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Comparison of Antioxidant Activity Between Aromatic Indolinonic Nitroxides and Natural and Synthetic Antioxidants

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In view of the possible employment of nitroxide compounds in various fields, it is important to know how they compare with other synthetic antioxidant compounds currently used in several industries and with naturally occurring antioxidants. To address this issue, the antioxidant activity of two aromatic indolinonic nitroxides synthesized by us was compared with both commercial phenolic antioxidants (BHT and BHA) and with natural phenolic antioxidants (α-hydroxytyrosol, tyrosol, caffeic acid, α-tocopherol). DPPH radical scavenging ability and the inhibition of both lipid and protein oxidation induced by the peroxyl-radical generator, AAPH, were evaluated. The results obtained show that overall: (i) the reduced forms of the nitroxide compounds are better scavengers of DPPH radical than butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) but less efficient than the natural compounds; (ii) the nitroxides inhibit both linolenic acid micelles and bovine serum albumin (BSA) oxidation to similar extents as most of the other compounds in a concentration-dependent fashion. Since the aromatic nitroxides tested in this study are less toxic than BHT, these compounds may be regarded as potential, alternative sources for several applications. The mechanisms underlying the antioxidant activity of nitroxides were further confirmed by UV–Vis absorption spectroscopy experiments and macroscale reactions in the presence of radicals generated by thermolabile azo-compounds. Distribution coefficients in octanol/buffer of the nitroxides and the other compounds were also determined as a measure of lipophilicity.

Keywords: Aromatic nitroxides; Antioxidants; Free radicals; Natural and synthetic antioxidants; Partition coefficients; Lipid and protein oxidation

INTRODUCTION

Nitroxide radicals are a group of compounds bearing an unpaired electron on the N—O function included in an aliphatic or aromatic ring system.[1] The original and still most popular use of these compounds is as spin labels and probes for understanding the structure and dynamics of membranes, proteins and other biopolymers.[2] Their use as contrast agents for nuclear and electron magnetic resonance imaging is also very common.[3] Moreover, they are gaining popularity as a distinct class of antioxidants since their protective effects against oxidative stress in a multiplicity of biological systems have now been widely established.[4–6] Of the many different classes of nitroxide radicals, we have been particularly interested in aromatic indolinonic nitroxides synthesized by us. These compounds efficiently react with a wide range of free radicals (alkyl, peroxyl, alkoxyl, hydroxyl, superoxide, thiyl and nitric monoxide)[7–12] that participate in the oxidation of biomolecules, and all reaction products and mechanisms have been chemically characterized and understood. In addition, nitroxides oxidize reduced metals, thereby inhibiting their participation in metal-catalyzed, free radical-generating reactions.[12,13] As confirmation of their scavenging activity and hence, antioxidant character, different biological systems exposed to various forms of oxidative stress have been protected

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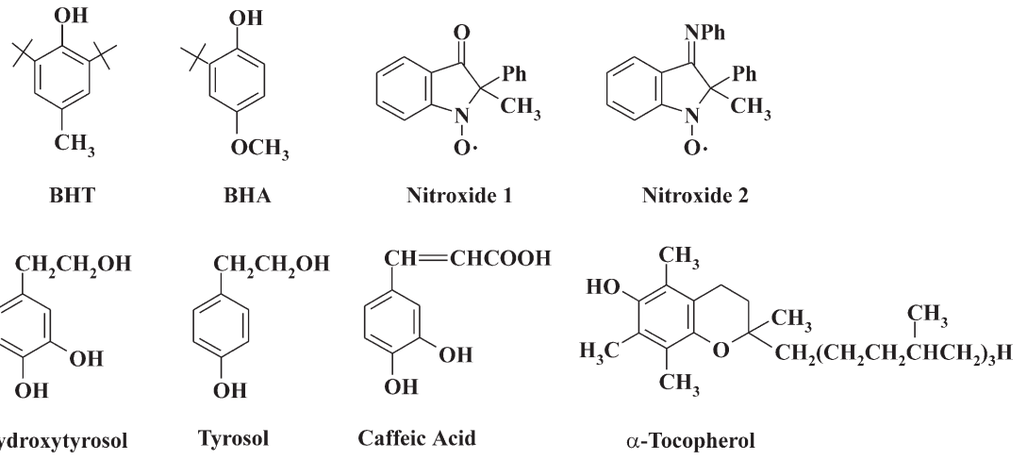

FIGURE 1 Chemical structures of the compounds studied.

by the presence of these compounds.[14–19] The use of these nitroxides as antioxidants on a large scale and for different purposes is now envisaged, so it is important to gain as much information on them as possible. For example, it is significant to know how they compare with other synthetic antioxidant compounds currently employed in the cosmetic and food industries or with naturally occurring antioxidant compounds. Therefore, the present study was undertaken to address this issue.

For this purpose, the antioxidant activity of indolinonic nitroxides **1** and **2** was compared with two commercial, synthetic and widespread phenolic antioxidants, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA)[20] and with the naturally occurring phenolic compounds α-hydroxytyrosol, tyrosol, caffeic acid, α-tocopherol (Fig. 1). These latter compounds were chosen as they are widely distributed in the plant kingdom and their beneficial effects is drawing much attention.[21–23] Three different experimental approaches were used to determine and compare the antioxidant potency of nitroxide radicals with the other compounds mentioned above: (a) DPPH radical scavenging ability, (b) lipid peroxidation, (c) protein oxidation. Distribution coefficients in octanol/buffer of the nitroxides and the other compounds were also determined as a measure of molecular lipophilicity since this is an important physicochemical parameter for many biological processes, such as membrane permeability.[24]

