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Formation of dehydrodiisoeugenol and dehydrodieugenol from the reaction of isoeugenol and eugenol with DPPH radical and their role in the radical scavenging activity

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ABSTRACT

The aim of this work was to investigate the products of the reactions between isoeugenol and eugenol with the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and their role in the radical scavenging mechanism. The reaction of isoeugenol and eugenol with the DPPH radical produced, as evidenced by GC–MS and HPLC–MS, a complex mixture of dimeric species in which dehydrodiisoeugenol and its adducts with methanol (reaction solvent) and dehydrodieugenol were the main reaction products, respectively. The antioxidant activity of dehydrodiisoeugenol, determined by the DPPH method, resulted lower than that of isoeugenol considering both the parameters Effective Concentration (EC_{50}) and Antiradical Efficiency (AE). In particular, due to its very slow kinetic behaviour ($T_{EC_{50}} = 201$ min), the possible contribution of dehydrodiisoeugenol to the DPPH radical scavenging activity of isoeugenol ($T_{EC_{50}} = 3.1$ min) was practically negligible. On the contrary, dehydrodieugenol had an antioxidant activity higher than that of eugenol and its lower $T_{EC_{50}}$ (85 min with respect to 126 min) made it possible to contribute to the DPPH radical scavenging activity of eugenol.

1. Introduction

Several analytical methods are routinely used to evaluate the antioxidant capacity of a large variety of compounds both in pure form and in complex mixture like herbs, spices, fruits and seeds extracts. Among them, the oxygen radical absorbance capacity (ORAC) assay, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay, the trolox equivalent antioxidant capacity (TEAC) assay and the ferric reducing antioxidant power (FRAP) assay are probably the most widely used. The ORAC method is based on the scavenging of peroxyl radicals generated by an azo compound, and fluorescein is used as target molecule. The decrease in fluorescence in the absence and in the presence of an antioxidant is monitored. The assay is carried out in phosphate buffer at pH 7.4, and the results are expressed as trolox equivalents (Cao, Alessio, & Cutler, 1993; Ou, Hampsch-Woodill, & Prior, 2001). The DPPH method is based on the scavenging of the stable DPPH radical by the antioxidant. The decrease in absorbance of the radical is monitored at 515 nm, in methyl alcohol, until the reaction reaches a steady state. The results are expressed by the parameters Effective Concentration (EC_{50}) that represent the [antioxidant]/[DPPH] concentration ratio, which decreases the initial DPPH concentration by 50%, the stoichiometry value (n) defined as the DPPH moles scavenged by one mole of antioxidant and the Antiradical Efficiency defined as $AE = 1/(EC_{50} \times T_{EC_{50}})$, where $T_{EC_{50}}$ represents the time needed to reach the steady state for EC_{50} (Brand-Williams, Cuvelier, & Berset, 1995; Sanchez-Moreno, Larrauri, & Saura-Calixto, 1998). With a similar approach, the Trolox Equivalent Antioxidant Capacity (TEAC) method is based on the ability of a compound to scavenge the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\bullet-}$) with respect to trolox C. The decrease in absorbance of the blue-green radical is monitored at 652 nm for 6–10 min. The assay is carried out in ethanol or in phosphate-buffered saline (PBS) at pH 7.4 and the results are expressed as trolox equivalents (Re et al., 1999). On the contrary, the FRAP method does not use free radical scavenging but is based on the reducing ability of the antioxidant versus the ferric-tripyridyltriazine complex. The increase in absorbance due to the formation of the coloured reduced ferrous complex is monitored and the results are expressed as trolox equivalents (Benzie & Strain, 1996). This brief description is enough to show the different approach and the different conditions of reaction time, reaction medium, temperature and treatment of the results used by these methods and the consequent difficulty to

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compare the results obtained for the same compounds. Foti and Ruberto (2001) observed a dependence of the antioxidant activity of some flavonoids and phenolic antioxidants on the hydrogen bond acceptor activity of the solvents. The effect of solvent and food components on the antioxidant activity obtained by applying the ORAC, DPPH, TEAC and FRAP methods to the mixture of catechin and gallic acid has been recently outlined by Perez-Jimenez and Saura-Calixto (2006). Also the oxidation processes involved are different: hydrogen atom transfer (HAT) in the case of the ORAC assay and single electron transfer (SET) in the other methods (Foti, Daquino, & Geraci, 2004; Huang, Ou, & Prior, 2005). These problems are even more enhanced by the possible occurrence of side reactions involving chemical species other than the antioxidant under testing. Studying the reaction mechanism of the DPPH radical with BHT, eugenol and isoeugenol, Brand-Williams et al. (1995) and Bondet, Brand-Williams, and Berset (1997) evidenced an inverse relationship between stoichiometry and reaction rates suggesting the intervention of a more complex reaction mechanism, eventually involving dimeric species, for the compounds with a slow kinetic behaviour such as eugenol. Recently, Ordoudi, Tsimidou, Vafiadis, and Bakalbassis (2006), studying the DPPH radical scavenging activity of guaiacol acid derivatives, reported the difficult to interpret the stoichiometry values of some compounds. Eklund et al. (2005) reported the formation of dimers in the reaction of lignans with DPPH radical. The EC_{50} of compounds with long reaction time can therefore reflect a radical scavenging capacity comprehensive not only of the contribution of the original antioxidant but also of other chemical species. Similar considerations were more recently outlined by Arts, Dallinga, Voss, Haenen, and Bast (2003) and Arts, Haenen, Voss, and Bast (2004) about the TEAC assay. In the reaction between $ABTS^{\cdot-}$ and the flavonoid chrysin, these authors reported the formation of a product with an antioxidant capacity higher than that of the parent compound. Hotta, Sakamoto, Nagano, Osakai, and Tsujino (2001) and Hotta, Nagano, Ueda, Tsujino, Koyama and Osakai (2002), studying the antioxidant capacity of natural compounds by column flow electrolysis, observed an increase of exchanged electrons by decreasing the flow and they attributed this effect to the oxidation of compounds formed by chemical reactions following the first oxidation process.

The aim of this work was to investigate the products formed by reaction of isoeugenol (**1**) and eugenol (**2**) with the DPPH radical under the same conditions used in the DPPH assay. The structural identification of these compounds, as well as the determination of their radical scavenging activity, may provide an insight into the antioxidant mechanism involved.

2. Materials and methods

2.1. Materials and reagents

All solvents were of analytical grade or LC–MS grade. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical, eugenol (**1**), isoeugenol (**2**; mixture of *E* and *Z* isomers), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and horseradish peroxidase (HRP, type II) were purchased from Sigma–Aldrich (Milan, Italy). Silica gel 60, 70–230 mesh ASTM and the thin-layer chromatography (TLC) silica gel plates, 0.25 mm thickness were provided by Merck (Darmstadt, Germany).

