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An analytical and theoretical approach for the profiling of the antioxidant activity of essential oils: The case of *Rosmarinus officinalis* L.

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ABSTRACT

The antioxidant constituents of essential oils (EOs) of *Rosmarinus officinalis* L. (α-pinene chemotype) were isolated at the flowering (A), post-flowering (B), and vegetative stages (C). GC–MS was used to analyze total chemical composition, Folin–Ciocalteau and Prussian blue methods for reducing substances. Radical scavenging capacity (DPPH test, IC_{50} 36.78 ± 0.38, 79.69 ± 1.54, 111.94 ± 2.56 μL) and anti-lipoperoxidant activity (TBARS, IC_{50} 0.42 ± 0.04, 1.20 ± 0.06 μL, 4.07 ± 0.05 μL) differed widely in the three stages. Antioxidant activity, identified after silica gel fractionation chromatography, was closely related (R^2 = 0.9959) to each EO's content of hydroxilated derivatives, (containing alcohols, phenols and 1,8 cineole): 15.26 ± 0.12%, 7.22 ± 0.06%, and 5.09 ± 0.10% in EOs from A, B, and C. Modeling the C, H, O terpenes in a simulated phospholipid bilayer indicated that anti-lipoperoxidant activity depended on the stability and rapidity of their interactions with the membrane bilayer components, and their positioning over its surface.

1. Introduction

In recent years, demand for essential oils (EOs) from medicinal plants has increased, particular for the oil from rosemary (*Rosmarinus officinalis* L.), on account of its widespread use as a natural food additive (listed by the FDA at the link http://www.cfsan.fda.gov/~dms/eafus.html), for food preservation thanks to its antimicrobial, antiviral, antimycotic [1], and antioxidant properties and, above all, its low cost and ease of availability.

Rosemary is an evergreen shrub that grows spontaneously in Mediterranean regions; its essential oil (EO) is usually extracted by the easy-to-handle, cheap, steam distillation procedure, which gives high yields of a product of appreciable quality on the basis of sensory (olfactory) evaluation, with remarkable functional properties – a safe alternative to synthetic antioxidants, which are suspected to have or to promote negative health effects.

Studies of the composition of EOs from different chemotypes of rosemary (α-pinene, 1,8 cineol, camphor, verbenone chemotypes) have focused on the analytical characterization of phytochemical constituents (non-oxygenated monoterpene and sesquiterpene hydrocarbons, oxygenated monoterpenes and sesquiterpenes, phenolic derivatives, and non-isoprenoidic components including volatile alcohols, aldehydes, and ketones [2,3]. However, only limited efforts have been made to characterize the profile of specific antioxidants in the EOs, and their activity, and particularly how their profile changes during the different stages of development of the shrub. In addition, findings on the antioxidant activity of rosemary EO are sometimes contradictory, and not very reliable because of the wide range of antioxidant assays used, based on different methodological approaches, and without following any rigorously standardized protocol.

These weaknesses might explain the contradictory reports on the antioxidant potency of the EO from *R. officinalis*, which complicates any attempt at direct comparison of the antioxidant activity tested by different methods, using different substrates.

With the aim of unequivocally establishing the antioxidant efficiency of the EO from *R. officinalis* of a specific chemotype, and to provide a tentative analytical protocol for its assessment, we have carried out an extensive study, following strictly selected standard methods, to chemically characterize the antioxidant and non-antioxidant constituents of the EOs of the α-pinene chemotype, obtained by steam distillation of the aerial parts of the plant in the flowering (A), post-flowering (B), and vegetative period(s) (C), representative of the whole year's growth, and to examine their antioxidant response.

In the first part of the study we (a) used GC–MS analysis to characterize the chemical composition of the EOs of *R. officinalis* at the three stages of development; (b) measured the content of reducing substances by conventional methods [Folin–Ciocalteau, and $FeCl_3/Fe(CN)_6$ tests]; (c) investigated the radical scavenging (DPPH test) and anti-lipoperoxidant activity (TBARS assay) in homogeneous and heterogeneous models; (d) fractionated the EOs by silica

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gel chromatography, investigated the antioxidant activity of the isolates, and quali-/quantitatively characterized the single components.

Finally, in view of the increasing need for new methods for a mechanistic understanding of the antioxidant activity of EOs, we made a molecular modeling investigation of how the antioxidant compounds interacted in a simulated membrane lipid bilayer, to clarify how their positions within the bilayer affected the EO antioxidant activities.

2. Material and methods

2.1. Plant material

R. officinalis at different stages of development was obtained from an organic cultivar with morenic soil near Lake Garda (Solferino, Mantua, Italy) between April and October 2009. Two voucher specimens were authenticated by Prof. G. Fico, from the Department of Biology, Faculty of Pharmacy, University of Milan, and deposited in the herbarium of the department. EOs were obtained from 25 kg of samples freshly collected on sunny days and immediately steam-distilled for 5–6 h in a conventional 270-L apparatus (La Solida S.r.L., Calcinato, Brescia, Italy). The yields in EO determined gravimetrically (mL/kg of plant material) were 2.53, 2.46, and 4.72 mL/kg respectively for EO from flowering aerial parts (A), from aerial parts with seeds and leaves (B), and from aerial parts with leaves only (C). All the samples were stored in the dark at 0–4 °C and left to stand for two to three months before processing.

2.2. Chemicals

Ethyl acetate, n-pentane, diethylether, thiobarbituric acid, trolox, gallic acid, caffeic acid, camphor, borneol, thymol, carvacrol, terpinen 4-ol, linalool, and 1-8 cineol were from Sigma–Aldrich (Milan, Italy). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), and Folin–Ciocalteau reagent were from Fluka (Buchs, Switzerland). $FeCl_3$, and $K_3Fe(CN)_6$ were from Carlo Erba (Milan, Italy). Acetate buffer solution, 100 mM, pH 5.5, was used in the DPPH reaction.

2.3. Animals

Male Wistar rats ($n = 5$; 200–250 g body weight) from Charles River (Calco, Como, Italy), were housed individually in a room with temperature of 22 °C and a daily 12 h light/dark cycles. Animals care was followed in accordance with the Laboratory Animal Welfare Act (publication no. 78–23), [4]. Food and water were given *ad libitum* until 24 h before sacrification. Animals were sacrificed after light ether anesthesia and liver microsomes isolated as previously described [5].

2.4. Spectrophotometric analysis

Spectrophotometric analysis was done using a Cary 50 Bio model instrument, from Varian, Leinì, Turin, using Cary WinUV software.

2.5. GC/MS identification

Qualitative and quantitative analysis of whole and fractionated EO was done using a Varian 3800 gas chromatograph, equipped with a Factor Four capillary column (VF-5 ms, 30 m; 0.25 mm i.d., film thickness 0.25 mm) coupled with a Saturn 2000 ion trap detector. The oven temperature was initially set at 60 °C (hold time 3 min), with a gradient from 60 to 150 °C (3.0 °C/min, hold 1 min), and from 150 to 240 °C (10 °C/min, hold 1 min); injector temperature 250 °C. Column flow 1.00 mL/min. Carrier gas helium 5.5; ionization energy 70 eV; the split/splitless ratio was set to 1:30 after 45 s.

GC–MS analyses were done by the standardized procedures previously reported [6,7]. Peaks were identified by comparing the retention times with those of authentic standard MS fragmentation patterns, and final confirmation by matching with the components of the commercial library NIST mass spectral database (vers. 6.41). The percentage composition of the oils was computed by the normalization method from the GC peak areas, calculated as the mean of three injections for each oil, without correction factors.