MATERIALS AND METHODS

Bovine serum albumin (BSA) (Fraction V, A-4503) was purchased from Sigma Chemical Co. (Milan, Italy), DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was purchased from Fluka Chemie (Zurich, Switzerland) while all other reagents, compounds and solvents were purchased from Aldrich Chemical Co. (Milan, Italy). Nitroxides **1** (1,2-dihydro-2-methyl-2-phenyl-3*H*-indol-3-one-1-oxyl) and **2** (1,2-dihydro-2-methyl-2-phenyl-3*H*-indol-3-phenylimino-1-oxyl) and their corresponding reduced forms (hydroxylamines) were synthesized as described previously by Berti *et al.*,[25] α-Hydroxytyrosol was synthesized according to the method reported in the literature with a few modifications.[26] In detail, 3,4-phenyldihydroxyacetic acid (0.5 g) was dissolved in 30 ml of anhydrous THF and $LiAlH_4$ powder (0.75 g) was added in small aliquots over a period of 30 min under vigorous magnetic stirring. The reaction was then refluxed for 3 h. Subsequently, the reaction was cooled and carefully and slowly poured into a beaker containing water and ice under magnetic stirring. The reaction was then acidified to pH 2–3 by 10% HCl and extracted with ethyl acetate (3 × 100 ml). The combined organic phases were washed with a saturated solution of sodium bicarbonate until reaching a permanent pH 8–9. The reaction was then dried with sodium sulphate, filtered and concentrated under reduced pressure. The compound was purified on a silica gel column (ethyl acetate/cyclohexane 1:1) from which an oil residue of 0.303 g (yield 66.0%) was obtained. The spectroscopic data were the same as those reported in the literature. The identity and purity of all the compounds synthesized were checked by thin-layer chromatography, by mass spectroscopy on a Carlo Erba QMD 1000 spectrometer in EI^+ mode, by NMR spectroscopy on a Varian Gemini 200 spectrometer in $CDCl_3$ or CD_3OD solutions and by electron spin resonance spectroscopy on a Varian E4 ESR spectrometer.

Scavenging of DPPH Free Radical

In an acetonitrile solution of DPPH (100 μM) test compounds dissolved in acetonitrile were added (20 μM), the reaction mixtures were shaken

vigorously and kept in the dark for 30 min. The absorbance of the remaining DPPH was then determined at 516 nm against a blank which lacked DPPH, on a UV Kontron 941 spectrophotometer. The scavenging activity was measured as the decrease in absorbance of DPPH, expressed as a percentage of the absorbance of a DPPH solution without test compounds [(A in the absence of compound − A in the presence of compound)/A in the absence of compound] × 100.

Peroxidation of Linolenic Acid Micelles

Linolenic acid micelles were prepared as follows: 20 μl linolenic acid were added to a round bottom flask containing 0.5 ml dichloromethane and the solvent was removed under a stream of nitrogen in an ice bath and then under vacuum for 30 min. The lipid film prepared was dispersed in 6 ml of 30 mM Tris-HCl buffer, pH 8.5 and vortexed for 15 min until a white, homogeneous, opalescent suspension was obtained. The final concentration of the resulting micellar suspension was 11 mM. Peroxidation of the micelles was followed by monitoring oxygen consumption on a Gilson oxygraph (Gilson Medical Electronics, Inc., Milwaukee, WI) with a Clark-type oxygen electrode (Clark Electromedical Instruments, Pangbourne, Essex, UK) in a final volume of 1.8 ml and at a constant temperature of 40°C after electrode stabilization. After 3 min from the introduction of the micelles (3 mM final concentration) in the reaction vessel containing 30 mM Tris buffer pH 8.5, 10 mM of the azo-initiator AAPH [2,2′-azobis(2-methylpropionamidine)dihydrochloride] was added to initiate the reaction. The reaction was followed for 12 min as this was the time it took for 100% consumption of oxygen in the presence of 10 mM AAPH. The effect of different concentrations of compounds (5–20 μM) on oxygen consumption in the presence of AAPH was then monitored by adding the appropriate amount of compound as acetonitrile solution (0.1% v/v) to the system above. The percentage of oxygen consumption in the presence of the compound tested during the 12 min time course of the reaction was considered an index of the extent of inhibition of linolenic acid peroxidation.

Protein Oxidation

Protein samples were prepared by dissolving 3 mg/ml of BSA in 50 mM potassium phosphate buffer, 0.1 mM EDTA, pH 7.4. The samples were then incubated at 50°C for 1 h in the presence or absence of 5 mM AAPH and/or different concentrations of compounds (50–100 μM). Appropriate amounts of compounds were added to the protein as acetonitrile solutions (2.5% v/v) and the mixture was vortexed prior to addition of AAPH for thorough incorporation.

The extent of protein oxidation was monitored by the method of Levine *et al.*, which uses the reaction of 2,4-dinitrophenylhydrazine (DNPH) with the carbonyl groups of oxidized proteins.[27] Briefly, 0.5 ml of 20 mM DNPH in 2.5 M HCl was added to 0.5 ml of each sample; blank samples lacked DNPH. Following 1 h of incubation at room temperature with continuous shaking, the protein was precipitated by addition of 2 ml 20% TCA and centrifuged at 3000*g* for 10 min. The protein was washed twice with ethanol/ethylacetate (1:1) and dissolved in 1 ml of 6 M guanidine HCl, pH 6.5. The absorbance was then read at 370 nm and protein carbonyls were evaluated using a molar absorption coefficient of 22,000 $M^{-1}\,cm^{-1}$.

Octanol/Buffer Distribution Coefficients

A solution of each compound (3 mM) in 1-octanol was kept at 60°C for an hour for complete dissolution. This solution was then diluted 100 times, a UV spectrum was run and the value of absorbance at the maximum was measured (A_0). Subsequently, equal volumes of organic solution and potassium phosphate buffer (0.1 M, pH 7.4) were mixed thoroughly by vortexing for 5 min at the highest speed and kept at room temperature for 30 min. The UV spectrum of the organic layer was then run again (A_x) and the partition coefficient (Log *P*) was calculated from the following relationship: $P = A_x/(A_o - A_x)$.[28] A solution of 1-octanol saturated with water was used as blank.

UV Experiments

In a final volume of 3 ml, the UV spectra of a mixture of 50 μM of nitroxide **1** or **2** in acetonitrile (2.5% v/v) and 5 mM AAPH in 50 mM phosphate buffer, 0.1 mM EDTA, pH 7.4, were recorded every 15 min for 1 h at 50°C.

