Conformational analysis: a tool for the elucidation of the antioxidant properties of ferulic acid derivatives in membrane models

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Abstract

With the aim to search and design more effective and safe antioxidant molecules to be used as functional ingredients in cosmetic formulations for UV protection, we evaluated the antioxidant/radical scavenging activities of ferulic acid and of some alkyl ferulates in both acellular and cellular systems. Ferulic acid esters, equipotent as antioxidant in homogeneous phase, showed when tested in membranous systems (rat liver microsomes, rat erythrocytes) marked differences in antioxidant potency. The n-C_{12} derivative was the most potent, followed by n-C_8, n-C_{16} and branched C_8, and then by ferulic acid.

A conformational study carried out by NMR and modelling, indicates that the different antioxidant activity of ferulates in membrane models is due to the different spatial conformation and arrangement of the side chain of the molecule, which governs the access and binding to the phospholipid bilayer, the modality of orientation of the scavenging/quenching nucleus (phenol moiety), and hence the overall antioxidant potency of the derivative.

These results emphasize the need of analytical studies (NMR and molecular modelling) addressed to the knowledge of the conformational parameters in combination with conventional antioxidant testings for understanding the antioxidant behaviour of a molecule in a biological membrane/system.
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1. Introduction

The risk of cutaneous sun damage has become dramatic during the last decades, due to an increasing sun exposure and to thinning of the ozone layer: acute and chronic exposure to sun rays promotes premature skin

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aging [1], erythema, inflammation, immunodepression and photo-carcinogenesis, and the cause of these diseases has been ascribed to the generation of free radicals [2]. In this context play a primary role reactive oxygen/nitrogen species, arising from the interaction of UV radiation with endogenous skin photosensitizers and an enhanced release in the skin of non-heme iron [3], a catalyst of free radical reactions. Recently a great deal of focus has been shed on the antioxidant potentialities of ferulic acid and its *n*-alkyl esters: this is due to their capacity to form after scavenging an highly stabilized phenoxy-radical by extensive electron delocalization on the aromatic nucleus substituted with two electron-donor groups. Ferulic acid is an high functional value polyphenol constituent, ubiquitous in nature, currently expected to prevent diseases such as inflammation [4], atherosclerosis and cancer or aging, caused by oxidative tissue imbalance. Ferulic acid occurs in rice, wheat, barley, oat, forage, tomatoes, asparagus, olives and many other plants; much of ferulic acid occurs as ester in many plants [5–8]. In seeds and leaves it is contained both in free and covalently bound form linked to lignin and other biopolymers.

Interestingly ferulic acid derivatives could be more strong antioxidant than acid by itself [9,10]. Studies on caffeic acid, dihydro caffeic acid and some of their corresponding esters, all structurally related to ferulic acid, have shown that their antioxidant activity in biological systems depends also on the ester chain, suggesting the importance of this modification [11].

Hence, pursuing our interest in the design and synthesis of new chemical entities with even more effective photoprotective properties, in this study we have carried out a detailed investigation on the antioxidant properties of ferulic acid and some alkyl-ferulates: *n*-octyl-, 2-ethyl-1-hexyl-, *n*-dodecyl- and *n*-hexadecyl-ferulates and their activity elucidated at the light of conformational properties. The prototypes were chosen on the following basis:

(i) 2-Ethyl-1-hexyl-ferulate is structurally related to octyl methoxycinnamate, one of the most important UV filter on the pharmaceutical/cosmetic market, (*n*-octyl ferulate was the reference compound with linear chain).

(ii) *n*-Dodecyl ferulate has a chain that, as we have previously demonstrated in several related UV

sunscreens, adopts a peculiar folded main conformation [12–14].

(iii) The *n*-hexadecyl ester is a derivative of ferulic acid largely represented in nature: its use can be of interest for understanding the effect of lengthening of the alkyl chain on antioxidant properties [7,15–17].