2.2. Melting point determination

Melting point determination was performed by differential scanning calorimetry (DSC) (Mettler TA 4000 system equipped with TC15 TA Controller, DSC 30 measuring cell and Star software version 8.10, Mettler, Greinfensee, Switzerland). Heat flow calibration was performed with indium, temperature calibration was done with *n*-hexane, distilled water and indium. Samples were placed into 40-μL aluminium pans that were hermetically sealed and scanned under 10 mL min^{-1} dry nitrogen flow to at least 10 °C above the endset of melting at a rate of 1 °C min^{-1}. The reported values were the average of two onset T_m.

2.3. ^1H and ^{13}C NMR spectra

^1H and ^{13}C NMR spectra were recorded on a Bruker AC-F 200 spectrometer at 200 and 50 MHz, respectively, using $CDCl_3$ as solvent at room temperature. NMR chemical shifts are reported as δ values from TMS.

2.4. Gas chromatography–mass spectrometry (GC–MS)

A Shimadzu gas chromatograph coupled to a quadrupole mass spectrometer QP-2010 (Shimadzu Corporation, Kyoto, Japan) was used. The column was a 30 m × 0.25 mm i.d., 0.25 μm film thickness, fused silica Equity 5 (Supelco, Sigma–Aldrich, Milan, Italy). The initial oven temperature was 60 °C for 2 min, then programmed to 280 °C at 15 °C min^{-1}. The injection was in splitless mode (2 min) with helium as carrier gas at a flow rate of 1 mL min^{-1}. The injector, transfer line and ion source temperatures were, respectively 280, 280 and 200 °C. The mass spectrometer operated in electron impact ionisation mode at 70 eV.

2.5. High performance liquid chromatography–mass spectrometry (HPLC–MS)

A Finnigan LXQ linear trap (Thermo Electron Corporation) was used for ESI-MS^n analysis in the positive and negative ion modes. The general conditions were capillary temperature 275 °C, sheath and auxiliary gas flows 25 and 5 (arbitrary units), respectively, source and capillary voltage in the positive polarity 4.0 kV and 2.0 V and 4.7 kV and −50.0 V in the negative polarity, respectively. Collision-induced dissociation (CID) multiple MS spectra (MS^n experiments) were acquired using helium as the collision damping gas in the ion trap at a pressure of 1 mTorr. Direct infusion of the standard solutions of pure dehydrodiisoeugenol (**3**) and dehydrodieugenol (**4**) and intact reaction mixtures diluted with MeOH or MeCN were performed with the aid of a syringe pump, using a flow rate of 5 μL min^{-1}. The LC-ESI-MS analyses were obtained with the same instrument coupled with a Finnigan Surveyor LC Pump Plus equipped with a Waters Spherisorb 5 μm ODS2 analytical column (4.6 × 250 mm) at 30 °C. Elution was carried out at a flow rate of 0.8 mL min^{-1} using an Accurate post-column flow splitter with a split ratio of 1:4 and MeCN/H_2O (65/35, v/v) as mobile phase.

2.6. DPPH radical scavenging method

The radical scavenging activity of the compounds was determined following the method used by Bortolomeazzi, Sebastianutto, Toniolo, and Pizzariello (2007). DPPH was dissolved in methanol at a final concentration of about 6×10^{-5} M. The exact concentration of DPPH was calculated from a calibration curve, ε = 11870 M^{-1} cm^{-1} at 515 nm. Different aliquots of a methanolic solution of the phenolic compounds were added to 2450 μL of DPPH solution and the volume was adjusted to a final value of 2500 μL with methanol. Five different concentrations were used for each assay. The decrease in the DPPH radical was followed at 515 nm until the reaction reached a steady state by using a Varian Cary 1E spectrophotometer equipped with a thermally controlled multicell block set at 25 °C. The concentrations of DPPH at the steady state, corrected for the natural disappearance of the DPPH radical, were plotted as a function of the molar concentration ratio

[AH]/[DPPH] to determine the Effective Concentration (EC_{50}). The time needed to reach the steady state for EC_{50} ($T_{EC_{50}}$) and the Antiradical Efficiency $AE = 1/(EC_{50} \times T_{EC_{50}})$ were also calculated.

2.7. Reaction of isoeugenol-DPPH radical

The conditions used to analyse the reaction products between isoeugenol (**1**) and DPPH were similar to those used in the DPPH assay. To 2450 μL of a methanolic DPPH solution were added 40 μL of methanol and 10 μL of an isoeugenol (**1**) methanolic solution. The final concentration was about 6×10^{-5} M and 4.4×10^{-5} M for DPPH and isoeugenol (**1**), respectively. After 5 min, the reaction mixture was reduced to dryness by a rotavapour at 40 °C, dissolved with 200 μL of acetonitrile and analysed by GC–MS. To the acetonitrile solution was then added 100 μL of BSTFA and was let to react for 1 h at room temperature before GC–MS analysis. The reaction was carried out also by using acetonitrile as solvent both for DPPH and isoeugenol (**1**) and analysed after 5 and 30 min.

2.8. Reaction of eugenol-DPPH radical

Similar conditions were used also for the analysis of the reaction products between DPPH radical and eugenol (**2**), final concentration was about 6×10^{-5} M and 4.7×10^{-5} M, respectively. The reaction was stopped after 10 min using methanol and after 30 min in the case of acetonitrile as solvents.

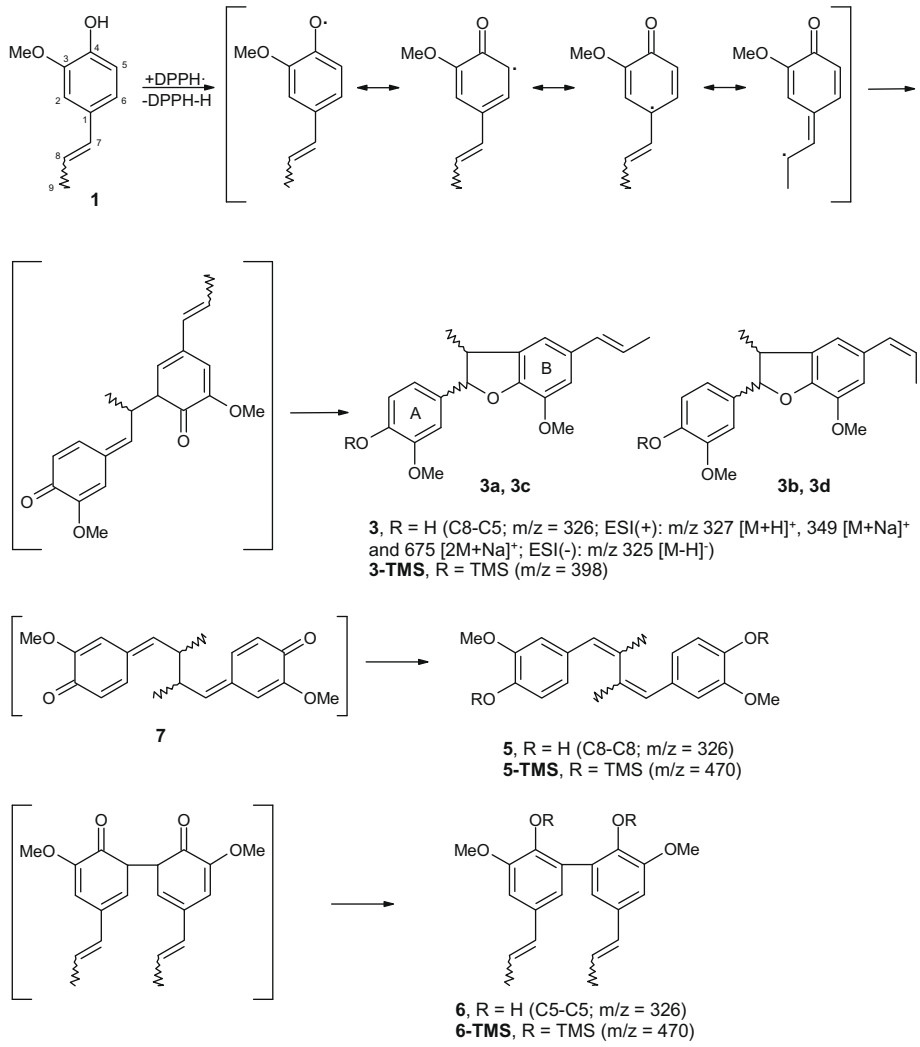
2.9. Synthesis of dehydrodiisoeugenol (*3*)