Reactions of Nitroxides with AIBN

Nitroxides **1** and **2** were each reacted with AIBN [2,2′-azobis(2-methylpropionitrile)] using a molar ratio of 1:5 (nitroxide/AIBN) in acetonitrile solution, under magnetic stirring and at 70°C. The reactions were monitored by thin layer chromatography eluting with cyclohexane/acetonitrile 8:2 and after 30 min the reaction solutions turned from red to yellow and were complete. After cooling and concentrating under reduced pressure, the reaction mixtures were both chromatographed on silica gel preparative plates using cyclohexane/acetonitrile 8:2. From the reaction with nitroxide **1**, 83% of the corresponding alkylated hydroxylamine [1-(2-cyanopropyloxy)-1,2-dihydro-2-methyl-2-phenyl-3-oxo-3*H*-indole] was obtained

as the only product, while from the reaction with nitroxide **2**, 74% of the corresponding alkylated hydroxylamine [1-(2-cyanopropyloxy)-1,2-dihydro-2-methyl-2-phenyl-3-phenylimino-3*H*-indole] was obtained together with 10% of the corresponding quinoneimine N-oxide. The two alkylated hydroxylamines were identified by their NMR and Mass spectra while the quinoneimine N-oxide was identified by comparison with an authentic product. ^{1}H-NMR for alkylated hydroxylamine **1**: 1.17 (3H, s, CH_3), 1.68 (3H, s, CH_3), 1.73 (3H, s, CH_3), 7.30 (6H, m, arom.), 7.56 (1H, m, arom.), 7.71 (2H, m, arom.); MS: 306 (M + , 6), 238 (100), 222 (69), 194 (76). ^{1}H-NMR for alkylated hydroxylamine **2**: 1.09 (3H, s, CH_3), 1.65 (3H, s, CH_3), 1.89 (3H, s, CH_3), 6.44 (1H, d, arom., $J = 9.1$), 6.77 (3H, m, arom.), 7.09 (1H, m, arom.), 7.40 (9H, m, arom.); MS: 381 (M + , 2), 313 (52), 297 (100), 211 (98).

Appropriate controls were carried out throughout all the experiments described above and the results reported are an average of at least three independent experiments. Statistical comparisons were performed using the student *t*-test and differences were regarded as statistically significant when p values were <0.05 (*), <0.01 (**) and <0.001 (***).

RESULTS

The model of scavenging the stable DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical is a popular method used to evaluate the free radical scavenging ability of various chemicals in a relatively short time with respect to other methods.[22,29] Therefore, to compare the anti-radical activity of the nitroxides with the other compounds, this assay was used as first experimental approach. However, since the effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen-donating ability, the hydroxylamine derivatives (>N—OH) corresponding to nitroxides **1** and **2** were used instead of the nitroxides. The scavenging order of the test compounds, as can be deduced from the absorbance results reported in Fig. 2 and from the % of DPPH radical scavenged by each compound (in brackets), was:

Caffeic acid (45%) = α-Tocopherol (41%) ⩾ α-Hydroxytyrosol (31%) ⩾ Nitroxide **2**-OH (18%) ⩾ Nitroxide **1**-OH (15%) > BHA (12%) ⩾ BHT = Tyrosol (0%).

Another common method for screening antioxidative activity is to evaluate the ability of test compounds to inhibit AAPH-induced lipid

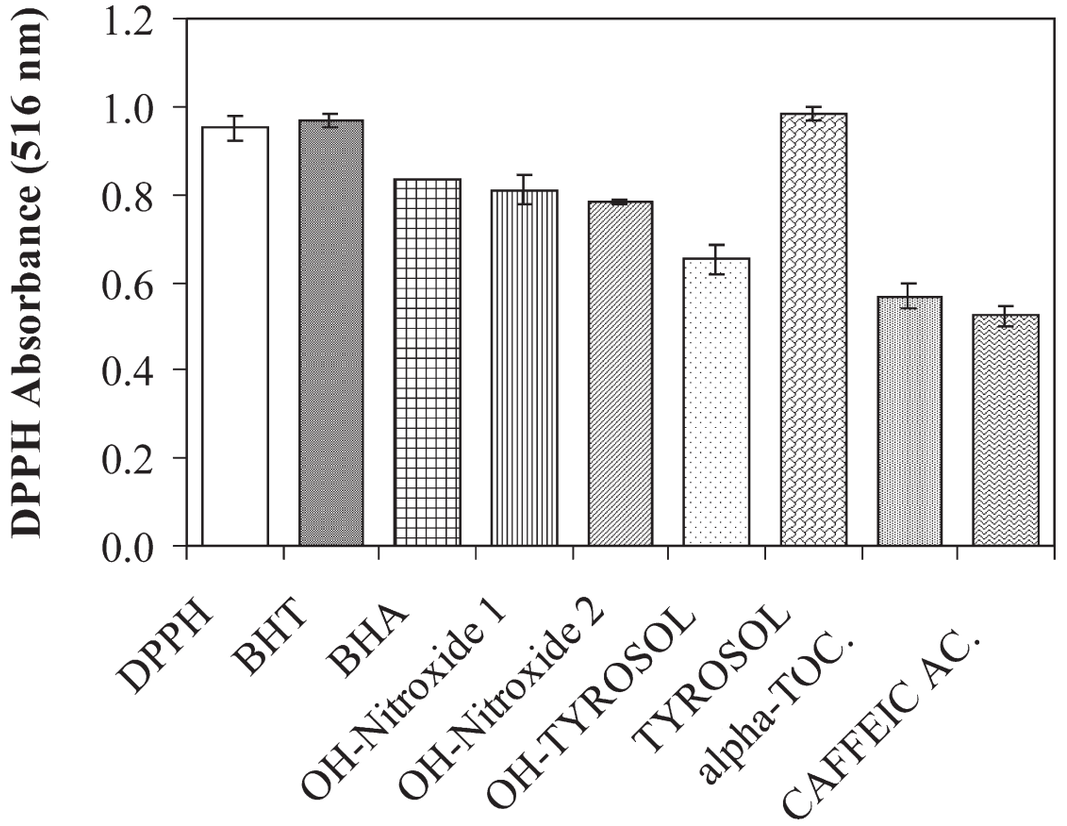

FIGURE 2 The effects of different compounds on the absorbance of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). In an acetonitrile solution of DPPH (100 μM), test compounds were added (20 μM) and the resulting absorbance at 516 nm was measured after 30 min. The first white bar indicates the absorbance of DPPH in the absence of test compounds (see Results for the % of DPPH radical scavenged).