2.6. Quantitative analysis by GC–MS

Linalool, verbenone, terpinen-4-ol, camphor, 1,8 cineole, and borneol in EOs from A, B, and C were quantified by comparison with calibration curves obtained by injecting increasing amounts (1.0 ng to 0.5 μg, injection volume 1.0 μL) of standard solutions obtained by dilution of a stock solution (1 μg/mL ethyl acetate). The concentrations of each compound in the three EOs were obtained by interpolation of the peak areas with those on the corresponding pure standard calibration curve, and expressed as mg/mL EO. The linearity of the calibration, in terms of correlation coefficient (R^2) obtained using a $1/x^2$ weighted quadratic fit, was always greater than 0.998. The calibration curve equations were: $y = 1{,}384{,}000x - 1{,}984{,}000$ for linalool, $y = 252{,}700{,}000x - 179{,}000$ for terpinen-4-ol, $y = 1{,}340{,}000x - 1{,}669{,}000$ for camphor, $y = 1{,}896{,}000x + 610{,}500$ for 1,8 cineole, $y = 150{,}600{,}000x - 42{,}050{,}000$ for thymol, $y = 1{,}506{,}002{,}110x - 42{,}050{,}200$ for carvacrol, $y = 240{,}600{,}000x + 3{,}073{,}000$ for verbenone, and $y = 1{,}665{,}000x - 1{,}580{,}000$ for borneol.

2.7. Physical constants

The three EOs were analyzed according to the standard methods of the Association Française de Normalisation (AFNOR). The physical constants were determined at 20 ± 0.2 °C. The refractive index (RI), measured with an Abbe optical refractometer (Ivymen System), was respectively 1.465, 1.4670 and 1.4666 for EO from A, B, and C [reference RI for rosemary EO 1.465–1.472 (Eur. Ph. V ed.)]; optical rotations were measured in ethanol (EtOH) solution with a D7 optical polarimeter (Bellingham & Stanley Ltd., Tunbridge Wells, Kent U.K.), and were respectively 0.61°, 0.82°, and 3.00°. Specific gravity was between 0.8940 and 0.9120 g/cm^3 for all the samples.

2.8. Sensory assessment

EOs from A, B and C were assessed by a panel of 20 EO sensory evaluation experts from the laboratories of Muller & Koster S.p.A. (Liscate, Milan, Italy).

2.9. Silica gel fractionation

Fractionation was done by solid phase extraction (SPE) according to the method described by Antonelli and Fabbri, with minor modifications [8]. Briefly, 500 μL of each EO was loaded onto an SPE cartridge (flash chromatography cartridge type SNAP KP-Sil, 10 g, Biotage®, Uppsala, Sweden), previously conditioned with 3 mL of n-pentane. The cartridge was eluted using a vacuum manifold at a velocity of 1 drop/s with the following solvent sequence:

- Fraction 1: 12 mL n-pentane;
- Fraction 2: 12 mL n-pentane/ethyl ether 95:5;

- Fraction 3: 12 mL ethyl ether;
- Fraction 4: 12 mL ethanol.

The solvent volume of the fraction was reduced to close to dryness under a gentle stream of helium (purity 5.5) and reconstituted to 500 μL, adjusting the volume with ethyl acetate for GC–MS analysis or with ethanol for analysis of antioxidant activity.

2.10. DPPH test

Radical scavenging using the DPPH radical is the main antioxidant assay used to investigate the mechanisms by which antioxidants act in food. We studied the free radical-scavenging activity of the EOs and their fractions by the original method of Blois [9]. We made the final test solution (3 mL) by adding 0.5 mL of DPPH in ethanol (500 μM) and 100 μL of ethanol containing different concentration (10–60 μL) of native EOs *in toto* or of the isolated fractions, to an acetate buffer (1 mL)/ethanol (1.4 mL) mixture.

In a parallel set of experiments, to assess the buffer's influence on the H/e^- transferring ability of the substrates to the DPPH radical, we progressively substituted the acetate buffer solution with pure ethanol. The mixtures were shaken vigorously then incubated at 25 °C in the dark. The absorbance of the unreacted DPPH was determined at different incubation times (15, 30, 60, 90 min) against a sample blank (reagents without DPPH) at λ_{max} = 517 nm. The scavenging activity was expressed as: (a) RSC% according to the formula: $RSC\% = [(A_{CTR} - A_{EO})/A_{CTR}] \times 100$; (b) IC_{50} (μL of EO quenching the DPPH by 50%); (c) trolox equivalent (TE, mg_{TE}/mL_{EO}). To plot a calibration curve, increasing amounts of the water-soluble analog of vitamin E, trolox (0.00, 0.05, 0.10, 0.50, 2.50, 5.00, 10.00 mg), were diluted in 100 μL of ethanol and analyzed as reported above. All analyses were done in triplicate. The trolox calibration curve was linear between 0.10 and 10.00 mg, and the equation was: $y = 3.116x + 10.27$, with a goodness of fit of 0.997.

2.11. Reducing substances

The total content of reducing substances content was determined using two methods:

(i) Folin–Ciocalteau assay with minor modifications [10], expressing the results as mg of gallic acid equivalent (GAE)/mL_{EO}. Briefly, different amounts (from 1 to 50 μL) of EO or the isolated fractions were added to 1 mL of Folin–Ciocalteau reagent previously diluted (1:10) with milliQ water. The mixture was vortexed for 2 min, and the spectrophotometric absorbance was measured at λ = 750 nm after 20 min incubation at room temperature. All determinations were done in triplicate. The GAE ($mg_{GAE}/mL_{EO} \pm$ S.D.) was calculated by comparison with a calibration curve plotted with a diluted stock solution (1 mg/mL) of gallic acid in $EtOH/H_2O$ (1:1). The calibration line ($y = 0.01162x + 0.04261$) was linear ($R^2 = 0.9997$) between 0.01 and 0.50 mg/mL.

(ii) the Prussian blue method [11], with minor modifications: 100 μL of ethanol containing different amounts of pure EO (from 1 to 50 μL) or of the isolated fractions were added to 3 mL of 0.1 M $FeCl_3$ in 0.1 N HCl and 3.0 mL of 8.0 mM of $K_3[Fe(CN)_6]$ and vigorously vortexed. The absorbance was determined spectrophotometrically at λ_{max} = 720 nm after 10 min incubation at room temperature. All analyses were done in triplicate. The results were expressed as mg_{GAE}/mL EO ± S.D. The GAE was calculated as described above, and the calibration line ($y = 0.03690x + 0.06004$) was linear ($R^2 = 0.9995$) between 0.01 and 0.50 mg/mL.

2.12. Anti-lipoperoxidant activity

The thiobarbituric acid reactive substances (TBARS) assay was done according to Sato et al. [12] with minor modifications, using rat liver microsomes prepared as previously described [5] as lipid-rich media (1 mg/mL microsomal protein suspension) [13]. Test solutions were prepared by dilution with DMSO of a stock solution of native EOs dissolved in DMSO (100 $\mu L_{EO}/mL_{DMSO}$), or of the fractions (dissolved in ethanol) or of the authentic standards in the same solvent, diluted as appropriate (0.05–1.00 μL in 10 μL of DMSO). For solid samples a stock solution of 10 mg/mL (DMSO) was prepared and diluted with DMSO as needed (from 1 to 100 μg/mL); 10 μL of each solution was employed.

