Free Radical Research, 2003 Vol. 37 (4), pp. 405–412

Antioxidant Activity of Galloyl Quinic Derivatives Isolated from *P. lentiscus* Leaves

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Accepted by Professor J. Vina

(Received 1 July 2002; In revised form 21 October 2002)

The antioxidant properties of galloyl quinic derivatives isolated from *Pistacia lentiscus* L. leaves have been investigated by means of Electron Paramagnetic Resonance spectroscopy (EPR) and UV-Vis spectrophotometry. Antioxidant properties have been also estimated using the biologically relevant LDL test. The scavenger activities of gallic acid, 5-*O*-galloyl, 3,5-*O*-digalloyl, 3,4,5-*O*-trigalloyl quinic acid derivatives, have been estimated against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, superoxide ($O_2^{\cdot-}$) radical, and hydroxyl (OH) radical. On the whole, the scavenger activity raised as the number of galloyl groups on the quinic acid skeleton increased. The half-inhibition concentrations (IC_{50}) of di- and tri-galloyl derivatives did not exceed 30 μM for all the tested free radicals. All the tested metabolites strongly reduced the oxidation of low-density lipoproteins (LDL), following a trend similar to that observed for the scavenger ability against OH radical.

Keywords: EPR; Hydroxyl and superoxide radicals; LDL; Spin trapping

Abbreviations: DEPMPO, 5-Diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide; DPPH, 1,1-Diphenyl-2-picrylhydrazyl; EPR, Electron paramagnetic resonance; LDL, Low density lipoproteins; NBT, Nitro blue tetrazolium; PBS, Phosphate-buffered saline; TBARS, Thiobarbituric acid reactive substances; X, Xanthine; XO, Xanthine oxidase

INTRODUCTION

Pistacia lentiscus L., an evergreen member of the Anacardiaceae family is largely distributed on "extreme" ecosystems of Mediterranean basin, which are characterized by nutrient and water scarcity and are exposed to long periods of high solar radiation and high temperatures.[1] Evergreen Mediterranean species usually exhibit low photosynthetic and growth rates and allocate most carbon into defensive compounds.[2] Polyphenols serve a central role in the acclimation mechanisms of evergreen sclerophylls to unfavourable environmental agents, since polyphenols may efficiently (i) attenuate the penetration of damaging UV radiation,[3] (ii) dissipate excess radiant energy[4,5] and at the same time (iii) scavenge reactive oxygen species.[5,6] A recent report by Briskin[7] has interestingly linked the role served by secondary metabolites in the responses of plants to environmental stresses with their role in the human health care. For example, the broad class of gallotannins, which has been recognized to protect plant tissues from pathogens and predators,[8] also exhibits relevant antioxidant properties.[9] A plethora of data has been produced on the role served by galloyl derivatives of both catechin and epicatechin, the major polyphenol constituents of green tea, in inhibiting free-radical mediated carcinogenic and mutagenic processes in animal cells.[10–12] Although pharmacological uses of other galloyl derivatives have received less attention than that devoted to gallocatechins,[9,13] galloylquinic derivatives have been found to be both potent antioxidants and inhibitors of HIV infection.[13,14]

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1071576031000068618

Recently, it has been shown by Romani *et al.*[15] that *P. lentiscus* leaves are very rich (6–7% of leaf dry weight) in low-molecular weight gallotannins, namely 5-*O*-, 3,5-*O*-di- and 3,4,5-*O*-trigalloyl quinic acid derivatives, which have been previously detected in some members of *Fagaceae* and *Combretaceae*.[16,17]

In this paper, the scavenger activities of such metabolites, as well as those of gallic acid, against 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide (O_2^-) and hydroxyl (OH) radicals have been determined using both Electron Paramagnetic Resonance (EPR) and UV-Vis spectrophotometry. Finally, the relative abilities of galloyl derivatives in inhibiting the chemically-induced oxidation of low-density lipoproteins (LDL), here used as a biologically relevant model system,[18] have also been tested.