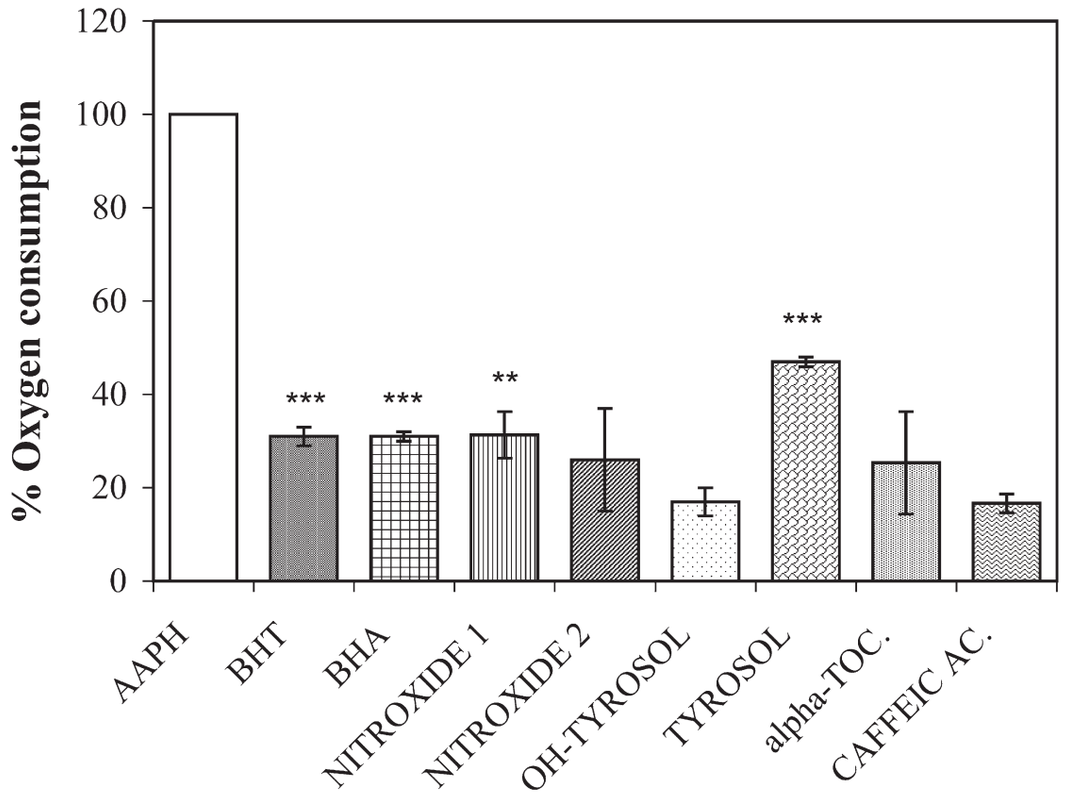

FIGURE 3 The effects of different compounds on oxygen consumption during peroxidation of linolenic acid micelles induced by AAPH. Oxygen consumption of linolenic acid micelles (3 mM) in 30 mM Tris-HCl buffer, pH 8.5, 37°C in the presence of 10 mM AAPH and 10 μM compounds was monitored for 12 min as this was the time it took for 100% consumption of oxygen in the presence of only AAPH (white bar).

peroxidation. The tertiary carbon radicals formed upon thermolysis of this azo-compound combine rapidly with oxygen generating a constant flux of peroxyl radicals which, in turn, induce lipid peroxidation by hydrogen abstraction.[30] Hence, during the early stages of this reaction there is rapid consumption of oxygen which can be easily monitored.[31] In this work, the percentage of oxygen consumption during the peroxidation of linolenic acid micelles in the presence and absence of compounds, was evaluated on a Clark-type oxygen electrode and the results are reported in Fig. 3. BHT, BHA, nitroxides **1** and **2** and α-tocopherol all inhibited oxygen consumption to similar extents (≈ 70% inhibition) at 10 μM concentration. Caffeic acid and α-hydroxytyrosol were more effective with almost 80% inhibition (statistical comparisons between data of these two compounds vs. the rest showed that they were significant except in the case of nitroxide **2** and α-tocopherol), whereas tyrosol was the least efficient exerting 60% inhibition of oxygen consumption. Figure 4 shows the effect of different concentrations of three selected compounds (BHT, representing a commercial antioxidant, α-hydroxytyrosol representing a natural antioxidant, and nitroxide **1** synthesized by us) on the inhibition of oxygen consumption during AAPH-induced linolenic acid peroxidation. Inhibition is clearly concentration-dependent in all three cases since at the highest concentration used (20 μM), inhibition of oxygen consumption is greater than that at 5 μM. At each concentration, the effects of BHT and nitroxide **1** were similar to each other, while α-hydroxytyrosol was more effective at 5- and 10 μM. At the highest concentration, all three compounds showed a similar degree of protection, although the differences between α-hydroxytyrosol and the other two compounds were statistically significant at all three concentrations.