The TBARS values were determined spectrophotometrically at 532 nm (absorbance of the pink chromogen due to the formation of MDA adducts and other secondary lipid peroxidation products to TBA).

The IC_{50} (the concentration inhibiting pigment formation by 50%) was calculated using the formula $[(T/C \times 100) - 100]$ where C is the absorbance of the fully oxidized control and T the absorbance of the test samples. They were also expressed as trolox equivalents (mg_{TE}/mL_{EO}). All analyses were run in triplicate.

2.13. In silico studies

To investigate the compounds' behavior in the bilayer, the initial systems were simulated in a phospholipid model membrane (l-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine, POPC), built with 200 POPC molecules and 5400 SPC water molecules (total number of atoms approximately 27 000) [14]. The lateral dimensions of the bilayer are 85 Å × 100 Å, and the distance between the bilayer surfaces, given by the average inner phosphorus to outer phosphorus distance, was 35 Å. Each water layer was up to 15 Å thick. After assembly of the system, the POPC bilayer membrane and each of the eight molecules were optimized in 250 steps, then simulated for 5 ns each, using the NAMD program. [15]. This software includes code developed by the Theoretical and Computational Biophysics Group in the Beckman Institute for Advanced Science and Technology at the University of Illinois at Urbana-Champaign. The bond distances and bond angles of water were constrained using the SETTLE algorithm and the coupling time was set to 1.0 ps with isothermal compressibility 4.6×10^{-5} bar^{-1}. The terpenes, terpenoids and solvent were independently coupled to a temperature of 298 K with a coupling time of 0.1 ps and the pressure was held at 1 bar, with a coupling time of 0.2 ps, using a Berendsen thermostat to maintain constant temperature and pressure. The time step was 1.0 fs and throughout the simulations periodic boundary conditions were used to reduce edge effects.

2.14. Statistical analysis

The results of the methods used to evaluate the antioxidant power of the EOs and their components are given as mean ± standard deviation. Analysis of variance was done by ANOVA using SPSS 14.0 for Windows. Significant differences between means were determined by Tukey's post hoc test. p Values less than 0.05 were considered significant.

In the correlation analysis between the % content in hydroxilated derivatives (alcohols/phenols/1.8 cineole), reducing substances, and antiradical or antilipoperoxidant activity in different essential oils from A, B, and C, a statistical dispersion of the three variables was employed (circle graph).

Table 1
Composition percentage of three essential oils of *R. officinalis*.

Compound	R.T.	EO from A	EO from B	EO from C
Tryciclene	6.449	0.365	0.435	0.246
α-Thujene	6.546	0.231	0.267	0.123
α-Pinene	6.836	25.962	35.026	37.655
Camphene	7.377	7.708	10.733	9.039
3-Carene	7.509	2.162	2.705	3.216
Sabinene	8.162	0.056	0.064	0.028
β-Pinene	8.351	4.771	5.007	3.321
4-Carene	8.554	0.039	0.000	0.060
β-Myrcene	8.759	3.206	3.968	3.980
Unidentified	9.313	0.061	0.000	0.000
α-Phellandrene	9.416	0.262	0.144	0.117
α-Terpinene	9.838	0.294	0.154	0.127
p-Cymene	10.200	1.212	1.422	1.352
d-Limonene	10.354	3.651	4.606	4.478
1.8 Cineole	10.523	6.405	3.310	1.825
τ-Terpinen	11.560	0.562	0.357	0.400
Terpinolene	12.725	0.593	0.498	0.624
Dehydro-p-Cymene	13.043	0.070	0.000	0.000
Chrisantenone	13.509	1.022	0.905	0.840
Linalool	13.606	1.480	0.750	0.761
cis-Carveol	13.851	0.045	0.000	0.086
Eucarvone	14.489	1.088	0.989	0.932
6-Camphenol	14.730	0.158	0.138	0.000
trans-Pinocarveol	15.436	0.098	0.066	0.052
Camphor	15.647	6.292	5.885	6.794
Isopinocarveol	15.913	0.023	0.000	0.000
Isopulegol	16.066	0.021	0.012	0.000
Isopinocamphone	16.229	1.117	1.072	1.031
Pinocarvone	16.315	1.014	0.840	0.840
Unidentified	16.527	0.065	0.000	0.000
α-Pinene oxide	16.721	0.176	0.077	0.000
Unidentified	16.829	0.009	0.000	0.000
Borneol	16.954	5.068	2.055	2.148
Terpinen-4-ol	17.185	0.404	0.212	0.021
Unidentified	17.397	0.002	0.000	0.000
α-Terpineol	17.949	0.400	0.294	0.106
Verbenone	18.491	4.560	4.531	5.257
cis-Geraniol	19.064	0.000	0.285	0.009
Methylcamphenoate	21.106	1.976	0.840	0.840
Bornylacetate	21.720	6.050	5.076	5.345
Thymol	22.504	0.450	0.000	0.000
Carvacrol	22.602	0.700	0.000	0.000
Unidentified	22.947	0.025	0.000	0.000
Ocimene	23.861	0.053	0.000	0.044
Isopulegol acetate	24.015	1.840	1.076	0.998
Dihydrocarveol	25.152	0.008	0.100	0.085
Dihydrocarveol acetate	25.427	1.992	1.044	1.006
α-Selinene	26.769	0.051	0.000	0.025
β-Caryophyllene	27.408	2.615	3.500	2.850
α-Caryophyllene	28.889	0.732	0.556	0.442
Alloaromadendrene	31.056	0.048	0.038	0.000
Unidentified	31.926	0.048	0.000	0.000
Caryophillene oxide	34.112	0.599	0.643	0.410
β-Guaiene	34.642	0.049	0.041	0.000
α-Bisabolene	35.097	0.039	0.053	0.000
β-Gurjunene epoxide	36.781	0.000	0.043	0.038
Isoaromadendrene epoxide	37.914	0.000	0.073	0.045

3. Results and discussion

3.1. Essential oil yield and chemical composition

The content of the EOs extracted by steam distillation from the freshly collected aerial apical parts of *R. officinalis* L. in the three different stages varied, with 2.53 mL/kg in A and 2.46 mL/kg in B, with the maximal yield during the vegetative phase (4.72 mL/kg in C).

3.2. GC/MS analysis

More than 50 compounds (Table 1) were identified in the three native EOs, amounting to 97–100% of the total oils. Chromatographic analysis of the three EOs showed the typical profile of the α-pinene chemotype of *R. officinalis*. Fig. 1 shows a representative GC profile of EO from A together and the percentages of non-oxygenated and oxygenated compounds in EO from A, B, and C are set out in Table 2. There were huge amounts of non-oxygenated terpenes, ranging from 54.73 ± 0.20% in EO from A (flowering aerial parts) to 69.57 ± 0.16% in EO from B (aerial parts with seeds and leaves) and 68.13 ± 0.43% in EO from C (aerial parts with leaves only) and smaller amounts of oxygenated derivatives (43.97 ± 0.16%, EO from A; 30.29 ± 0.12%, EO from B; 30.94 ± 0.24%, EO from C). GC–MS analysis of the oxygenated fraction indicated that it consisted of ketones, esters, oxides and peroxides in approximately the same amounts for all three EOs; there was also a substantial amount of hydroxilated derivatives, (containing alco-