Our results show that both gallic acid and galloylquinic derivatives efficiently scavenge the tested free radicals, with a major activity showed by 3,5-*O*-di and 3,4,5-*O*-trigalloyl quinic derivatives. In addition, galloyl derivatives extracted from *P. lentiscus* leaves are effective in protecting LDL from oxidation in a low-concentration range.

MATERIALS AND METHODS

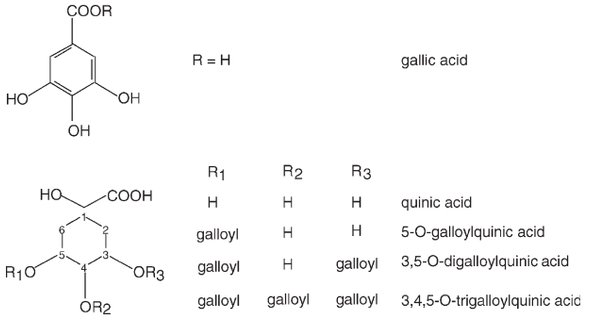
Xanthine (X), xanthine oxidase (XO), nitro blue tetrazolium (NBT) and DPPH were purchased from Sigma. The 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) was synthesized by us according to Frejaville *et al.*[19] Galloyl derivatives were extracted and isolated following the protocol previously reported.[15] The galloyl derivatives were stable in the solid state and soluble in ethanol, methanol and acetonitrile. Fresh solutions of galloyl derivatives and quinic acid were prepared for each measurement.

The chemical structures of galloyl derivatives are shown in Scheme 1.

EPR spectra were recorded on a Bruker 200D SRC X-band spectrometer. Frequency was measured using a XL Microwave Model 3120 Counter (Jagmar, Krakow, Poland). The spectrometer was interfaced to a PS/2 Technical Instrument computer and data recorded using a Stelar (Mede, Italy) data acquisition program.

The 1.0 × 1.2 (mm) I.D. × O.D. quartz tubes (Wilmad) have been used for EPR experiments. All measurements have been repeated three times in order to obtain a good reproducibility of experiments.

Spectrophotometric measurements were performed using a Hewlett Packard 8453 spectrophotometer.



SCHEME 1

Scavenger Activity Against DPPH Radical

The scavenger activities of galloyl derivatives against the DPPH radical were measured following a protocol similar to that reported by Hatano *et al.*[20] 300 μl of 0.1 mM ethanol solution of DPPH were mixed with 100 μl of acetonitrile (blank) or with an equal volume of galloyl derivative solution, in the concentration range 0.005–0.1 mM. The following operating conditions were used to record the EPR spectra at 298 K: microwave frequency 9.573 GHz, centre field 340 mT, scan width 10 mT.

The UV-Vis assay was performed monitoring the absorbance of DPPH at 521 nm in the absence and in the presence of galloyl derivatives.

Scavenger Activity Against O_2^- Radical

EPR Assay

Superoxide radicals were generated following the protocol of Sichel *et al.*[21] 100 μl of H_2O_2 0.3 M and 70 μl of 0.4 mM KOH (blank) or an equal volume of galloyl derivatives (in KOH, 0.4 mM) in the concentration range 0.075–0.5 mM were added to 130 μl of KOH 0.4 mM. Reaction was started after the addition of 100 μl of spectroscopic grade acetone (Merck). EPR operating conditions at 120 K were: microwave frequency 9.565 GHz, centre field 335 mT, scan width 50 mT.

UV-Vis Assay

Superoxide radicals were generated using the X/XO system and the scavenger activity was measured spectrophotometrically at 560 nm using the NBT assay.[22–24] In phosphate buffer 0.1 M (pH 7.4), 300 μl of 0.1 mM xanthine, 300 μl of 0.08 u/ml of xanthine oxidase and 300 μl of NBT 0.6 mM were mixed. Then, 100 μl of acetonitrile (control) or 100 μl of metabolites in acetonitrile, in the concentration range 0.25–0.1 mM, were added to start the reaction. The solution was incubated at 35°C for 30 s and

absorbance at 560 nm was measured during a 150 s period.