Inhibition of protein oxidation by antioxidants is another approach which gives information on the antioxidant power of compounds. Here, BSA was oxidized by means of AAPH, in the presence and absence of compounds and the carbonyl content (an index of protein oxidation) was determined with DNPH.[27] This compound forms a hydrazone with carbonyl groups that can be easily quantified at 370 nm. Figure 5 shows the effects of 100 μM compounds on protein oxidation induced by 5 mM AAPH. As can be observed, the increase in oxidative modification of BSA during incubation with AAPH is reduced in the presence of 100 μM compounds by

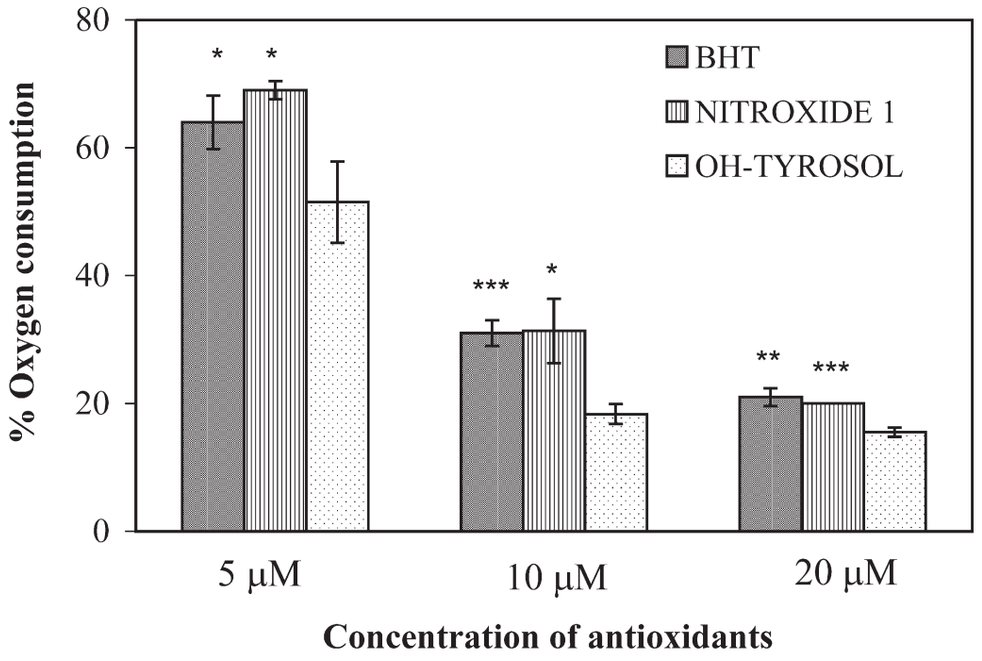

FIGURE 4 The effects of different concentrations of BHT, nitroxide **1** and α-hydroxytyrosol on oxygen consumption during peroxidation of linolenic acid micelles induced by AAPH. Oxygen consumption of linolenic acid micelles (3 mM) in 30 mM Tris-HCl buffer, pH 8.5, 37°C in the presence of 10 mM AAPH and different concentrations of compounds was monitored for 12 min as this was the time it took for 100% consumption of oxygen in the presence of only AAPH.

almost 50%. All the compounds protected protein oxidation with the exception of α-tocopherol whose protection was not statistically significant. Statistical data are reported vs. nitroxide **1** whose extent of protection compares with that of tyrosol and caffeic acid. On comparing the effects of BHT, nitroxide **1** and α-tocopherol at different concentrations, interesting results were obtained (Fig. 6). Inhibition is

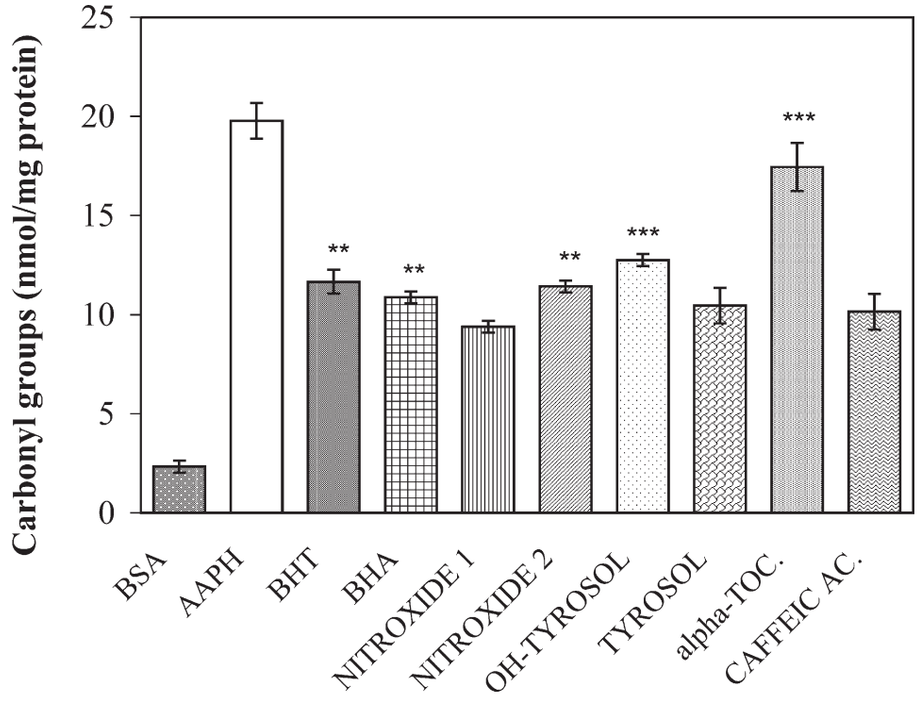

FIGURE 5 The effects of different compounds on protein carbonyl levels in BSA oxidized with AAPH. Carbonyl groups in BSA (3 mg/ml) were evaluated after 1 h incubation at 50°C in the presence of 5 mM AAPH alone (white bar) and in the presence of AAPH and 100 μM compounds in 50 mM phosphate buffer, 0.1 mM EDTA, pH 7.4.