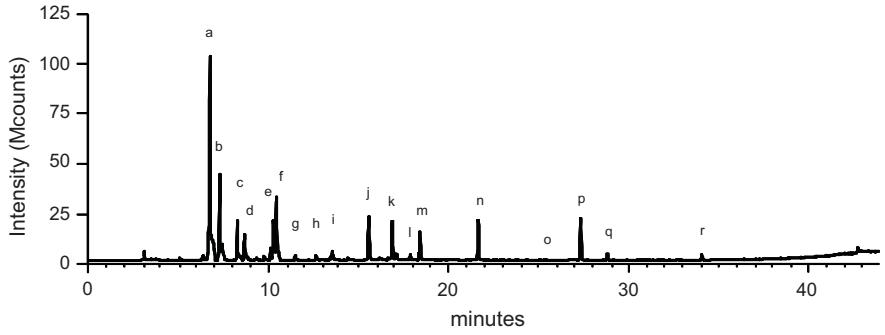

Fig. 1. Representative GC–MS chromatogram of *R. officinalis* essential oil in the flowering stage (α-pinene chemotype). Some prominent compounds: (a) α-pinene; (b) camphene; (c) β-pinene; (d) pseudolimonen; (e) limonen; (f) 1,8 cineole; (g) τ-terpinene; (h) terpinolene; (i) linalool; (j) camphor; (k) borneol; (l) terpinen-4-ol; (m) verbenone; (n) bornyl acetate; (o) l-aromadendrene; (p) β-caryophillene; (q) α-caryophillene; (r) caryophillene oxide.

hols, phenols and 1,8 cineole) which were maximal in native EOs from flower aerial parts (A), somewhat less in the apical part with leaves and seeds (B), and less again in the parts containing only leaves (C).

3.3. Sensory assessment

Differences in sensory profiles (not shown) between EO from A, B, and C were barely detectable in view of the similar composition of the three oils. Only the EOs from flowering aerial parts (Table 2) had a fragrance of fresh flowers (cool, fresh, and sweet) attributable to the larger content of the highly odoriferous alcohols, most valuable in terms of their contribution to the fragrance of the essential oil. By contrast EO from B and C gave off the typical smell of monoterpene hydrocarbons, which contribute less to the fragrance than oxygenated compounds.

3.4. Antioxidant activity in homogeneous phase of the EOs in toto

First we studied the radical scavenging capacity (RSC) of the three EOs by the original DPPH test of Blois [9]. All the EOs extracted during the flowering, post-flowering and vegetative periods showed definite increases in RSC, in line with the oil concentration (Table 3). The EO from the flowering aerial parts was the most active (IC_{50} 36.78 ± 0.38 μL; TE 0.34 mg/mL_{EO}; S.D. < 5% for all the means), followed by the oils from the aerial parts with seeds and leaves (IC_{50} 79.69 ± 1.54 μL; TE 0.15 mg/mL_{EO}) and then from aerial parts with leaves only (IC_{50} 111.94 ± 2.56 μL; TE 0.11 mg/mL_{EO}).

Lowering the concentrations of acetate buffer substantially reduced the RSC% of EO from A (Fig. 2), indicating this aqueous solvent's fundamental role: by solvation of the polar groups of the active constituents it facilitates H/e^- transfer to the DPPH radical in the quenching reaction.

This assumption is confirmed by the fact that using the same DPPH concentration and pure ethanol as solvent system, the RSC percentages were negligible and fairly similar for all three oils – 10.97 ± 0.24%, 8.85 ± 0.36%, and 6.09 ± 0.31% for 30 μL (prototype concentration) of EO from A, B, and C, whereas the RSC% for the same amount(s) in the original test were 44.18 ± 0.14, 19.09 ± 0.80, and 12.00 ± 0.82.

The reducing substances, in the Folin–Ciocalteau assay, were 0.705 mg_{GAE}/mL for EO from A, 0.492 mg_{GAE}/mL for EO from B and 0.362 mg/mL for EO from C. The Prussian blue assay (Fig. 3) gave lower concentrations (0.491 mg_{GAE}/mL for EO from A, 0.208 mg_{GAE}/mL for EO from B, 0.019 mg_{GAE}/mL for EO from C, S.D. < 5% for all the means), very likely because of the higher specificity of this assay. Interestingly, whatever the method used to determine reducing substances in the oils, the content was always maximal in EO from A, lower in that from B and lowest in that from C.

These findings and the GC–MS quantitative analysis of the constituents profiles of the three EOs (Fig. 4a) suggest a tentative correlation (R^2 = 0.9409) between the DPPH response, the content in reducing substances in the three phytocomplexes at different concentrations, and the concentration of hydroxylated derivatives (i.e. linalool, terpineol, terpinen-4-ol, 6-camphenol, borneol, isopulegol, geraniol, phenols, and 1,8 cineole).

Table 2
% Composition of three different rosemary EO (means of three replicates).

Compounds % in EO	EO from A	EO from B	EO from C
Alcohols/Ethers	14.11 ± 0.12	7.22 ± 0.06	5.09 ± 0.10
Ketones/Aldehyds	15.09 ± 0.01	14.22 ± 0.04	15.69 ± 0.08
Phenols	1.15 ± 0.00	Traces	Traces
Esthers	11.86 ± 0.07	8.04 ± 0.02	8.19 ± 0.04
Oxides and peroxides	0.78 ± 0.01	0.84 ± 0.00	0.49 ± 0.02
Total oxygenated compounds	43.97 ± 0.16	30.29 ± 0.12	30.94 ± 0.24
Terpenes non oxygenated	54.73 ± 0.20	69.57 ± 0.16	68.13 ± 0.43
Unidentified compounds	0.21 ± 0.02	–	–
Total	97.93 ± 0.39	99.89 ± 0.27	97.60 ± 0.65

Table 3
Increase in the DPPH scavenging ability increasing the EO concentration.

EO (μL)	DPPH RSC% (n = 3)		
	EO from A	EO from B	EO from C
10	24.48 ± 1.21	3.18 ± 0.11	1.25 ± 0.01
20	38.55 ± 2.64	10.00 ± 1.06	9.22 ± 0.41
30	44.18 ± 0.14	19.09 ± 0.80	12.00 ± 0.82
40	54.14 ± 1.87	23.92 ± 1.34	16.69 ± 1.06
50	59.53 ± 1.91	29.25 ± 0.15	21.19 ± 1.00
60	69.47 ± 1.74	38.61 ± 1.67	25.55 ± 0.97
Calibration curve	$y = 0.85x + 18.47$	$y = 0.68x - 3.28$	$y = 0.46x - 2.21$
	$r^2 = 0.9841$	$r^2 = 0.9912$	$r^2 = 0.9849$
IC_{50}	36.78 ± 0.38 μL	79.69 ± 1.54 μL	111.94 ± 2.56 μL

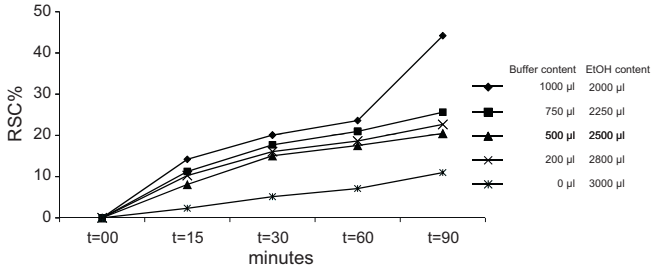

Fig. 2. Effect of the lowering of buffer solution in the original DPPH assay: EO (from flowering aerial parts). Prototype oil concentration: 30 μL.