Scavenger Activity Against OH Radical

Hydroxyl radicals were generated by the system copper(II) sulphate and hydrogen peroxide and were detected by DEPMPO spin trap.[25] In detail, 80 μl of $CuSO_4$ 2 mM (in water) were mixed with 80 μl of DEPMPO 80 mM (in water) and 80 μl of pure acetonitrile or 80 μl of metabolites in acetonitrile in the concentration range 0.025–0.5 mM. The reaction was started by the addition of 80 μl of H_2O_2 9.8 mM. Galloyl derivatives were dissolved in acetonitrile, since solvents like ethanol or methanol strongly interfered with hydroxyl radical formation. EPR spectra of the DEPMPO–OH adduct were recorded 1 min after the addition of H_2O_2, using the following operating conditions at 298 K: microwave frequency 9.568 GHz, centre field 340 mT, scan width 20 mT.

Quantification of Scavenger Activities and Calculation of Half-inhibition Concentration (IC_{50})

The scavenger activity of galloyl derivatives was calculated by the EPR spectra as:

$$R = [(H_0 - H_x)/H_0] \times 100$$

where R is the normalized scavenger ratio,[26] H_0 and H_x are the EPR signal intensities of samples without or with galloyl derivatives, respectively. H_0 and H_x were measured on the fourth EPR peak for DEPMPO–OH adduct, and on the central most intense line for the synthetic DPPH radical. The IC_{50}s of galloyl derivatives were calculated by plotting the scavenger ratios vs. final metabolite's concentration.[27] Raw data were fitted by means of 2nd order linear regression equations (Sigma Plot 2000, SPSS Science, Chicago, USA).

LDL Test

LDL were isolated by sequential ultracentrifugation from plasma obtained by healthy, normolipidemic human volunteers. Prior to their use, LDL (d = 1.019–1.055 g ml^{-1}) were separated by sequential ultracentrifugation from plasma, which was previously added with 0.1% EDTA.[28] LDL were dialysed against degassed phosphate-buffered saline medium (PBS at pH 7.4) during a 48-h period. Mobility and purity of samples were checked by agarose gel electrophoresis, stored at 4°C in the dark, and used within 24 h. Protein content was measured according to Lowry.[29] LDL were diluted to 200 μg protein ml^{-1} with PBS and incubated at 37°C in a shaking bath throughout the experiment. The samples were incubated for 30 min with either the galloyl derivatives or the vehicle prior the addition of 5 μM $CuSO_4$ (final concentration) and the incubation was stopped after 3 h. At the end of the incubation, TBARS were spectrophotometrically quantified at 532 nm, according to Balla *et al.*[30] Galloyl derivatives were tested in the concentration range (final concentration) 2.5–25 μM and half-inhibition concentrations (IC_{50}) calculated as reported above.

RESULTS

Three different reactive species were chosen to test the antioxidant activity of galloyl derivatives isolated from *P. lentiscus* leaves: DPPH, superoxide and hydroxyl radicals. Since, DPPH radical is not biologically relevant the DPPH assay was performed as a preliminary study to estimate the direct free radical scavenging abilities of galloyl derivatives.[20,27] The DPPH assay was performed either by EPR spectroscopy and by UV-Vis spectrophotometry. The antioxidant activity of galloyl derivatives against superoxide radicals was estimated by chemical and enzymatic assays.

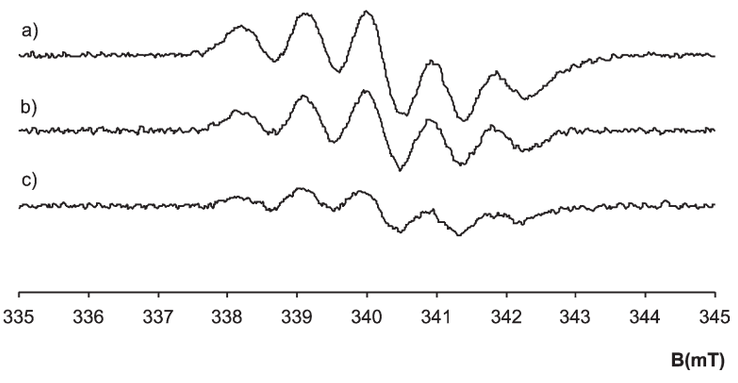


FIGURE 1 Room temperature EPR spectrum of DPPH radical (a) in the absence of the antioxidant, (b) in the presence of 5-*O*-galloyl quinic derivative and (c) 3,4,5-*O*-trigalloyl quinic derivative, both at a final concentration of 6.25 μM.