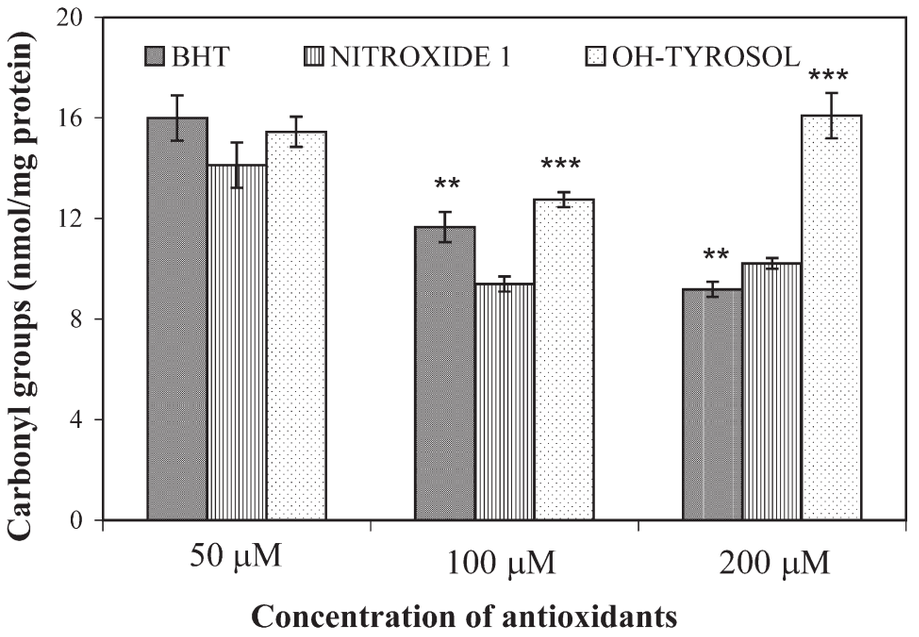

FIGURE 6 The effects of different concentrations of BHT, nitroxide **1** and α-hydroxytyrosol on protein carbonyl levels in BSA oxidized with AAPH. Carbonyl groups in BSA (3 mg/ml) were evaluated after 1 h incubation at 50°C in the presence of 5 mM AAPH and different concentrations of compounds in 50 mM phosphate buffer, 0.1 mM EDTA, pH 7.4.

concentration-dependent for BHT which shows maximum inhibition at the maximum concentration used (200 μM). For nitroxide **1**, maximum inhibition is achieved at 100 μM which is similar to that obtained using the highest concentration (200 μM), whereas the most effective concentration for α-hydroxytyrosol was 100 μM: increasing the concentration leads to less inhibition of protein carbonyl formation. Furthermore, BHT and nitroxide **1** at the two highest concentrations used are better antioxidants in this experimental system than the natural compound α-hydroxytyrosol. The statistical data are reported at all concentrations vs. nitroxide **1**.

The interaction of a compound with biomembranes, or the uptake of a compound into membranes, is strongly related to its lipophilicity expressed as the partition coefficient (Log *P*). This parameter may influence the bioavailability of the compound and hence its antioxidant activity and therefore, it should be determined.[24,28] The higher the partition coefficient, the more lipophilic the compound since its concentration in the 1-octanol layer is higher. From the results reported in Table I, it is clear that the most lipophilic compounds are the nitroxides **1** and **2** followed by α-tocopherol, while the catechol derivatives are the least lipophilic. The partition coefficients of the commercial synthetic antioxidants BHA and BHT fall in between these two groups of compounds. The results are an average of at least two determinations that differed by $< 16\%$.

TABLE I Partition coefficient of compounds studied

Compound	Log *P*
Caffeic acid	−0.65
Tyrosol	−0.10
α-Hydroxytyrosol	−0.05
BHA	0.42
BHT	0.59
α-Tocopherol	0.74
Nitroxide **2**	1.06
Nitroxide **1**	1.10

Log *P* was calculated from the following relationship: $P = A_x/(A_o - A_x)$, where A_o = absorbance of compound in octanol, A_x = absorbance of compound in equal volumes of octanol/phosphate buffer (0.1 M, pH 7.4) after 30 min (see "Materials and Methods" section).

In order to provide support to the radical scavenging mechanisms of the nitroxide compounds, the levels of the nitroxides **1** and **2** during their reaction with AAPH in phosphate buffer pH 7.4 was monitored spectrophotometrically and the results are reported in Fig. 7. The UV–Vis absorption spectra of the nitroxides (thick black line) changes after 1 h incubation with AAPH at 50°C. From the resulting spectra obtained, we deduce that nitroxide **1** is transformed into the corresponding alkylated hydroxylamine (thick grey line, Fig. 7A). This is based on the fact that when the lipid soluble azo-initiator AIBN is reacted with nitroxide **1**, this is the main product obtained (see "Materials and Methods" section). Unfortunately, it was impossible to carry out a macroscale reaction between nitroxides and AAPH due to the insolubility of AAPH in organic solvents. Nitroxide **2** (Fig. 7B) is instead transformed into the corresponding quinoneimine N-oxide (thick grey line) and confirmed by comparison of the UV spectrum with the authentic sample

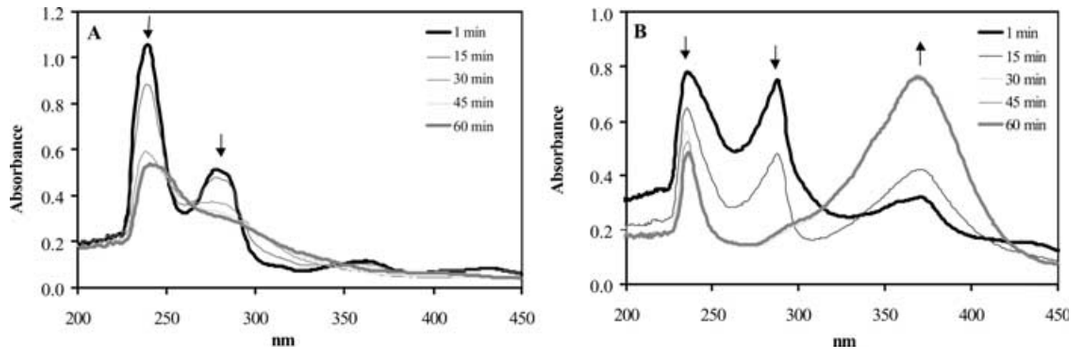

FIGURE 7 UV spectral changes of nitroxide **1** (A) and nitroxide **2** (B) in the presence of AAPH. The nitroxides (50 μM) were each incubated with 5 mM AAPH in 50 mM phosphate buffer, 0.1 mM EDTA, pH 7.4 at 50°C for 1 h and recordings were taken every 15 min.

obtained during the macroscale reaction with AIBN (see "Materials and Methods" section).