The antioxidant potency of these esters was evaluated both in an acellular model using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical [18], and in different cellular models constituted by:

1. rat liver microsomes—thiobarbituric acid (TBA) test [19,20],
2. rat erythrocytes—cumene hydroperoxide (CuOOH) induced hemolysis [21].

The conformational analysis was based on NOESY experiments. The restraints found with the NOE measurements were applied in a subsequent molecular dynamics study.

From the results of this work it becomes clear that only from the combination of antioxidant testings in vitro and physico-chemical informations we can achieve a clear insight into the antioxidant potency of new compounds to be used for skin protection toward the oxidative stress.

2. Materials and methods

2.1. Apparatus

Spectrophotometric studies were carried out in a computer aided Perkin-Elmer Lambda 16 spectrophotometer (Perkin-Elmer, Monza, Italy). NMR analysis was performed on a 200 MHz Bruker AC-200 (Bruker Biospin, Milan, Italy).

The conformational study was carried out in vacuo using the AMBER force field and the AMBER software [22] running on a Linux workstation equipped with a Pentium CPU.

2.2. Chemicals

Alkyl ferulates, compounds **2**–**5** (Fig. 1), were synthesized according to standard procedures by reaction of ferulic acid with the corresponding alkanol [6].

1: R = H
2: R = n-octyl
3: R = 2-ethyl-1-hexyl
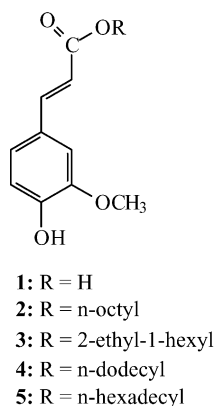**4:** R = n-dodecyl
5: R = n-hexadecyl

Fig. 1. Structures of ferulic acid (**1**) and its derivatives (**2–5**).

The crude products were purified on a silica gel column and identified by TLC, UV and NMR analyses [6,7,17].

All the organic solvents used were of analytical grade (Aldrich, Milan, Italy). Ferulic acid was supplied by Tsuno Rice Fine Chemicals, Wakayama, Japan. *n*-Octyl, 2-ethyl-1-hexyl, *n*-dodecyl and *n*-hexadecyl alcohols, DPPH, ferric chloride, adenosine-5′-diphosphate disodium salt (ADP), ascorbic acid and TBA reagent and dimethyl sulfoxide (DMSO) were purchased from Aldrich (Milan, Italy).

2.3. Isolation of microsomes

Male Wistar rats (Charles River, Calco, LC, Italy; 220 ± 10 g body weight) were maintained in compliance with the policy on animal care expressed in the National Research Council guidelines (NRC 1985). Rat liver microsomes were isolated from the liver as previously described [23].

Animals were killed by decapitation, livers were quickly removed and washed with isolation medium (ice-cold 0.25 M sucrose containing 10 mM Tris–HCl, pH 7.4). A 10% liver homogenate was made in isolation medium. Microsomes were isolated by differential centrifugation, washed twice with 10 mM phosphate buffer (pH 7.4) and suspended in the same buffer. All operations were carried out at 0–4 °C. The proteins were estimated by the Lowry method [19]. In the lipid peroxidation experiments microsomes were diluted with 10 mM phosphate buffer pH 7.4 (2.0 mg protein/ml).

2.4. DPPH assay

0.1 ml of methanol solutions of each single compound (final concentration 5.0, 15.0, 25.0 and 50.0 µM) were added to 0.1 mM DPPH methanol solution (final volume 1.5 ml) and the loss in absorbance at 517 nm read after 90 min incubation of the mixtures at room temperature in the dark. DPPH solution in methanol served as the control. The radical scavenging activity of the samples was expressed as percentage of inhibition of DPPH absorbance. It was calculated as $100 - (As/Ac \times 100)$ where As and Ac are, the absorbance values of the samples in the presence and the absence of the inhibitors, respectively (mean $\pm$ S.D. of five determinations).