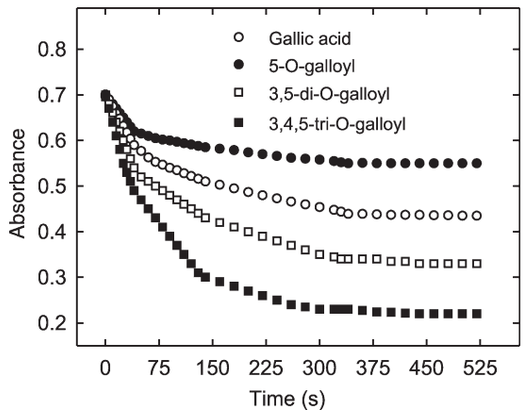


FIGURE 2 Effect of galloyl derivatives on the reduction of DPPH absorbance signal at 521 nm, estimated over a 525-s period, using a final metabolite's concentration of 6.25 μM.

The chemical assay using H_2O_2, KOH and acetone[21] to produce superoxide radical is just a semiquantitative test and the reaction system is far from biological conditions.[31] On the other hand, the enzymatic assay (X/XO system) is more strictly related to physiological conditions. The OH radical is the most reactive oxygen species found in both plant and animal cells. Since $OH^{\bullet}$ has a very short half-life time, the EPR spin trapping technique has been used to monitor the scavenger ability of the metabolites against this radical produced by the Cu(II)–H_2O_2 system.

In Fig. 1, the DPPH–EPR signal (blank, Fig. 1a) was compared to that of DPPH in the presence of 5-*O*-galloyl (Fig. 1b) and 3,4,5-*O*-trigalloyl quinic acid derivatives (Fig. 1c). The scavenger ratio of individual gallotannins against the DPPH radical was estimated by comparing the height of the central, most-intense line, of the DPPH–EPR spectrum in the absence or in the presence of the antioxidant. Experiments on the scavenger activities of galloyl derivatives against the DPPH radical by UV–Vis spectrophotometry were also performed, monitoring the absorbance at 521 nm (Fig. 2).

The scavenger abilities of secondary metabolites against superoxide radicals generated in the KOH/acetone/H_2O_2 system have been reported in Fig. 3. The EPR signal of the superoxide radical (blank, Fig. 3a) was reduced in the presence of 5-*O*-galloyl quinic derivative (Fig. 3b) and to a markedly greater extent by 3,4,5-*O*-trigalloyl quinic acid (Fig. 3c), confirming the results showed by the DPPH assay.

The EPR spectrum of the stable DEPMPO–OH adduct ($A_P = 47.7$ G, $A_N = 14$ G, $A_H = 13.4$ G, $g_{iso} = 2.006$), resulting from the hydroxyl-generating Cu^{2+}/H_2O_2 system and DEPMPO spin trap, has been reported in Fig. 4. The intensity of the DEPMPO–OH adduct signal (Fig. 4a) was markedly reduced after the addition of both gallic acid (Fig. 4b) and 3,5-*O*-digalloyl quinic acid derivative (Fig. 4c).

A more detailed analysis of the scavenger ability of galloyl derivatives against the tested radicals has been reported in Figs. 5–7, by plotting the normalized scavenger ratios against the final gallotannin concentrations, and fitting the raw data using 2nd order linear regression equations, for all the tested metabolites. The half-inhibition concentrations (IC_{50}) have then been calculated and reported in Table I.

Data of Fig. 5 clearly show that the scavenger ratio against DPPH radical raised as the number of galloyl groups (and hence OH groups) increased, and the half-inhibition concentration of 3,4,5-*O*-trigalloyl quinic derivative was 2.9 and 1.8 times smaller than that of gallic acid and 3,5-*O*-digalloyl derivative, respectively (Table I). Furthermore, the addition of the quinic acid skeleton on a pyrogallol moiety markedly reduced the scavenger activity, since 5-*O*-galloyl quinic acid had an IC_{50} 1.8 times superior than that of gallic acid (Table I).