DISCUSSION

The purpose of this work was to compare the antioxidant efficacy of aromatic nitroxide compounds synthesized by us with both commercial, synthetic antioxidants and naturally occurring ones shown in Fig. 1. From the results reported above it is clear that the antioxidant activity of nitroxides **1** and **2** is comparable with all the other compounds tested, in all the systems studied.

With regard to lipid peroxidation, both nitroxides inhibit AAPH-induced oxygen consumption in linolenic acid micelles just as well as BHT and BHA and only slightly less with respect to the natural compounds, with the exception of tyrosol (Fig. 3). This pattern still holds when different concentrations were used for representatives of the three classes of compounds: aromatic nitroxides (nitroxide **1**), commercial phenolic antioxidants (BHT), natural phenolic antioxidants (α-hydroxytyrosol) (Fig. 4). Tutour *et al.*, have previously shown that α-hydroxytyrosol was more effective than BHT and α-tocopherol in inhibiting thermal initiated oxidation of methyl linoleate and the results obtained in the present work confirm these findings.[32] However, he also observed that tyrosol had neither antioxidant nor prooxidant activity, while here we demonstrate that tyrosol is capable of inhibiting oxygen consumption in linolenic acid micelles. Other comparative studies performed by others on natural and synthetic antioxidants also substantiate the trend of our findings since it is reported that α-hydroxytyrosol is more efficient than α-tocopherol in inhibiting bulk olive oil peroxidation[28] and that the addition of caffeic acid or α-tocopherol in lard and corn oil significantly extends the induction time of lipid oxidation more than BHT does.[22] The resistance of low density lipoproteins to oxidation is also greatly reduced by α-hydroxytyrosol compared to its mono-hydroxy counterpart, tyrosol.[33]

The mechanism underlying the antioxidant activity of all the phenolic compounds tested shown in Fig. 1, is that of the well-known classical hydrogen donation to peroxyl radicals, here generated by the well-known water-soluble azo-initiator, AAPH. The peroxyl radical is neutralized and propagation of lipid peroxidation is inhibited. Instead, from the chemical reactivity of aromatic nitroxide compounds, the mechanism by which these compounds act as antioxidants is that of trapping the carbon-centred radicals initially generated upon thermal decomposition of AAPH at the nitroxide function to give alkylated hydroxylamines,[7] or by trapping the peroxyl radical (formed by coupling of the carbon-centered radical with oxygen) on the conjugated benzene ring to give the quinoneimine N-oxide,[8] as shown in Fig. 8. To confirm this, UV spectra were run and changes in the absorbance of the nitroxides in the presence of AAPH were recorded. The spectra shown in Fig. 7A,B seem to point out that the rate constant for the reaction of nitroxide **1** with carbon-centred radicals derived from AAPH to give the alkylated hydroxylamine is greater than the rate of reaction of these radicals with oxygen, whereas the rate constant for the reaction of nitroxide **2** with carbon-centred radicals is probably slower. In fact, the resulting transformation product between nitroxide **2** and AAPH is the quinoneimine N-oxide which usually forms when nitroxide radicals trap peroxy radicals. The rate constant for the reaction of most carbon-centered radicals with oxygen is almost diffusion controlled ($10^9\,M^{-1}\,s^{-1}$) which is the same order of magnitude for the reaction of most alkyl radicals with nitroxides.[34] Therefore, it would seem that nitroxide **1** competes with oxygen for alkyl radicals better than nitroxide **2** does. Confirmation of this data comes from the macroscale reaction performed in air between the nitroxide and the lipid-soluble

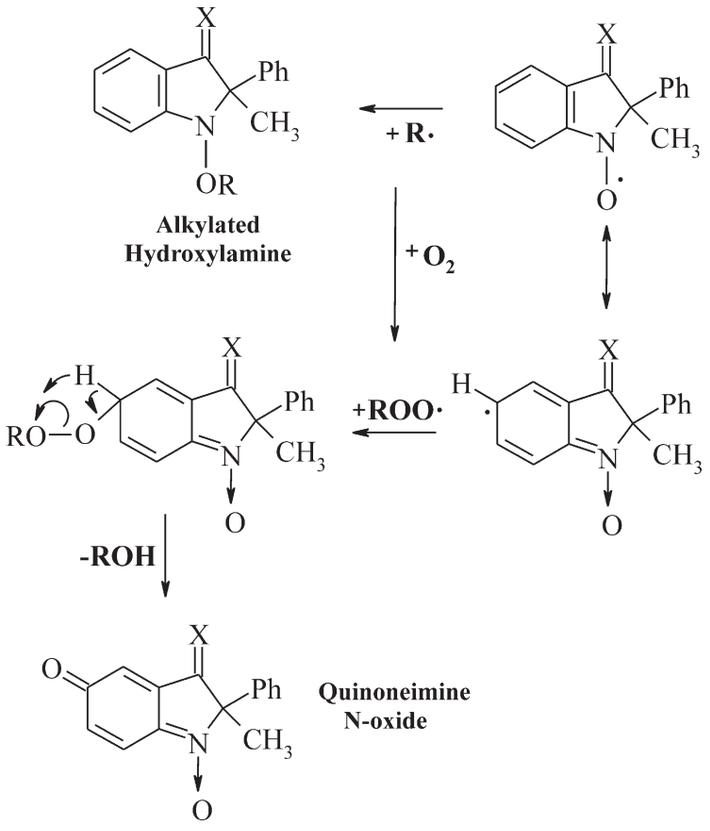

FIGURE 8 Scheme showing reactivity of indolinonic nitroxides with alkyl (R•) and peroxyl radicals (ROO•).

azo-initiator AIBN, where the only product obtained in the case of nitroxide **1** was the alkylated hydroxylamine while with nitroxide **2**, the quinoneimine N-oxide was also obtained in small quantities. However, the differences in behaviour between the two nitroxides in aqueous solution may be due to their different oxidation potentials rather than to actual trapping of peroxyl radicals on the conjugated benzene ring. In fact, nitroxide **2** has a lower oxidation potential (+0.905 V vs. SCE in DMF/H_2O) than nitroxide **1** (+1.010 V),[35] therefore oxidation to the corresponding oxoammonium cation by the peroxyl radicals derived from AAPH may be feasible. Through a series of steps involving addition of water and oxidation, the oxoammonium cation is transformed into the quinoneimine N-oxide as reported previously.[36] From the above facts it is clear that the main outcome is the evidence for scavenging/neutralizing free radical species by nitroxide compounds which lies at the basis of their antioxidant activity in biological systems.