Dehydrodiisoeugenol (**3**) was prepared by oxidative coupling of isoeugenol (**1**) using the peroxidase-H_2O_2 system, following the method reported by Nascimento, Lopes, Davin and Lewis (2000) with minor modifications. To a solution of isoeugenol (**1**) (1.01 g, 6.2 mmol) in 30 mL of MeOH were added 275 mL of citrate–phosphate buffer (20 mM, pH 3) and horseradish peroxidase (8.5 mg, 1500 U) in 10 mL of buffer. 0.31 mL of H_2O_2 (3.05 mmol) was then added dropwise over 10 min and under magnetic stirring. The reaction mixture was stirred for an additional 10 min and then transferred in a separatory funnel and extracted with 100 mL of ethylacetate. The organic phase was washed with water, dried over anhydrous Na_2SO_4 and reduced to dryness. The crude product was loaded into a silica chromatography column (20 cm length × 2 cm i.d.) and eluted with *n*-hexane/ethylacetate mixture from an initial 8.5/1.5 (v/v) ratio. The chromatographic separation was monitored by TLC. The fractions containing the product (Rf 0.41, *n*-hexane/ethylacetate, 8/2, v/v) were combined, reduced to dryness (268 mg, 27%) and crystallised from MeOH/H_2O. m.p. 132.5 °C [lit. 128–130 °C (Shiba, Xiao, Miyakoshi, & Chen, 2000); 129–132 °C (Juhász, Kürti, & Antus, 2000)]; calcd. for $C_{20}H_{22}O_4$: C, 73.60; H, 6.79; found C, 73.56; H, 6.71. EIMS *m*/*z* (rel. int.): *m*/*z* 326 (M^+, 100), 311 (M^+-CH_3, 16), (1 × TMS) *m*/*z* 398 (M^+, 100), 383 (M^+-CH_3, 14), 368 ($M^+-2 \times CH_3$, 10). ESI (+)-MS (infusion, MeOH), *m*/*z*: 327 $[M + H]^+$ (MS^3 transitions 327 > 203 > 171), 349 $[M + Na]^+$, 675 $[2\,M + Na]^+$. ESI (−)-MS (infusion, MeOH), *m*/*z*: 325 $[M-H]^-$ (MS^5 transitions 325 > 310 > 295 > 277 > 249). ^1H NMR ($CDCl_3$/TMS): δ 1.37 (d, 3H, *J* = 6.8 Hz, ACH$_3$-9), 1.86 (dd, 3H, *J* = 6.4, 1.5 Hz, BCH$_3$-9), 3.37–3.52 (m, 1H, ACH-8), 3.86 (s, 3H, AMeO-3), 3.88 (s, 3H, BMeO-3), 5.09 (d, 1H, *J* = 9.5 Hz, ACH-7), 5.67 (br s, 1H, OH), 6.10 (dq, 1H, *J* = 15.6, 6.5 Hz, BCH-8), 6.36 (dd, 1H, *J* = 15.6, 1.5 Hz, BCH-7), 6.76 (br s, 1H, BCH-6), 6.78 (br s, 1H, BCH-2), 6.87–6.93 (m, 2H, ACH-5, ACH-6), 6.97 (br s, 1H, ACH-2). ^{13}C NMR ($CDCl_3$): δ 17.5 (AC-9), 18.3 (BC-9), 45.5 (AC-8), 55.8 (AMeO-3), 55.9 (BMeO-3), 93.7 (AC-7), 108.8 (AC-2), 109.1 (BC-2), 113.2 (BC-6), 114.0 (AC-5), 119.9 (AC-6), 123.4 (BC-8), 130.8 (BC-7), 132.0 (BC-1), 132.1 (AC-1), 133.2 (BC-5), 144.1 (BC-3), 145.7 (AC-4), 146.5 (BC-4), 146.6 (AC-3) (Scheme 1).

The GC–MS analysis of the crystallised product evidenced the presence of four compounds with practically the same mass spectra and M^+ at *m*/*z* 326. The relative retention times (RRTs) to the most abundant compound were 0.96, 0.97, 1, 1.01 and the corresponding percent chromatographic areas, calculated on the basis of the *m*/*z* 326 ion signal, were 2.1, 0.1, 95.7 and 2.1%, respectively. The HPLC–MS analysis of the same product evidenced the presence of three compounds with *m*/*z* 327 $[M + H]^+$ and the same fragmentation patterns till MS^3. Sarkanen and Wallis (1973) reported the formation of (*E*)-1-[(2*RS*,3*SR*)-2,3-dihydro-2-(4-hydroxy-3-methoxy phenyl)-7-methoxy-3-methyl-1-benzofuran-5-yl]-1-propene [(*E*)-(±)-*trans*-dehydrodiisoeugenol] (**3a**) and (*Z*)-1-[(2*RS*,3*SR*)-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-3-methyl-1-ben zofuran-5-yl]-1-propene [(*Z*)-(±)-*trans*-dehydrodiisoeugenol] (**3b**) by peroxidase catalysed oxidation of (*E*)- and (*Z*)-isoeugenol, respectively. Due to the presence in isoeugenol (**1**) standard of both the (*E*)- and (*Z*)-isomers in the proportion of about 93% and 7%, respectively, the formation of both the dehydrodimers **3a** and **3b** would be expected. On this basis the structure of (*E*)-(±)-*trans*-dehydrodiisoeugenol (**3a**) has been assigned to the most abundant compound and the structure of (*Z*)-(±)-*trans*-dehydrodiisoeugenol (**3b**) to one of the other three compounds. Regarding the other two, the following structures (*E*)-1-[(2*RS*,3*RS*)-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-3-methyl-1-ben zofuran-5-yl]-1-propene [(*E*)-(±)-*cis*-dehydrodiisoeugenol] (**3c**) and (*Z*)-1-[(2*RS*,3*RS*)-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-3-methyl-1-benzofuran-5-yl]-1-propene [(*Z*)-(±)-*cis*-dehydrodiisoeugenol] (**3d**) have been hypothesised (Scheme 1). The ^1H and ^{13}C NMR spectra were in agreement with the structure of (*E*)-(±)-*trans*-dehydrodiisoeugenol (**3a**) (Barbosa-Filho, Leitão da-Cunha, & Sobral da Silva, 1998; Juhász et al., 2000; Shiba et al., 2000) which purity was about 95.7%. The structure-numbering system used in the ^1H and ^{13}C NMR data does not follow the actual compound names but is based on the numbering of the monomer so as to maintain consistency.