2.5. CuOOH-induced hemolysis

Erythrocytes from male Wistar rats isolated and washed from white blood cells and platelets by standard techniques [24], were suspended in phosphate buffered saline, pH 7.4 (125 mM NaCl and 10 mM sodium phosphate buffer (PBS)), stored a 4 °C and used until 4 days old. For experiments, an aliquot of the erythrocyte suspension was drawn from the stock, centrifuged at $1000 \times g$ for 5 min and 0.1 ml of the pellet diluted to 50 ml with PBS to obtain a 0.2% erythrocyte suspension (approximately 3×10^7 cells/ml).

3.5 ml aliquots of the 0.2% red blood cell (RBC) suspension were incubated at 37 °C for 150 min in the presence of CuOOH (dissolved in ethanol; final concentration 50 µM) and the cellular integrity determined turbidimetrically at 710 nm [25] at 30-min intervals.

Ferulic acid derivatives dissolved in 0.1% DMSO (final concentration 0.1–5.0 µM) were preincubated for 30 min with RBCs before the addition of CuOOH (blanks were RBCs added with DMSO).

The percentage hemolysis was determined setting as a 100% hemolysis the absorbance value determined in RBC suspensions sonicated for 5 s at 50% power. Mean values of five determinations were used for the calculation.

2.6. Thiobarbituric acid assay

An aliquot of microsomal suspension was incubated for 15 min at 37 °C with test compounds (**1–5**) at different concentrations (5–50 µM).

Then the reaction mixture was incubated for 1 h with 150 μM ferric chloride, 3 mM ADP and 100 μM of ascorbic acid.

The extent of lipid peroxidation was assessed by measuring the content of malondialdehyde (MDA) in the incubation mixture by the TBA assay [20]. Briefly, the reaction mixture was treated with 1 ml of TBA reagent consisting of 0.37% TBA, 15% of trichloroacetic acid (TCA) in 0.5 N HCl and placed in a boiling water bath for 15 min, cooled and centrifuged at $4000 \times g$ for 10 min, the supernatants read at 532 nm converting to nanomoles of MDA with an absorption coefficient of 156 mM^{-1} cm^{-1}. Blanks were the overall reaction mixtures without TBA. Mean values of five determinations were used for the calculation.

2.7. Conformational analysis

2.7.1. NMR analysis

2D-NOE (NOESY) spectra were recorded for 0.01 M solutions in CDCl$_3$ with standard sequences.

2.7.2. Molecular modelling

The conformational study was carried out in vacuo using the AMBER force field and the AMBER software [22]. The structures of the compounds were built from scratch and proton-proton distance restraints were calculated according to the observed NOE's intensities: distance values of 3.0, 4.0 and 5.0 Å were used for strong, intermediate and weak NOE's, respectively. A constant temperature dynamics run with periodic temperature jumps [26] followed, according to the protocol: 8 ps MD at 300 K, temperature jumps at 600 K for 4 ps to provide enough energy to pass conformational barriers, four repetitions of this cycle. A 75 ps MD run at 300 K followed. The same protocol was applied to all molecules.

2.8. Statistical analyses

Data are showed as mean $\pm$ S.D. from five independent experiments. The statistical significance of differences between compounds was assessed by Student's *t*-test and recognized to be significant when *P*-value was 0.05 or lower.

3. Results and discussion

3.1. DPPH assay

As a preliminary screening for the study of the scavenging ability of ferulic acid and its derivatives (Fig. 1), we tested their quenching activity against DPPH in homogeneous phase.

DPPH is a stable free radical used to evaluate the antioxidant activity of different compounds which act by electron or hydrogen transfer. The scavenging effects of tested compounds are reported in Table 1. They are active on DPPH radical (EC$_{50}$) at micromolar concentrations (20–27 μM). All the compounds showed a dose dependent activity: the quenching ability of *n*-octyl ferulate is more similar to that of ferulic acid followed by hexadecyl ferulate, by branched C$_8$ and by *n*-C$_{12}$ derivatives.

The fact that all the compounds showed fairly similar radical scavenging activities indicates that in homogeneous solution different structural features of the side chain (length, branching, etc.) do not affect the quenching ability of the phenol nucleus towards the stable DPPH radical.