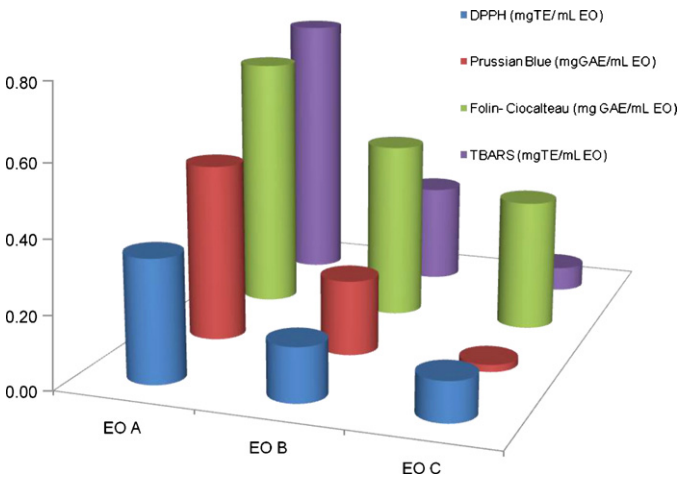

Fig. 3. Antioxidant activity of the EOs from *R. officinalis* at different stages of development (A, B, and C) in four different assays. Data are the mean ± standard deviation (S.D. < 5%) of three independent measurements.

3.5. Anti-lipoperoxidant activity

Our next step was to investigate the anti-lipoperoxidant activity of the three native EOs using rat liver microsomes as model lipid membrane, since their composition is close to that of foodstuffs. The free radical promoter was ADP/Fe^{3+}/ascorbic acid, specific for the formation of •OH radicals and subsequently in the lipid medium of ROO• and RO• radicals. The results, shown in Fig. 3, indicated an IC_{50} of 0.420 ± 0.04 μL for EO from A (0.76 ± 0.05 mg_{TE}/mL_{EO}), 1.202 ± 0.06 μL (0.278 ± 0.02 mg_{TE}/mL_{EO}) for EO from B and 4.070 ± 0.05 μL for EO from C (0.068 ± 0.03 mg_{TE}/mL_{EO}). In particular, EO from A had significantly greater anti-lipoperoxidant activity than that from B and C (confirmed by the higher TE content than in B and C, $p > 0.001$); this agrees with the predictive DPPH quenching activities of the EOs and their content in reducing substances. Here again we found a good correlation for each native oil, between reducing substances, hydroxylated derivatives content and anti-lipoperoxidant activity (Fig. 4b; $R^2 = 0.9814$).

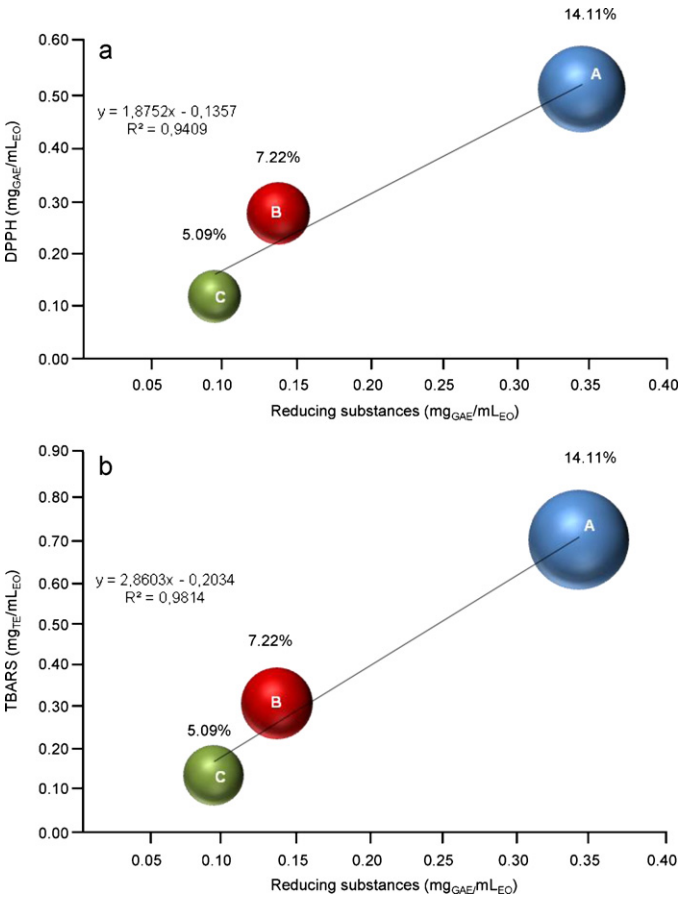

Fig. 4. Correlations between: % content in hydroxylated derivatives (alcohols, phenols and 1,8 cineole), reducing substances (*Prussian blue test*), and antiradical activity (*DPPH assay*) in different essential oils from A, B, and C. The diameters of the circles are used as quantitative index of the different content of hydroxylated derivatives in the three essential oils from (A) 15.26 ± 0.12%, from (B) 7.22 ± 0.06%, and from (C) 5.09 ± 0.10%. % content in hydroxylated derivatives (*circles*), reducing substances (*Prussian blue test*), and anti-lipoperoxidant activity (*TBARS assay*) in the three essential oils from A, B, and C.

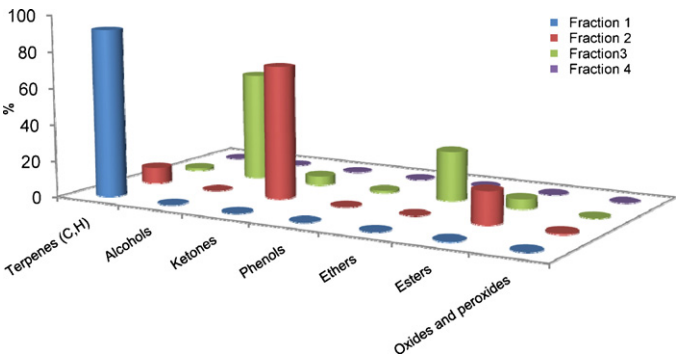

Fig. 5. Chemical classes of compounds identified in the EO fractions from flowering aerial parts (A).

3.6. EO fractionation (GC–MS analysis)

In the second part of the study the three EOs were chromatographically fractionated on silica gel using different solvents with increasing polarity, to identify the components responsible for the antioxidant activity and explain the different RSC% and anti-lipoperoxidant profiles of EOs from A, B, and C. Each of the isolates was first screened by GC–MS to characterize the constituents, then their antioxidant activity was investigated in the DPPH and TBARS tests.

Elution of essential oils from A, B, and C with n-pentane (fraction 1) showed mainly non-oxygenated monoterpene species which accounted for 49.01 ± 2.16% of the total components of native EOs for EO from A, 63.54 ± 1.97% for EO from B, and 61.95 ± 2.47% for EO from C (Fig. 5). Elution with n-pentane/diethylether 95:5 (fraction 2) gave 14.50%, 13.92% and 14.24% of ketones for EOs from A, B and C, 11.80%, 7.34%, and 7.25% of esters, and small amounts of residual non-oxygenated monoterpene hydrocarbons (5.00%, 4.14%, and 3.30%). Diethylether elution (fraction 3) gave mainly ethers and alcohols (Fig. 6). EO from A accounted for 14.11% in the native oil, of which there was 1,8 cineol (6.41%), and borneol, linalool, terpinen-4-ol, terpineol, 6-camphenol, geranyl alcohol, carveol, isopulegol, dihydrocarveol, (7.71%), plus 1.15% of phenols (carvacrol 0.70% and thymol 0.45%); EO from B gave a total of 7.14%, and EO from C 5.05%. There were also residual amounts of ketones (1.40%, 2.56%, and 5.01% for EOs from A, B, and C, respectively). Elution with ethanol (fraction 4) did not recover other polar species.