2.10. Synthesis of dehydrodieugenol (*4*)

Dehydrodieugenol (**4**) was prepared by oxidative coupling of eugenol (**2**) using potassium ferricyanide as oxidising agent following the method reported by De Farias Dias (1988). To a solution of eugenol (**2**) (1.0 g, 6.1 mmol) in an acetone/H_2O mixture (2:1, v/v, 30 mL) 18 mL of aqueous NH_4OH (28%) was added and the mixture was stirred for 10 min. To the mixture a saturated aqueous solution of $K_3Fe(CN)_6$ (2.0 g, 6.1 mmol) was then added dropwise over a period of about 5 h and then 18 mL of aqueous NH_4OH (28%) was added. The mixture was stirred for an additional 16 h at room temperature and then neutralised with HCl 10%. There was the formation of a solid precipitate which was filtered, washed with water and dried (720 mg, 72%). The crude product was crystallised from ethanol after decolourisation with activated charcoal. m.p. 105.8 °C, [lit. 105–106 °C, (De Farias Dias, 1988)]; calcd. for $C_{20}H_{22}O_4$: C, 73.60; H, 6.79; found C, 73.10; H, 6.84. EIMS *m*/*z* (rel. int.): *m*/*z* 326 (M^+, 100), 297 (M^+-HCO, 34), 285 ($M^+-CH_2CHCH_2$, 6), 284 [$M^+-(CH_2CHCH_2 + H)$, 7], 267 [$M^+-(CH_2CHCH_2 + H_2O)$, 6], 253 [$M^+-(CH_2CHCH_2 + H + CH_3O)$, 22], 244 ($M^+-2 \times CH_2CHCH_2$, 11), 221 [$M^+-(CH_2CHCH_2 + H + CH_3O + CH_3OH)$, 9]; (2 × TMS) *m*/*z* 470 (M^+, 100), 455 (M^+-CH_3, 46), 440 ($M^+-2 \times CH_3$, 36). ESI (+)-MS (infusion, MeOH), *m*/*z*: 327 $[M + H]^+$, 349 $[M + Na]^+$ (MS^3 transitions 349 > 308 > 277), 675 $[2\,M + Na]^+$. ESI (−)-MS (infusion, MeOH), *m*/*z*: 325 $[M-H]^-$ (MS^5 transitions 325 > 310 > 295 > 254 > 226). ^1H NMR ($CDCl_3$/TMS): δ 3.36 (d, 4H, *J* = 6.7 Hz, CH_2-7, CH_2-7′), 3.89 (s, 6H, 2 × OMe), 5.01–5.18 (m, 4H, CH_2-9, CH_2-9′), 5.87–6.09 (m, 2H, CH-8, CH-8′), 6.05 (br s, 2H, OH), 6.72 (d, 2H, *J* = 1.9, CH-2, CH-2'), 6.75 (d, 2H, J = 1.9, CH-6, CH-6′). ^{13}C NMR ($CDCl_3$): δ 39.9 (C-7, C-7′), 56.0



Scheme 1.

(2 × OMe), 110.6 (C-2, C-2′), 115.6 (C-9, C-9′), 123.0 (C-6, C-6′), 124.4 (C-5, C-5′), 131.8 (C-1, C-1′), 137.6 (C-8, C-8′), 140.8 (C-4, C-4′), 147.2 (C-3, C-3′). The ^{1}H and ^{13}C NMR spectra were in agreement with the data reported in literature (De Farias Dias, 1988).

3. Results and discussion

3.1. Reaction products of isoeugenol (*1*)

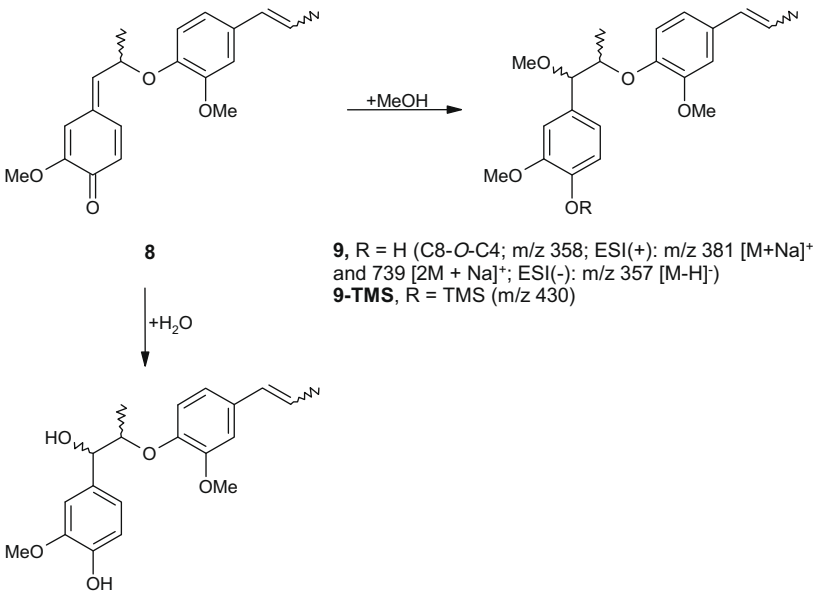
3.1.1. GC–MS analysis

The GC–MS chromatogram of the reaction mixture of isoeugenol (**1**) and DPPH radical in methanol evidenced the presence of two groups of compounds characterised by the M_r 326 and 358, respectively. The first group was composed of six compounds with M^+ at m/z 326 (Scheme 1), four of which had the same retention times of those present in the GC–MS chromatogram of the synthesised dehydrodiisoeugenol (**3**). In particular, the compound present in the highest amount (83.0%) had the same RT and mass spectrum of (*E*)-(±)-*trans*-dehydrodiisoeugenol (**3a**). The other two compounds with m/z 326 accounted for only 2.0% of the total chromatographic area.

The other group was composed of three compounds characterised by the same mass spectrum with M^+ at m/z 358. Compounds with M_r 358, which corresponded to an increase of 32 u with respect to the M_r 326, can be considered as the methanol adducts **9** arising from the addition of methanol to the C8–*O*–C4 quinone methide intermediate **8** (Scheme 2).

