was measured as a percentage against a blank containing no test compounds.

6.3. Determination of scavenging effects on hydroxyl radicals

Scavenging effects of isolated *abeo*-abietanes on hydroxyl radicals were performed, as described by Halliwell and Gutteridge[32] 1.0 mL of KH_2PO_4/KOH (10.0 mM, pH 7.4) containing H_2O_2 (100.0 μM), EDTA (100.0 μM) and $FeCl_3$ (100.0 μM), 2-deoxyribose (2.8 mM) and ascorbic acid (100.0 μM) was added to rates of test compounds in order to obtain samples with final concentration equal to 37.5, 75.0, 125.0, 250.0 μM. Mixtures were incubated at 37 °C for 1 h. The extent of 2-deoxyribose degradation was tested by using the TBA method. TBA (1.0 mL; 1.0%, w/v in NaOH 50 mM) and TCA (1.0 mL; 2.8%, w/v) were added to the mixture, which was heated in a water bath at 90 °C for 30 min. The absorbance of the mixture was read spectrophotometrically at 532 nm. Analyses were carried out in triplicate ($n = 3$). Detected activities are reported as antioxidant capacity percentage versus blank.

6.4. Determination of effects on protein oxidation

The effects of metabolites on protein oxidation were determined according to the method reported by Suji and Sivakami.[33] Samples were added to 0.2 mL of a solution containing H_2O_2 (1.0 mM), $FeSO_4$ (0.5 mM), and bovine serum albumin (10 mg/mL). After shaking (10 min), the protein was precipitated at 4 °C with TCA (10.0% w/v). After centrifugation at 11,000*g* for 3 min, the pellets are treated with DNPH (10 mM, 0.5 mL) in HCl 2 N. The reaction was followed by stirring for 1 h at room temperature. Thus to each sample 0.5 mL of cold TCA (20.0%, w/v) was added. The samples were left on ice for 15 min and then centrifuged at 11,000*g* for 3 min. The protein precipitate was first washed three times with 1.0 mL EtOAc/EtOH (1:1, v/v) and then dissolved in 1.5 mL of 6 M guanidine–HCl (pH 6.5) for 3 h. The absorbance of the sample was read at 370 nm. The analyses were carried out in triplicate ($n = 3$). Detected activities are reported as antioxidant capacity percentage versus blank.

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