GC–MS analysis of the single fractions provided unequivocal structural confirmation of the quali-quantitative composition of the constituents found in unfractionated EOs. The fractions analyzed were used in the antioxidant assays.

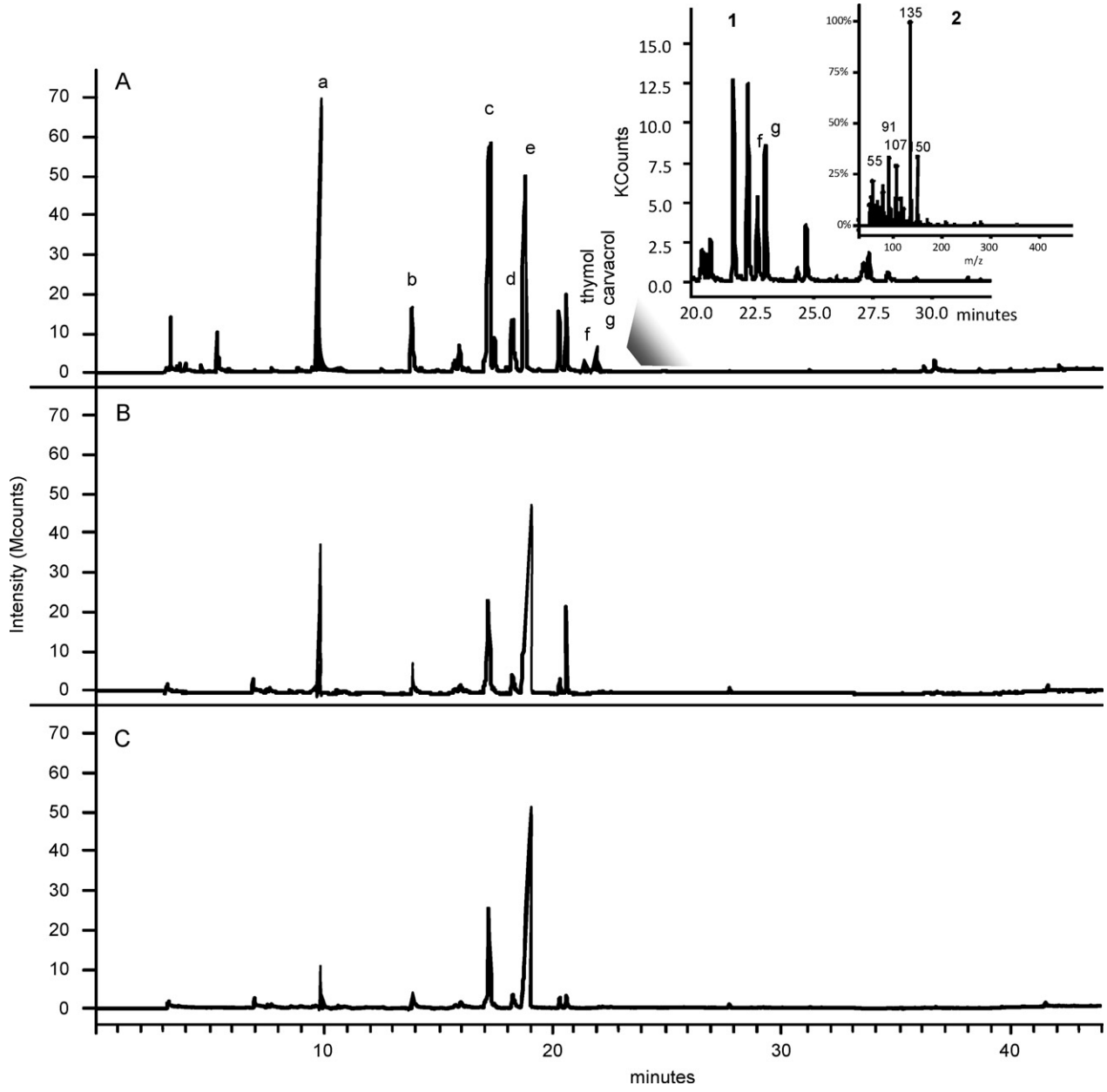

Fig. 6. GC–MS chromatogram of the ethylether fraction (fraction 3) of the three EOs. (a) 1.8 cineole; (b) linalool; (c) borneol; (d) terpinen-4-ol; (e) verbenone; (f) thymol; (g) carvacrol. Inserts in panel A: (1) expansion of the chromatogram between R.T. 20 and 30 min; (2) mass spectrum of thymol. See the loss/decrease in active constituents in EOs from B and C (panel B and C of the figure).

3.7. EO fractions: antioxidant activity in homogeneous and heterogeneous phases, and reducing substances

Fractions 1 and 2 of EOs from flowering aerial parts had little activity in the DPPH quenching reaction (IC_{50} 128.38 ± 3.51, and 124.92 ± 2.81 μL; 0.048 ± 0.002, and 0.060 ± 0.003 mg_{TE}/mL_{fract}), and the same held for fractions 1 and 2 of EO from B and C, which gave virtually negligible DPPH responses (RSC < 1.40%). However, fraction 3 of EO from A gave a strong reaction with DPPH (IC_{50} 69.53 ± 2.43 μL; 0.138 ± 0.005 mg_{TE}/mL_{fract}), while the same fraction from B and C a weaker response (IC_{50} 104.28 ± 3.95, 109.48 ± 3.62 μL; 0.022 ± 0.001, and 0.016 ± 0.001 mg_{TE}/mL_{fract}).

The lower DPPH quenching activity of fractions 1 and 2 of EOs from A, B, and C was closely paralleled by a lower content in reducing substances (less than 0.091 mg_{GAE}/mL_{EO} calculated by the Prussian blue method (Table 4). Fraction 3 of EO from A had the highest content in reducing substances: 0.398 ± 0.003 mg_{GAE}/mL_{EO} compared to 0.117 ± 0.001, and 0.014 ± 0.000 mg_{GAE}/mL_{EO} for EO from B and C (Prussian blue method).

In the experiments in heterogeneous phase (TBARS assay), fractions 1 and 2 from EOs from A, B, and C did not give any response, with only mild pro-oxidant activity, more evident in fraction 1 of EOs from B and C (15.3% more than the chromogen basal value). Only the diethylether fractions of EOs from A, B, and C had an appreciable IC_{50}, maximal for that from A (0.48 ± 0.01 μL), close to that of native EO (0.42 ± 0.02 μL), and lower for those from EOs from B and C (1.80 ± 0.54 and 5.04 ± 0.31 μL). For the diethylether isolates there was a close correlation (R^2 = 0.9951) between the concentration of the hydroxylated derivatives and the anti-lipoperoxidant action, expressed as IC_{50} (Fig. 7).

From the results in the homogeneous and heterogeneous phases we can draw convincing evidence that the components responsible for the antioxidant activity of the three EOs were the CHO derivatives present in the diethylether fraction, previously struc-

Table 4
Reducing substances content (Folin–Ciocalteau and Prussian blue tests) and antioxidant activities (DPPH and TBARS assays) of fractions 1, 2, 3 from EOs at different stage of development. Data are the mean ± standard deviation (S.D. < 5%) of three independent determinations.