The formation of two diastereomeric forms of **9** was previously reported by Miller (1972) among the dimeric compounds obtained by 2,4,6-tri-*tert*-butylphenoxyl radical oxidation of (*E*)- or (*Z*)-isoeugenol in benzene/methanol solution. Also Chioccara et al. (1993) reported the formation of a mixture of *erythro* and *threo* diastereomers **9** by addition of methanol to a quinone methide intermediate formed by C8-*O*-C4 coupling of two phenoxyl radicals during the reaction of (*E*)-isoeugenol with hydrogen peroxide and HRP in aqueous buffer and methanol from 10% to 90%. The same authors observed an increase in the yields of these compounds with increasing methanol content. The addition of methanol to a quinone methide structure formed by reaction of lignans and DPPH radical was recently reported by Eklund et al. (2005). To confirm these structures, the reaction mixture was acetylated (Ac_2O, pyridine) and analysed by GC–MS. The analysis evidenced the presence of four compounds with m/z 400 (M^+) and fragment ions at m/z 237, 236, 209, 195, 194, 167 and 164. The M_r and the fragmentation patterns were in agreement with those reported by Chioccara et al. (1993) for the acetate derivatives of the methanol adducts of the C8–*O*–C4-coupled dimers **9**.

The GC–MS chromatogram of the TMS derivatives of the reaction products of isoeugenol (**1**) and DPPH radical in methanol is shown in Fig. 1A. Some compounds were detectable only after



Scheme 2.

TMS derivatisation probably due to a better chromatographic behaviour of the TMS derivatives and, in particular, of the compounds present in very low amount. The GC–MS analysis evidenced the presence of four compounds with M^+ at m/z 398 corresponding to 326 + TMS, four compounds with M^+ at m/z 430 corresponding to m/z 358 + TMS, three compounds with M^+ at m/z 470 corresponding to 326 + 2 × TMS and three compounds with M^+ at m/z 502 corresponding to 358 + 2 × TMS. The mixture of products resulting from the reaction between DPPH radical and **1** was very complex, also due to the presence of (*E*)- and (*Z*)-isoeugenol, and can be rationalised by considering basically two groups of compounds characterised by M_r 326 and 358, respectively. The compounds with M_r 326 (Scheme 1) are dimeric form of **1** which, after silylation, produced two separate classes of isomers. The first class had originally one phenolic hydroxy group and the M_r of 398 corresponds to a mono-TMS derivative. Among the dimers with one phenolic hydroxy group, the most abundant was that corresponding to (*E*)-(±)-*trans*-dehydrodiisoeugenol (**3a**) arising from a C8–C5 coupling process (Scheme 1).

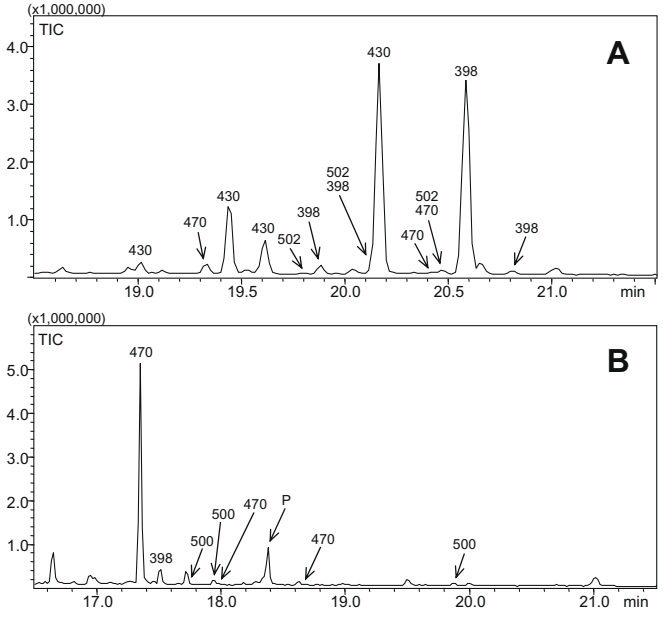
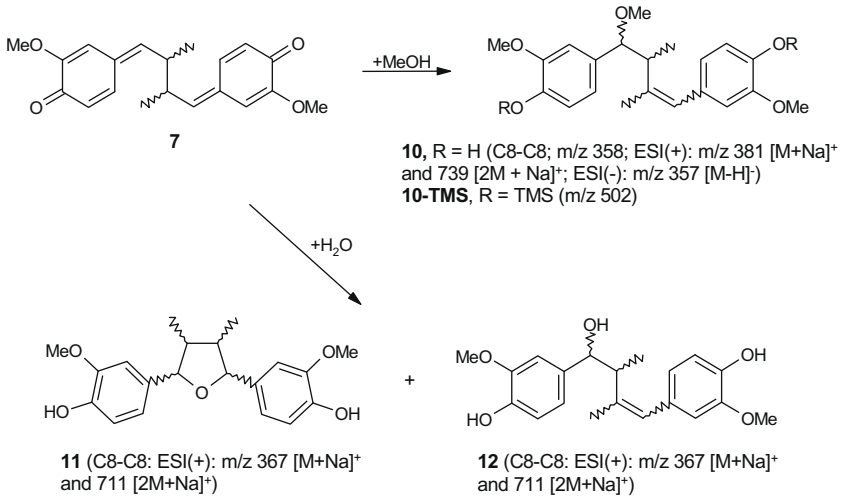


Fig. 1. GC–MS chromatograms of the dimer regions of the silylated reaction mixtures of isoeugenol (**1**) and eugenol (**2**) with DPPH radical in methanol. The number above the peaks represents the m/z ratio of the molecular ion of the corresponding compound. (**A**) isoeugenol – DPPH radical (for m/z 398 and 470 refer to Scheme 1, for m/z 430 refer to Scheme 2, for m/z 502 refer to Scheme 3); (**B**) eugenol – DPPH radical (for m/z 470 and 398 refer to Schemes 4 and 5, for m/z 500 refer to Scheme 6); P = phthalate contaminant.

The second class had two phenolic hydroxy groups and the M_r of 470 corresponded to a bis-TMS derivative. The dimers with two phenolic hydroxy groups **5** and **6** were present at a very low amount compared to **3a** and all the other compounds and can be originated from the C8–C8 and C5–C5 coupling processes, respectively, as hypothesised in Scheme 1. Sarkanen and Wallis (1973) reported the formation of compounds arising from the addition of water to a bisquinone methide system, such as **7** shown in Scheme 1, formed by a C8–C8 coupling process in the case of the enzyme-catalysed oxidation of both (*E*)- and (*Z*)-isoeugenol. Although the same authors did not report the formation of the C8–C8-coupled dimer **5** (Scheme 1), its formation in very low amount by aromatisation of bisquinone methide **7**, before the addition of water, cannot be excluded. Regarding the C5–C5-coupled dehydrodimer **6** (Scheme 1), Sarkanen and Wallis (1973) did not report its formation among the oxidation products of both (*E*)- and (*Z*)-isoeugenol. On the other hand, Ralph, Quideau, Grabber and Hatfield (1994) and Hatfield, Ralph and Grabber (1999) reported both the presence of C8–C8 and low amounts of the C5–C5 dehydrodimers of ferulic acid in plant cell wall extracts.

Like the compounds with M_r 326, also the compounds with M_r 358 produced, after silylation, two classes of isomers. The first class, which comprehended the methanol adducts of the C8–*O*–C4-coupled dimers **9**, had originally one phenolic hydroxy group and the M_r of 430 (326 + 32 + 72) corresponded to the mono-TMS derivatives **9-TMS** (Scheme 2).