3.2. CuOOH-induced hemolysis

The method is based on the evaluation of the protective effect of the title compounds (**1**–**5**) against hemolysis of an erythrocyte suspension induced by the free radical promoter CuOOH. The choice of the erythrocyte as a model is due to the fact that it is a complete cellular system that presents structural characteristics (high content of polyunsaturated fatty acids) close to

Table 1
Scavenging activity of different compounds on DPPH radical. Data are shown as EC$_{50}$ (concentration, μM, of a title compound which decreases by 50% the DPPH absorbance at 517 nm after 90 min of incubation)

Compound	EC$_{50}$
1	23.55 ± 1.25
2	24.04 ± 1.35
3	25.55 ± 1.18
4	26.22 ± 1.49
5	25.27 ± 1.35

Values are mean $\pm$ standard deviation (S.D.) of five determinations. Values are not significantly different at $P < 0.05$.
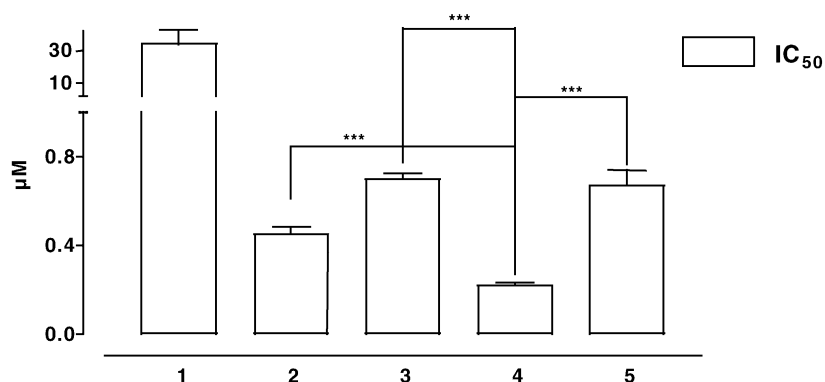
Fig. 2. Protective effect of different compounds on CuOOH induced hemolysis of rat erythrocytes. Data are shown as IC_{50} (μM) values calculated after 90 min of incubation and are means $\pm$ S.D of five determinations. Statistical analysis was done by the Student's t-test. Compound "**4**" is significantly different from **2**, **3**, **5**, **1** (*** $P < 0.0001$).

liver microsomes. Moreover, the membrane contains cytoskeleton proteins (spectrin, ankirin, band 3, band 4.1, glycophorin, actin) that, intercalating with lipids and phospholipids, create the typical flexible structure of the erythrocyte [27].

The hemolytic process is modulated by two factors: (i) a process of lipoperoxidation of the phospholipid bilayer, (ii) a process linked to the oxidation and degradation of cytoskeleton proteins.

The liphophilic cytotoxic agent CuOOH, once inside the membrane generates a lipid peroxidation burst which propagates to cytoskeleton proteins. They lose stability and the erythrocyte membrane undergoes the hemolytic shock.

We tested the protective effect of the ferulic acid derivatives at a concentration level below 5 μM (0.1–5 μM); for ferulic acid from 0.5 to 50 μM. The protective effect was expressed as IC_{50} value calculated after 90 min of incubation in the presence of the compounds (Fig. 2) [28].

The results show that all ferulic acid esters are much more active than the acid itself with the following order of potency: n-C_{12} ($IC_{50} = 0.22 \pm 0.01$) > n-C_8 ($IC_{50} = 0.45 \pm 0.03$) > n-C_{16} ($IC_{50} = 0.67 \pm 0.06$) > branched C_8 ($IC_{50} = 0.70 \pm 0.02$) > ferulic acid ($IC_{50} = 34.14 \pm 1.05$).