	Fractions	Folin–Ciocalteau ($mg_{GAE}/mL_{fraction}$)	Prussian blue ($mg_{GAE}/mL_{fraction}$)	DPPH ($mg_{TE}/mL_{fraction}$)	TBARS (IC_{50})
EO from A	1 (n-Pentane)	0.052	0.007	0.048	Not detectable
	2 (n-Pentane/ethylether)	0.110	0.019	0.060	Not detectable
	3 (Ethylether)	0.597	0.398	0.138	0.48 μL
EO from B	1 (n-Pentane)	0.048	0.002	0.016	Not detectable
	2 (n-Pentane/ethylether)	0.090	0.091	0.018	Not detectable
	3 (Ethylether)	0.323	0.117	0.022	1.80 μL
EO from C	1 (n-Pentane)	0.016	0.001	0.009	Not detectable
	2 (n-Pentane/ethylether)	0.030	0.003	0.010	Not detectable
	3 (Ethylether)	0.226	0.014	0.016	5.04 μL

turally elucidated by GC–MS analysis, as alcohols, phenols, and 1.8 cineole.

3.8. Radical scavenging and anti-lipoperoxidative activity of standard hydroxylated derivatives

We then tested the RSC and anti-lipoperoxidant activities of the main hydroxylated derivatives, to investigate their antioxidant behavior, first alone, then in combination. We checked the individual standards (borneol, linalool, 1,8 cineole, terpinen-4-ol, verbenone, carvacrol and thymol) and then a mixture of the standards dissolved in ethanol (1 mL final volume), whose composition was established according to the percentages of the components in EOs from A, B, and C. We did not consider the contributions to the antioxidant activity from terpineol, 6-camphenol, geranyl alcohol, carveol, isopulegol, or dihydrocarveol since their concentrations, calculated from the peak areas, were extremely low and – above all – of the same order of magnitude in all the oils.

In the DPPH assay the standards gave the following IC_{50} (Table 5): 105.54 ± 3.44 mg borneol; 226.24 ± 0.66 μL linalool; 330.31 ± 2.64 μL 1,8 cineole; 115.28 ± 1.64 μL terpinen-4-ol; 261.16 ± 4.91 μg carvacrol, 284.00 ± 10.63 μg thymol, 97.48 ± 2.55 μL verbenone. The most active were thymol and carvacrol, followed by borneol and the α,β-unsaturated ketone, verbenone, then linalool and 1,8 cineol.

The TBARS assay gave the following IC_{50}: 74.22 ± 2.01 μg borneol; 0.74 ± 0.01 μL linalool; 3.41 ± 0.02 μL 1,8 cineole; 2.87 ± 0.02 μL terpinen-4-ol; 5.03 ± 0.27 μg carvacrol, 5.85 ± 0.01 μg thymol, and 0.74 ± 0.00 μL verbenone. Table 5 shows the concentrations of these compounds in the native oils from A, B, and C. The lower levels of DPPH and of lipid peroxidation inhibition for the individual standards compared to the native EOs is not surprising [16], since it indicates a synergistic interaction between the components of the mixture of the phytocomplex.

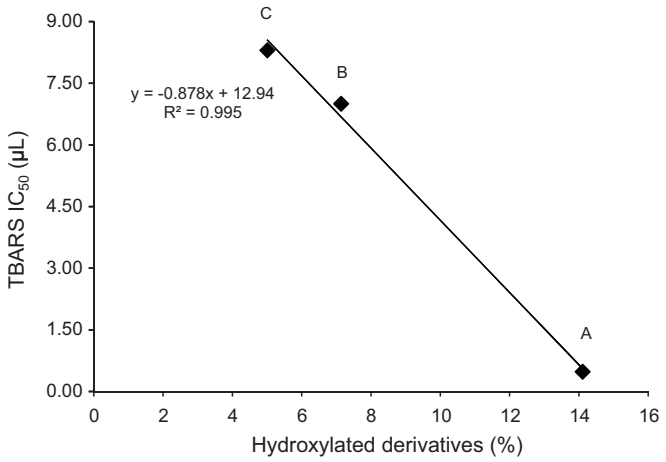

Fig. 7. Correlation between the % content of hydroxylated derivatives and the anti-lipoperoxidant activity in the diethylether isolates of the three EOs.

The IC_{50} for the DPPH and TBARS assays were respectively 39.11 ± 0.98 and 0.49 ± 0.03 μL, close to those of EO from A; for EO from B the IC_{50} were 81.45 ± 2.64 μL and 1.36 ± 0.05 μL, and for EO from C 120.21 ± 1.88 μL and 4.22 ± 0.11 μL.

From these results we can conclude that the difference in the radical scavenging and anti-lipoperoxidant activity in EOs from A, B, and C is due to the different concentrations of hydroxylated derivatives in the native EOs.

3.9. Modeling study

The final part of the study was a molecular modeling investigation to clarify the localization of the main C, H, O terpenes, which contribute significantly to the overall anti-lipoperoxidant activity of the EOs from *R. officinalis* (terpinen-4-ol, linalool, borneol, 1,8 cineole, and verbenone), and define their mechanism of antioxidant action better. We also investigated another ketone, camphor, which in our hands was inactive in the DPPH and TBARS tests (data not shown), but was shown to be an effective antioxidant by Bozin et al. [3], and two widely represented species, the non-oxygenated monoterpenes camphene and α-pinene, which had no antioxidant activity and were used as negative controls.

Of these eight compounds, only camphene and α-pinene were able to cross the membrane, reaching the core of the interface between the two POPC layers (Fig. 8). After the initial phase of the simulation, in which camphene and α-pinene were located near the POPC–water interface, both compounds moved toward the center of the POPC bilayer, reaching their final positions in 3.4 and 3.8 ns, respectively. Once reached the core of the bilayer, they made only small movements, remaining in much the same position until the end of the simulation.

This behavior (Fig. 8) can be explained by the two compounds' high lipophilicity, which gives them a strong propensity to interact exclusively with the apolar portion of the membrane. Under the strong conditions of the TBARS assay (100 °C, 15 min, highly acidic pH), they can act jointly to disrupt the lipid membrane bilayer and exert the mild pro-oxidant effect observed in the n-pentane fraction of EOs from B and C.

The other six molecules in the modeling investigation were positioned at the interface with the aqueous layer, and can be divided into two sets on the basis of the location of their polar group in relation to the POPC–water interface:

(I) Terpinen-4-ol, linalool and verbenone: these three terpenes had almost identical interaction dynamics. In the early stages of the simulation, they began to position at the interface between the phospholipid and aqueous layers, with very limited longitu-

Table 5
Antilipoperoxidant (TBARS) and radical scavenging (DPPH) activities of pure standards, and concentration of the standards in the three different EOs.