The second class, present in very low amount, had two phenolic hydroxy groups and the M_r of 502 (326 + 32 + 2 × 72) corresponded to a bis-TMS derivative. The addition of methanol can take place only to the quinone methide intermediate **7** formed by the C8–C8 coupling process yielding **10** (Scheme 3). This consideration



Scheme 3.

suggested that the dimers with two hydroxy groups were probably of the C8–C8 type (Scheme 1). The necessity of the presence of methanol to produce the adducts was demonstrated by carrying out the reaction in acetonitrile. In this case neither compounds with M_r 358, due to methanol addition, nor compounds with M_r 430 and 502 after silylation were observed.

Besides the dimers, other peaks corresponding to unreacted isoeugenol (**1**) and DPPH radical thermodegradation products were present in the GC–MS chromatogram, in particular diphenylamine and 2,4,6-trinitroaniline, which were the most abundant compounds in the reaction mixture as identified on the basis of the mass spectrum and comparison with the NIST library.

3.1.2. HPLC–MS analysis

Due to the possible risk of artifacts formation in the hot injection port of the gas chromatograph, the reaction mixture between isoeugenol (**1**) and DPPH radical was analysed also by HPLC–MS. The HPLC–MS analysis evidenced the presence of three compounds with ESI (+)-MS at m/z 327 $[M + H]^+$, 349 $[M + Na]^+$ and 675 $[2\,M + Na]^+$ and ESI (−)-MS at m/z 325 $[M - H]^-$ with the same retention times and fragmentation patterns of the synthesised dehydrodiisoeugenol (**3**). Moreover, three compounds with ESI (+)-MS at m/z 381 $[326 + 32 + Na]^+$ and 739 $[2 \times (356 + 32) + Na]^+$ and ESI (−)-MS at m/z 357 $[326 + 32 - H]^-$ which corresponded to the methanol adducts **9** and/or **10** were present (Schemes 2 and 3). These results confirmed the previous findings obtained by GC–MS apart from the number of isomers detected which can depend by the different resolving powers of the two chromatographic techniques. HPLC–MS analysis showed six other compounds with m/z at 367 $[326 + 18 + Na]^+$ and 711 $[2 \times (326 + 18) + Na]^+$ which probably corresponded to the addition of water to the quinone methides **7** and **8** with the formation of **11** and **12** (Scheme 3) and **13** (Scheme 2), respectively.

The formation of **11** (Sarkanen and Wallis, 1973) and **13** (Sarkanen and Wallis, 1973; Shiba et al., 2000) was previously reported in the case of the enzyme-catalysed oxidation of **1** moreover, like the addition of methanol leading to **10**, the addition of water to **7** could afford **12** (Scheme 3). The formation of water adducts could be due to the dilution of the methanolic reaction mixture with water to a final concentration of about 35% like the mobile phase prior to HPLC–MS analysis.

The HPLC–MS analysis of the reaction mixture carried out in acetonitrile and diluted with water, before analysis, evidenced the formation of the dimers of **1** and the water adducts, but not the formation of the methanol adducts, confirming the results obtained by GC–MS. The reactions carried out in acetonitrile were visibly slower than those carried out in methanol.

Neither termination products by coupling of **1** and DPPH radicals nor compounds arising by the abstraction of two hydrogen atoms from **1** were detected under our experimental conditions.

From the data obtained it can be concluded that the main products formed by reaction of isoeugenol (**1**) and DPPH radical in methanol were the (*E*)-(±)-*trans*-dehydrodiisoeugenol (**3a**) and the methanol adducts **9**.

*3.2. Reaction products of eugenol (**2**)*

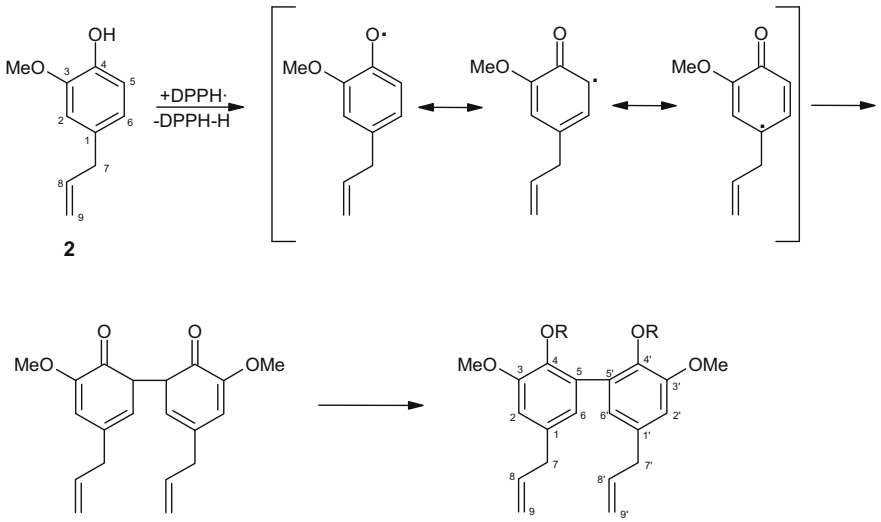
3.2.1. GC–MS analysis

The GC–MS chromatogram of the TMS derivatives of the reaction products of eugenol (**2**) and DPPH radical in methanol is reported in Fig. 1B. The GC–MS analysis evidenced the presence of three peaks with M_r 470, one peak with M_r 398 and three peaks with M_r 500. The compounds with M_r 470 ($326 + 2 \times$ TMS) corresponded to the bis-TMS derivatives of eugenol dimers and the most abundant had the same retention time and mass spectrum of the TMS derivative of the dehydrodieugenol (**4**) synthesised, derived from a C5–C5 coupling process (Scheme 4). The other two compounds, which accounted for only 0.2% of the chromatographic area, could arise from the addition of eugenol-5-radical **14** at the 7 and 9 position of the quinone methide intermediate **15** affording **16** and **17**, respectively, as hypothesised in Scheme 5.

The compound with M_r 398 (326 + TMS) could be interpreted as mono-TMS derivative of eugenol dimer with only one hydroxy phenolic group, a C5–*O*–C4 coupling process could be the mechanism leading to compound **18** (Scheme 5). The formation of a compound formed by a C5–*O*–C4 coupling process was reported by Sy and Brown (1998) in the case of the oxidative coupling of 4-allylphenol.

The compounds with M_r 500 ($326 - 2 + 32 + 2 \times$ TMS), present in very low amount, could have structures **19** and **20**, corresponding to the bis-TMS derivatives of the addition products of methanol to the quinone methide **21**, as hypothesised in Scheme 6. As in the case of isoeugenol (**1**), the necessity of the presence of methanol to produce these adducts was demonstrated by carrying out the reaction in acetonitrile, in this case no compounds with M_r 500 were observed.

Unreacted eugenol (**2**) and DPPH radical thermodegradation products were also present in the GC–MS analysis of the reaction mixture.



Scheme 4.