It is also worth pointing out that the strong inhibition of oxygen consumption induced by the thermal decomposition of AAPH (10 mM) by the relatively low quantity of test compounds (10 μM) shown in Fig. 3 is most probably due to the fact that the half life of AAPH at 37°C in neutral aqueous solutions is 175 h, therefore during the time course of the experiments presented here (12 min), very little AAPH will have decomposed and that amount which does, is efficiently trapped by the antioxidants present according to the mechanisms described above. In this way, the initiation and propagation of lipid peroxidation is slowed down and oxygen consumption is reduced.

The nitroxides also reduced protein oxidation induced by AAPH to similar extents as the other compounds tested. In actual fact, nitroxide **1** at 100 μM was significantly more effective than most of the compounds with the exception of tyrosol and caffeic acid. Interestingly, tyrosol exerts the same degree of protection as caffeic acid and nitroxide **1**, hence it seems to be more efficient at inhibiting protein oxidation than lipid oxidation. At higher concentrations, α-hydroxytyrosol (200 μM) is not as protective towards oxidative modifications in BSA as at 100 μM. This could be due to some pro-oxidant effect, possibly of the quinone oxidized form, which may overwhelm the antioxidant effect of this compound at high concentrations in this protein system. Most antioxidant studies on the ubiquitous, natural compounds α-hydroxytyrosol, tyrosol, caffeic acid have focused on the oxidation of low density lipoproteins and other model lipid systems

as well as on DNA,[33,37–40] but data on their actual interaction and effect on just protein oxidation using a simple model system such as the one used here is scarce, if any. Therefore the findings reported here add additional information on the antioxidant behaviour of these compounds and further investigations should be performed to fully understand better their effects in all kinds of biological systems.

On comparing the efficacy towards scavenging of DPPH radical, the hydroxylamine derivatives of nitroxides **1** and **2** are more potent than BHT and BHA but less potent than the natural antioxidants tested. These differences may likely be due to the different bond dissociation enthalpies (BDE) of the O—H bond: BDEs of BHT and BHA are 80.70 and 77.61 kcal mol^{-1}, respectively[41] whereas the BDEs of indolinonic nitroxides are 70–71 kcal mol^{-1}.[42] However, the BDE of α-tocopherol is 78.93 kcal mol^{-1} and therefore one would expect it to be a poorer DPPH radical inhibitor than the two hydroxylamines and BHA, but this is not observed (Fig. 2). In fact, other factors such as the greater or lesser stabilizing effects of the resulting radical species formed after hydrogen donation also have a role in determining antioxidant activity. This can be further confirmed by comparing the results obtained between α-hydroxytyrosol and caffeic acid. Both compounds possess two phenolic hydroxyl groups in their ring which lowers the O—H bond dissociation enthalpy and increases the rate of hydrogen donation leading to an *ortho*-quinoid structure. But the unsaturated 2,3-double bond on the side chain of caffeic acid also maximizes the stabilization of the phenolic radical compared to α-hydroxytyrosol explaining its greater inhibitory effect towards DPPH.[39]

The hydrophobicity or partition coefficient of compounds also influences antioxidant behaviour. From the results obtained, it is clear that the most hydrophobic compounds are the nitroxides followed by α-tocopherol, therefore these are expected to penetrate phospholipid structures easier and faster than the other compounds tested. α-Tocopherol, as expected, is much more hydrophobic than the polyphenol derivatives which occupy the higher positions in the table and this order of hydrophobicity agrees well with the data reported in the literature.[28] Taking into account the Log *P* values reported in Table I, one would expect that the actual level of nitroxide **1** for example, accumulating in the micelles is 20-fold higher than that of α-hydroxytyrosol. Nevertheless, the inhibitory effect against lipid peroxidation of this latter compound is greater. This suggests that the nitroxides interact deeper within the micelles which might result in their lower interaction with the radicals generated from water-soluble oxidants such as AAPH used here. In fact, α-hydroxytyrosol and caffeic acid have lower partition coefficients and therefore would be more readily capable of intercepting the water-soluble radicals generated from AAPH. It would be interesting to observe how these same antioxidants behave using lipid-soluble oxidants where the initiating free radicals are generated within a hydrophobic compartment, and this is now a matter of future work.

In conclusion, the use of different experimental approaches (DDPH radical scavenging ability, lipid peroxidation, protein oxidation) to evaluate the potency of antioxidant activity is, therefore, important because as the results here demonstrate, activity in one test does not necessarily correlate with activity in another. This activity depends on several factors and not just on the chemical reactivity towards radicals (BHT and tyrosol do not react with DPPH but they are still efficient antioxidants) and hydrophobicity (nitroxides are the most hydrophobic but they are not the best lipid antioxidants in our model lipid system). Other factors such as chemical stability, mobility of the antioxidant at the microenvironment, fate of the antioxidant derived radical, hydrophobicity/hydrophilicity of the oxidant all play their role, and the net effect is the result of all these factors combined in the experimental system under investigation.

On examining the results overall, the aromatic nitroxides are efficient inhibitors of both protein and lipid oxidation just as the commercial antioxidants BHT and BHA are. Therefore, they may be regarded as new, alternative, synthetic antioxidant sources for possible applications in various fields (cosmetics, foods, polymers, plastics, paints). BHT and BHA are widely used in the food industry as direct food additives to protect against both protein and lipid oxidation of food but they are also used in indirect addition to food products through diffusion from polymeric films of the package.[43] Because the aromatic nitroxides tested in this study are less toxic than BHT, applications at this regard may be envisaged.[44]

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