The scavenger activity of tested metabolites against the superoxide radical confirmed the highest

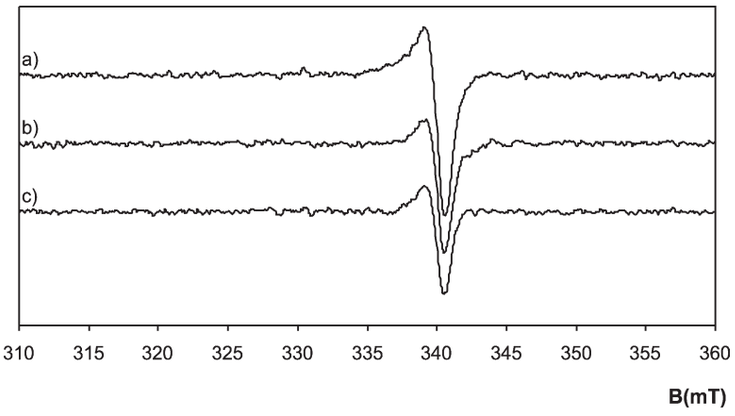


FIGURE 3 Low temperature (120 K) EPR spectrum of superoxide radical generated by KOH/acetone/H_2O_2 system (a) in the absence of the antioxidant, (b) in the presence of 5-*O*-galloyl quinic derivative and (c) 3,4,5-*O*-trigalloyl quinic derivative, both at a final concentration of 12.5 μM.

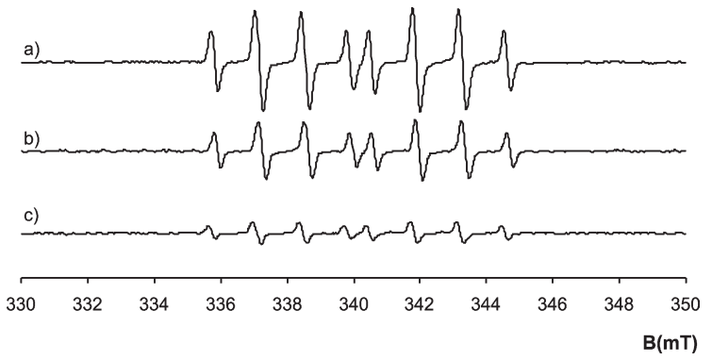


FIGURE 4 (a) EPR spectrum of DEPMPO–OH adduct formed by Cu(II)/H_2O_2 system in the absence of antioxidant, (b) in the presence of gallic acid and (c) in the presence of 3,5-*O*-digalloyl quinic derivative, both at a final concentration of 30 μM.

efficiency of 3,4,5-*O*-trigalloyl quinic derivative. Furthermore, monogalloyl and digalloyl derivatives did not substantially differ for their scavenger abilities (Fig. 6, Table I). The similarities between the IC_{50}s estimated by the EPR and the enzymatic X/XO system (Table I) were consistent with an almost exclusive action of galloyl derivatives as quenchers of superoxide anion.

Interesting results were observed on the scavenger properties of galloyl-derivatives against the Cu(II)-mediated generation of hydroxyl radicals (Fig. 7). It was observed that gallic acid showed a markedly higher ability to quench OH radical than the 5-*O*-galloyl quinic acid (as previously detected for DPPH radical, Fig. 5) and, above all, that 3,5-*O*-digalloyl was a better scavenger against $OH^{\bullet}$ than 3,4,5-*O*-trigalloyl derivative (Table I).

The antioxidant properties of galloyl derivatives estimated using the relevant biologically LDL system (Table II), showed a close relation between the scavenger activity against the OH radical and the relative abilities to protect low-density-lipoproteins from oxidation. Although the IC_{50}s for all the tested gallotannins were within a narrow concentration range, 3,5-*O*-digalloyl quinic acid was, respectively, three and two times more effective than 5-*O*-galloyl quinic acid and gallic acid in inhibiting the LDL oxidation. Remarkably, the IC_{50} of 3,4,5-*O*-trigalloyl quinic acid was approximately 50% higher than that of 3,5-*O*-digalloyl quinic acid (Table II).