3.2.2. HPLC–MS analysis

The HPLC–MS analysis of the reaction mixture of eugenol (**2**) and DPPH radical evidenced the presence of a compound with ESI (+)-MS at *m/z* 327 $[M + H]^+$, 349 $[M + Na]^+$ and 675 $[2\,M + Na]^+$ and ESI (−)-MS at *m/z* 325 $[M - H]^-$ corresponding, as retention time and mass spectrum, to the synthesised dehydrodieugenol (**4**). Moreover, two compounds with *m/z* 379 $[326 - 2 + 32 + Na]^+$ and 355 $[326 - 2 + 32 - H]^-$ which could correspond to **19** and **20** (Scheme 6) were present. These results confirmed the previous findings obtained by GC–MS.

As in the case of isoeugenol (**1**), the reactions carried out in acetonitrile were visibly slower than those carried out in methanol, and neither termination products by coupling of **2** and DPPH radicals nor compounds arising by the abstraction of two hydrogen atoms from **2** were detected under our experimental conditions.

*3.3. DPPH radical scavenging activity of dehydrodiisoeugenol (**3**) and dehydrodieugenol (**4**)*

The DPPH radical scavenging activity of **3** and **4** was determined in order to evaluate their possible contribution to the activity of the corresponding parent compounds, isoeugenol (**1**) and eugenol (**2**), respectively. Only these two dimeric species have been considered from the point of view of the antioxidant activity being the most abundant among the products originated from the reactions of **1** and **2** with the DPPH radical. The results expressed as EC_{50}, stoichiometry (*n*), $T_{EC_{50}}$ and AE are reported in Table 1 together with the radical scavenging activity of **1** and **2** previously determined by Bortolomeazzi et al. (2007) under similar conditions of analysis.

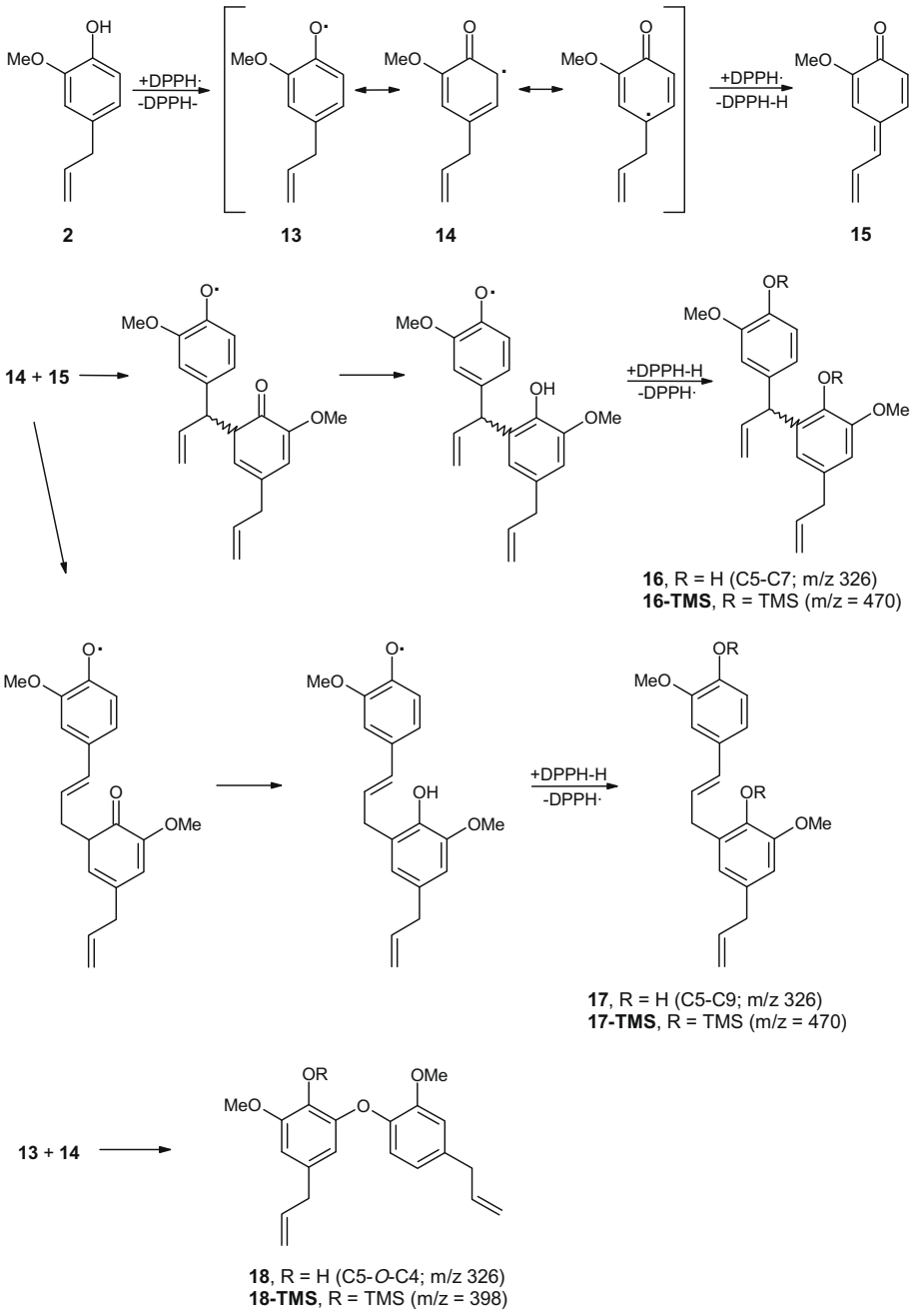
The EC_{50} of dehydrodiisoeugenol (**3**) was slightly higher than that of isoeugenol (**1**) and one mole of both compounds roughly scavenged one mole of DPPH, with a stoichiometry value of 0.7 and 0.9, respectively, corresponding to the number of phenolic OH present in the molecular structure. On the contrary, their kinetic behaviour was completely different with **3** acting much more slowly ($T_{EC_{50}} = 201$ min) than **1** ($T_{EC_{50}} = 3.1$ min). The very rapid kinetic of **1** has been explained by the presence, at the para position relative to phenol, of a propenyl group with the double bond conjugated to the benzene ring. This allows a better stabilisation of the phenoxyl radical by extending the delocalisation and lowers the Bond Dissociation Enthalpy (BDE) of the phenolic O–H bond (Barclay, Xi, & Norris, 1997; Wrigth, 2002; Wrigth, Johnson, & Di Labio, 2001). This structure was lost in the formation of **3** and the eventually stabilising electron-donating (+I) effect of the aliphatic part of new heterocyclic moiety was moreover impaired by the −I effect of the oxygen linked to the ᴀC7 in **3** (Scheme 1).

The radical scavenging activity of the methanol adducts **9** was not determined, due to the lack of the pure compounds; however, on the basis of their structure it was sensible to hypothesise an antioxidant activity similar to that of **3**.

Sanchez-Moreno et al. (1998) outlined the presence of compounds with comparable EC_{50} but with very different kinetic behaviour. These authors introduced the parameter AE that takes into account both the radical scavenging activity (EC_{50}) and the kinetic behaviour ($T_{EC_{50}}$). On the basis of the AE values, isoeugenol (**1**) resulted about 90 times more active than its corresponding dimer dehydrodiisoeugenol (**3**) (Table 1).