Samples	IC_{50} (TBARS)	IC_{50} (DPPH)	Concentration of standards in EOs (mg/mL)		
			A	B	C
Borneol (μg)	74.22 ± 2.01	$105.54e^{03}$ ± 3.44	61.23 ± 1.11	26.58 ± 0.21	25.93 ± 1.04
Linalool (μL)	0.74 ± 0.01	226.24 ± 0.66	19.88 ± 0.50	9.51 ± 0.37	9.05 ± 0.35
Eucalyptol (μL)	3.41 ± 0.02	330.31 ± 2.64	61.43 ± 1.00	33.61 ± 1.52	24.70 ± 1.24
Terpinen-4-ol (μL)	2.87 ± 0.02	115.28 ± 1.64	1.50 ± 0.00	0.06 ± 0.00	0.05 ± 0.01
Verbenone (μL)	0.74 ± 0.00	97.48 ± 2.55	$0.242e^{-03}$ ± 0.00	$0.241e^{-03}$ ± 0.00	$0.207e^{-03}$ ± 0.00
Carvacrol (μg)	5.03 ± 0.27	261.16 ± 4.91	2.00 e^{-06} ± 0.03	–	–
Thymol (μg)	5.85 ± 0.01	284.00 ± 10.63	1.35 e^{-06} ± 0.03	–	–
Reconstructed fraction A (μL)	0.49 ± 0.03	39.11 ± 0.98			
Reconstructed fraction B (μL)	1.36 ± 0.05	81.45 ± 2.64			
Reconstructed fraction C (μL)	4.22 ± 0.11	120.21 ± 1.88			

dinal movements; then almost immediately they moved their lipophilic chain toward the apolar layer. This was confirmed in the subsequent simulation steps, where the three terpenes gradually inserted their apolar portion inside the lipid bilayer, creating a kind of virtual cavity within the POPC bed. This conformation was characterized by the interaction of their polar groups with the solvent and with the phosphate heads of lipids, with the oxygen preferentially oriented toward water (Fig. 8). Terpinen-4-ol was the first to reach this dynamic equilibrium position within the bilayer (2.3 ns), followed by linalool (2.7 ns) and finally verbenone (3.7 ns), and this pattern was maintained until the end of simulation.

(II) Borneol, camphor and 1,8 cineole: here the interaction patterns differed in their dynamic properties in relation to the bilayer. As in the previous case, they were located at the POPC–water interface, but differently from terpinen-4-ol, verbenone, and linalool, they all moved their polar groups toward the POPC polar heads, orienting their lipophilic chain toward the water layer. Borneol and 1,8 cineole reached their dynamic equilibrium positions in 2.9 and 3.3 ns, and maintained this position until the end of the simulation (Fig. 8). Camphor showed greater mobility, that hampered the molecule in reaching its equilibrium position within the bilayer. Camphor was the only compound that even after 5 ns of simulation was unable to find a stable dynamic equilibrium position above the membrane, and continued to fluctuate between the water and the phospholipid phase. This difficulty in achieving dynamic equilibrium probably explains why we could find no antioxidant activity.

This model of simulated phospholipid bilayer therefore suggests that to exert anti-lipoperoxidant action a molecule needs to achieve stable interactions with the POPC bilayer and must reach the dynamic equilibrium position within the membrane bilayer in a short time (<3.8 ns).

The orientation of the polar groups of terpinen-4-ol, verbenone and linalool toward the aqueous layer gives them the chance to directly quench the •OH radicals and interact through a chelating mechanism [17] with the promoter (Fe^{3+}) of the free radical cascade, thus forming a first line of defense. By contrast, borneol and 1,8 cineole, projecting their oxygen-containing groups close to the POPC polar heads, and their lipophilic portion toward the water layer, can synergistically cooperate with terpinen-4-ol, linalool and verbenone by entrapping the highly mobile RO• and ROO• lipid radicals escaped from the lipid surface or formed within the lipid

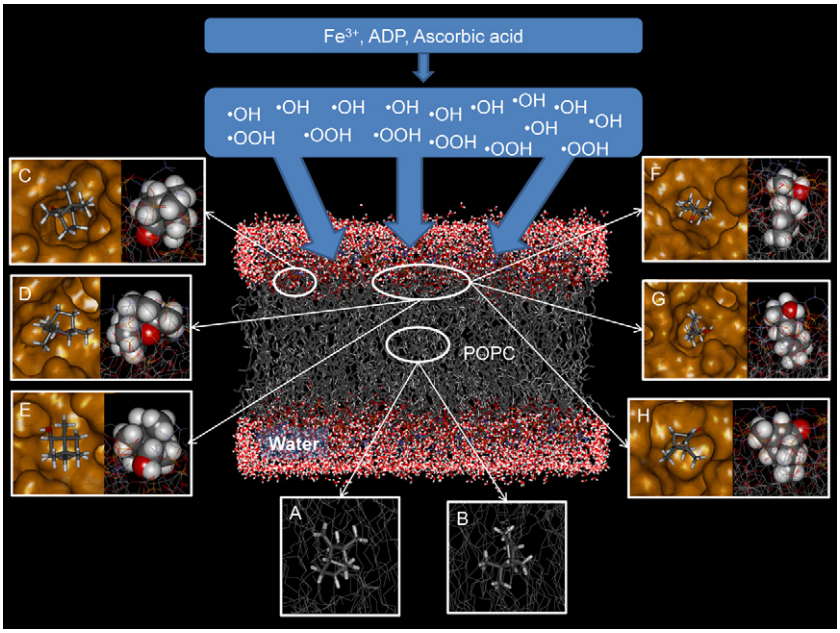

Fig. 8. Positioning of some of the main non-oxygenated and oxygenated terpenes compounds in a simulated lipid bilayer. Schematic illustration representing the position of the MD simulation results inside the hydrated l-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) membrane model. Camphene and pinene (A and B, stick) are located toward the center of the POP bilayer. Camphor, 1,8 cineole and borneol (C, D, E, stick left and CPK right) lie over the POPC solvent-accessible surface. The same holds for terpinen-4-ol, linalool and verbenone (F, G and H). These last three terpenes (F, G, and H) were characterized by interaction of the polar group with both the solvent and the lipid phosphate heads, while the lipophilic portion was inserted into the polar part of the POPC layer. The orientation of the polar group of F, G, H, enables them to interact with the promoters Fe^{3+}, and/or products of the free-radical cascade, thus forming the first line of anti-lipoperoxidative defense.

bilayer through a hydrogen-donating mechanism (second line of defense).

4. Conclusions

These results illustrate the differences in the antiradical and anti-lipoperoxidant activities of *R. officinalis* EO from the aerial parts of the plants in the various phases of development. They are related to the contents of the main C, H, O derivatives of the native EO, borneol, linalool, 1,8 cineole, terpinen-4-ol, carvacrol and thymol, which peak in the flowering stages of the plant between March and April (A), diminishing first after the flowering period (B) then much more in the vegetative stage (C). Hence we can reasonably assume that in the flowering period, the insect-attracting properties of the flowers [18] require greater emission of these secondary metabolites (C, H, O terpenes), and their repellent properties act as anti-feedants and oviposition deterrents, protecting the plant from pests and parasites (aphids, worms, spiders, fleas, cockroaches, etc.).

The modeling study in a simulated membrane bilayer indicated that for anti-lipoperoxidant action, and consequently a preservative effect on a foodstuff, the components of an EO must interact fast and stably with the phospholipid bilayer, and also preferentially project the oxygen-containing polar groups toward the external face of the membrane bilayer.

Finally, beside the need for information on the geographical origin, chemotype, soil type, irrigation conditions, etc., the findings show how important it is to clearly specify the stage of plant development since this dictates the nature and the amount of active ingredients present in the phytocomplex. In addition, rigorously standardized protocols are obviously essential for any evaluation of the antioxidant efficiency of an EO, taking into account not only the conventional chemical characterization of its components but also the sequential evaluation of: (a) content in reducing substances, (b) RSC%, (c) anti-lipoperoxidant action, and (d) the quali/quantitative determination of the specific chemical constituents responsible for the antioxidant response. As recently reported by Bounatirou [19] for the essential oils from Tunisian *Thymus capitatus* Hoff. et Link, when considering our results from an applicability point of view, this study shows that the oil collected from *R. officinalis* L. during the flowering phase attained the best activity to prevent lipid oxidation and to act as biocide to combat bacterial pathogens.

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