DISCUSSION

On the whole, our data show that the *P. lentiscus* galloyl derivatives were very effective scavengers against the tested radical species. On the contrary, the antioxidant activity for quinic acid was absent. In this regard, the di- and tri-galloyl derivatives were highly superior than the monogalloyl derivatives, thus confirming that the number of galloyl moieties determines the antioxidant properties of hydrolysable tannins and largely control their biological activities.[9,14,32,33] It should be also pointed out that results for scavenger abilities against the DPPH radical are in good agreement with previous data

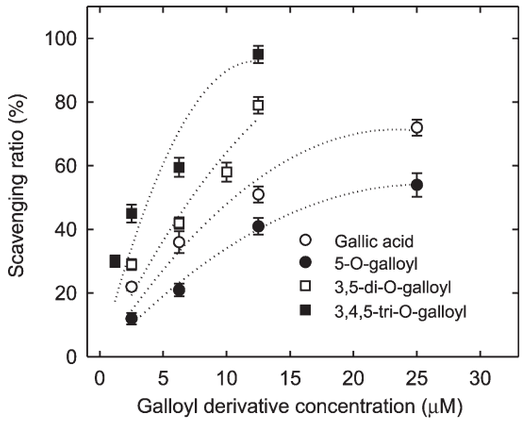


FIGURE 5 Scavenger ratio against DPPH radical vs. different concentrations of galloyl quinic derivatives. Raw data were fitted using a 2nd order linear regression ($r^2 > 0.98$ for all the tested metabolites). Data point are means ± SD of $n = 4$.

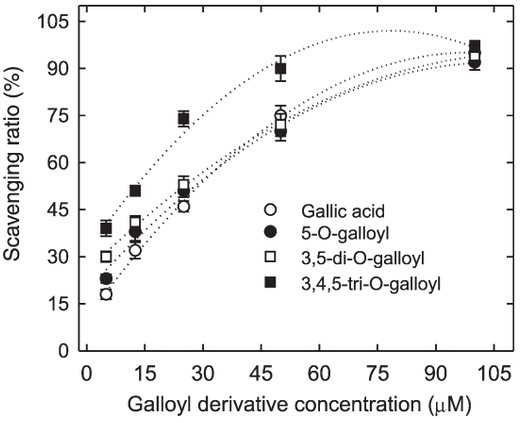


FIGURE 6 Scavenger ratio against superoxide radical vs. different concentrations of galloyl derivatives. Superoxide radical was generated in the KOH/Acetone/H_2O_2 system. Data management and statistics as reported in Fig. 5.

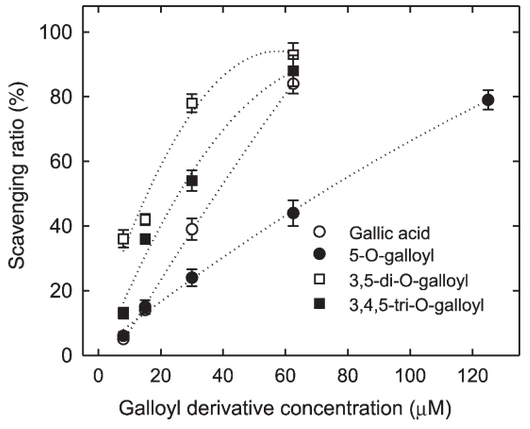


FIGURE 7 Scavenger ratio against hydroxyl radical vs. different concentrations of galloyl derivatives. Hydroxyl radical was generated in the Cu(II)/H_2O_2 system and EPR spectra of the DEPMPO–OH adduct were analysed. Data management and statistics as reported in Fig. 5.

TABLE II Antioxidant activities of galloyl derivatives extracted from *P. lentiscus* leaves against chemically-induced LDL oxidation*

Metabolite	IC_{50} (μM)
Gallic acid	10.2 ± 0.4
5-*O*-galloyl quinic	18.7 ± 1.6
3,5-*O*-digalloyl quinic	5.4 ± 0.2
3,4,5-*O*-trigalloyl quinic	7.5 ± 0.3

* Metabolites were tested in the range 2.5–25.0 μM (final concentration) and IC_{50} values were estimated by fitting the raw data by individual second-order linear regressions curves ($r^2 > 0.98$ for all the tested compounds).

reported for gallic acid and galloyl glucoses.[32,34] Furthermore, IC_{50}s of both 3,5-*O*-digalloyl and 3,4,5-*O*-trigalloyl quinic derivatives (Fig. 5) are in the same concentration range (5–10 μM) as previously reported for epigallocatechin and epigallocatechin gallate,[35] the highly antioxidant components of green tea.