Dehydrodieugenol (**4**) had an EC_{50} value lower than that of eugenol (**2**) and one mole can scavenge about 2.7 mol of DPPH, whereas **2** scavenged 2 mol of DPPH radical. Also the $T_{EC_{50}}$ value was lower and, on the basis of the AE parameter, the antioxidant activity of **4** resulted about twice that of **2** (Table 1). The higher activity of **4** with respect to **2** can be explained by the presence of two phenolic OH groups and an ortho benzene ring which allowed an extensive conjugation stabilising the phenoxyl radical of **4**.

The $T_{EC_{50}}$ values of the compounds analysed deserve some more comments. The $T_{EC_{50}}$ of isoeugenol (**1**) was very low in comparison to those of dehydrodiisoeugenol (**3**) and eugenol (**2**) attributing this effect to the lower BDE of the phenolic OH bond in **1** as a consequence of the extended conjugation. A similar consideration has been applied also to **4** although, in this case, the substituent is attached to the ortho position, relative to the two phenolic OH. Wrigth et al. (2001) reported that a vinyl group in the ortho position relative to phenol showed a stabilising electronic effect only slightly lower than in the para position. On this basis, the kinetic behaviour of dehydrodieugenol (**4**) could be expected to be more rapid with a $T_{EC_{50}}$ more similar to that of isoeugenol (**1**). The slow reaction between **4** and DPPH radical can, however, be explained

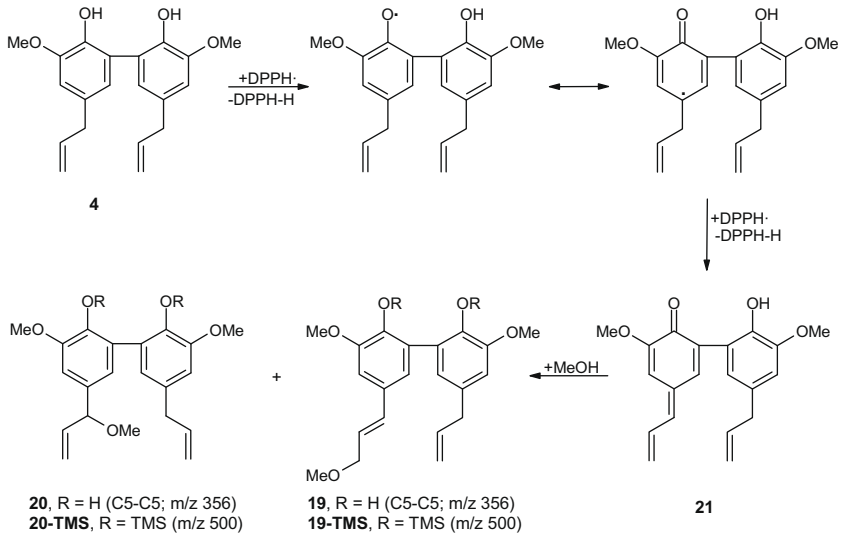


Scheme 5.

by the intervention of steric hindrance which can interfere with the planar conformation of **4** necessary to achieve optimal stabilisation of the phenoxyl radical by resonance as hypothesised by Barclay et al. (1997) in the case of 2,2′-dihydroxy-3,3′-dimethoxy-5,5′-dimethoxymethylbiphenyl.

Brand-Williams et al. (1995) and Bondet et al. (1997) reported that in the case of compounds reacting very quickly, the number of DPPH molecules reduced equals the number of hydroxyl groups present in the antioxidant molecule ($n = 1$). On the contrary, in the case of slow reacting compounds the stoichiometric value is, in general, larger than one ($n > 1$). This aspect was evidenced by the behaviour of isoeugenol (**1**) and eugenol (**2**) which had a kinetic fast (0.5 min) and slow (120 min) and $n = 1.1$ and 1.9, respectively. These authors explained the finding of stoichiometric values larger than one by hypothesising three reaction pathways after the initial formation of the antioxidant radical, such as donation of a second hydrogen atom to DPPH radical, dimerisation by coupling of two antioxidant radicals and coupling of antioxidant and DPPH radicals. In particular, for **2**, these authors suggested a dimerisation mechanism, while in the case of **1** none of these reactions would take place.

The results obtained in our work evidenced that, contrary to the hypothesis of Brand-Williams et al. (1995) and Bondet et al. (1997), the phenoxyl radical of isoeugenol (**1**) underwent extensive dimerisation and, moreover, the solvent methanol was involved in the reaction mechanism. The 0.9 stoichiometric value of **1** (Table 1) can, however, be explained by the very low kinetic behaviour of dehydrodiisoeugenol (**3**) with respect to **1**. When the DPPH method was applied to **1**, the time of the analysis was too short for the formed **3**, and eventually the methanol adducts **9**, to reveal their contribution to the total radical scavenging activity of **1**.



Scheme 6.

Table 1
Effective Concentration (EC_{50}), stoichiometry (n), $T_{EC_{50}}$ and antiradical efficiency (AE) obtained with the DPPH assay. (The values are the mean of three determinations ± standard deviation).

Compound	EC_{50}[a]	n[b]	$T_{EC_{50}}$[c]	AE × 10^3[d]
Isoeugenol (**1**)	0.54[e] ± 0.04	0.93 ± 0.07	3.12 ± 0.06	592 ± 45[e]
Dehydrodiisoeugenol (**3**)	0.74 ± 0.02	0.67 ± 0.02	201 ± 3	6.7 ± 0.3
Eugenol (**2**)	0.26[e] ± 0.01	1.94 ± 0.07	126 ± 8	31 ± 2[e]
Dehydrodieugenol (**4**)	0.184 ± 0.002	2.72 ± 0.03	85 ± 1	64 ± 1

[a] Mol AH/mol DPPH.
[b] Mol DPPH/mol AH.
[c] Min.
[d] Mol DPPH/(mol AH × t); AH, phenolic antioxidant.
[e] Bortolomeazzi et al. (2007).

In the case of eugenol (**2**), the formation of dehydrodieugenol (**4**), as the main reaction product, confirmed the hypothesis of Brand-Williams et al. (1995) and Bondet et al. (1997) regarding the dimerisation of **2**. The 1.9 stoichiometry value of **2** (Table 1) can then be explained by the formation of the dimer and its reaction with DPPH radical. In fact, considering the $T_{EC_{50}}$ of **2** and **4**, the time window of the reaction between DPPH radical and **2** can overlap with that of the reaction between DPPH radical and **4** which can then contribute to the radical scavenging activity of eugenol (**2**).

The formation of a complex mixture of dimeric compounds originated by different coupling processes and finding that also the solvent, methanol, and eventually water can be involved, evidenced that the reaction mechanism can be more complex than previously hypothesised. The possible contribution of the reaction products to the radical scavenging activity of a compound limits the use of the DPPH method to evaluate structure–activity relationship and the possibility to correlate the results with the antioxidant activity obtained by other methods.

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