The result that gallic acid, monogalloyl and 3,5-*O*-digalloyl derivatives did not substantially differ for the scavenger abilities against the superoxide radical (Fig. 6) is in agreement with previous findings that showed no substantial differences between gallic acid and polygalloyl derivatives as scavengers of O_2^-.[20,36,37] Furthermore, the similarity of IC_{50}s calculated from both the KOH/Acetone and X/XO superoxide generating systems, strongly suggests that galloyl derivatives are negligible inhibitors of xanthine oxidase.[38,39]

The finding that the number of galloyl groups was not related to the antioxidant properties of *P. lentiscus* gallotannins against the hydroxyl radical generated in the Cu-catalysed "Fenton-like reaction" (Fig. 7, Table I) seems to require some additional speculations. It has been previously reported that in metal-catalysed reactions leading to reactive oxygen generation, polyphenols may directly quench free radicals and inhibit free radical generation through the formation of Cu(II)-polyphenol complex, but also have a prooxidant behaviour.[31,40–43] In our experiment, EPR and UV-Vis measurements[43,44] did not reveal the formation of Cu(II)-galloyl complexes, and this result confirms the substantial inability of galloyl moieties to complex Cu(II).[44] Nevertheless it may be deduced that galloyl derivatives isolated from *P. lentiscus* may have a prooxidant behaviour in the presence of Cu(II), as previously reported for flavonoids.[31] Our results seem to indicate that the quenching ability against $OH^\bullet$ and the prooxidant activity of gallotannins increase by increasing the number of OH groups.

Galloyl derivatives were highly efficient antioxidants using the biologically relevant model of chemically induced LDL oxidation (IC_{50}s in the range 5.4–18.6 μM, Table II) and showed a close similarity with the ability to scavenge hydroxyl radicals (Table I). Since LDL test was also performed in the presence of Cu(II) ions, these data further support the idea that galloyl derivatives may either scavenge and generate OH radicals when metal ions are involved in the reaction system.

In conclusion, the galloyl-quinic derivatives, an unusual class of hydrolysable tannins isolated from *P. lentiscus* leaves, are highly efficient free radical scavengers and may serve important pharmacological roles, linked to their antioxidant behaviour, as those related to the prevention of atherosclerotic lesions and coronary heart diseases, induced by oxidation-modified LDL. As a consequence

TABLE I Half-inhibition concentration (IC_{50}) of galloyl derivatives against DPPH, superoxide and hydroxyl radicals*

Metabolite	DPPH	IC_{50} (μM) O_2^- (KOH/Acetone)	O_2^- (X/XO)	$OH^\bullet$
Gallic acid	11.2 ± 0.9	26.7 ± 2.5	37.1 ± 3.6	42.6 ± 5.1
5-*O*-galloyl	18.7 ± 2.1	25.4 ± 2.1	35.2 ± 3.2	71.3 ± 4.7
3,5-*O*-di	7.1 ± 0.8	22.6 ± 1.9	29.7 ± 3.5	16.0 ± 3.8
3,4,5-*O*-tri	3.9 ± 0.6	11.5 ± 1.5	16.8 ± 2.4	26.6 ± 2.9

* IC_{50}s for superoxide radical are reported using both KOH/acetone system (EPR spectroscopy) and X/XO enzymatic system (UV-Vis spectrophotometry). IC_{50}s were calculated from the 2nd order linear regression equations reported in Figs. 5–7. Data are the means ± SD of $n = 4$.

the present results may encourage additional and more in-depth studies on the pharmacological properties of *P. lentiscus* tissue preparations, whose beneficial effects have been from a long-time recognized in the Mediterranean ethnomedicine.

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