3.3. Microsomal lipid peroxidation

Five levels of concentrations were tested in the range of 5–50 μM for each derivative, and for ferulic acid from 5 to 500 μM. The concentrations that inhibit by 50% the peroxidation process are reported in Fig. 3. The results confirm the efficacy of all the compounds as anti-lipoperoxidant. Among the ferulic acid derivatives the n-C_{12} (**4**) showed the strongest inhibitory potency ($IC_{50} = 10.70 \pm 0.14$) followed by the n-C_8 (**2**, $IC_{50} = 12.36 \pm 0.19$), by n-C_{16} (**5**, $IC_{50} = 23.47 \pm 1.34$) and branched C_8 (**3**, $IC_{50} = 32.20 \pm 0.99$). Also in this model ferulic acid showed a less marked antioxidant activity ($IC_{50} = 243.79 \pm 2.95$). It is interesting to observe that the trend of anti-lipoperoxidant activity in liver microsomes perfectly matches that observed in rat erythrocytes (Fig. 4): this indicates that the n-alkyl ferulates act with a similar mechanism in both the membrane models and that the side chain is primarily involved in the
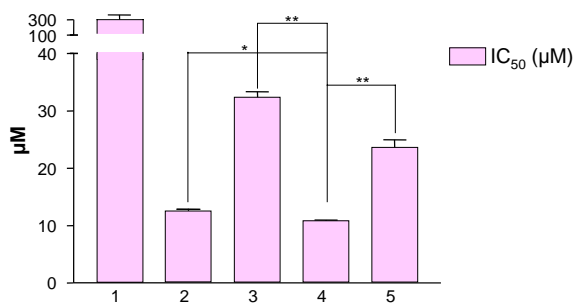

Fig. 3. Anti-lipoperoxidant activity of different compounds. IC_{50} (μM) values are means $\pm$ S.D. of five independent experiments. Statistical analysis was done by the Student's t-test (* $P < 0.05$; ** $P < 0.01$).
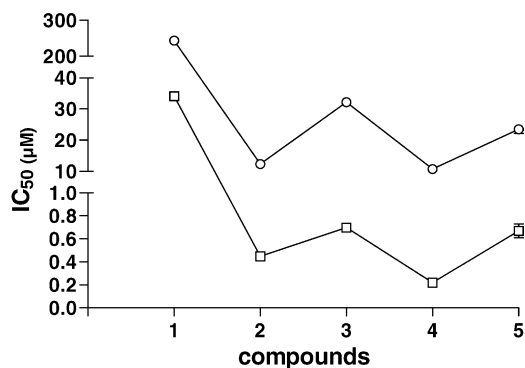
Fig. 4. Comparative trend of the antioxidant activity of ferulic acid and its derivatives in different membrane models (($\square$) erythrocytes, ($\bigcirc$) microsomes).

interaction with the phospholipid bilayer of the membrane. Ferulic acid, active in homogeneous phase, becomes inactive in heterogeneous phase since side chain ionization in physiological medium hampers binding to the biological membrane.

3.4. Conformational analysis

3.4.1. NMR analysis

The conformational analysis in solution was performed by 2D NOE (NOESY) measurements. The 2D NOE analyses were done for 0.1 and 0.01 M solutions in CDCl$_3$. Since the results were the same for the two concentrations levels tested the NOEs found were due basically to intramolecular interactions. The experimental NOEs are reported in Table 2.

The NOE effects within aromatic and ethylenic moieties, OCH$_3$ $\leftrightarrow$ H3 and H3 $\leftrightarrow$ H8, are present in all compounds. Thus the aromatic ring and the ethylenic moiety adopt closely related conformations in all compounds.

The branched C$_8$ derivative (**3**) has a NOE interaction, OCH$_3$ $\leftrightarrow$ H9, which contributes, together with the previous effects, to give a peculiar orientation to the side chain. Since there are no other interactions, we can reasonably assume that the alkyl chain adopts a linear conformation sharply distant from the aromatic moiety, compound **3** (a, b), Fig. 5. Also in the linear C$_8$ ester (**2**) we have found an OCH$_3$ $\leftrightarrow$ H9 interaction and thus the conformation of this part of the molecule is the same in both compounds. However,
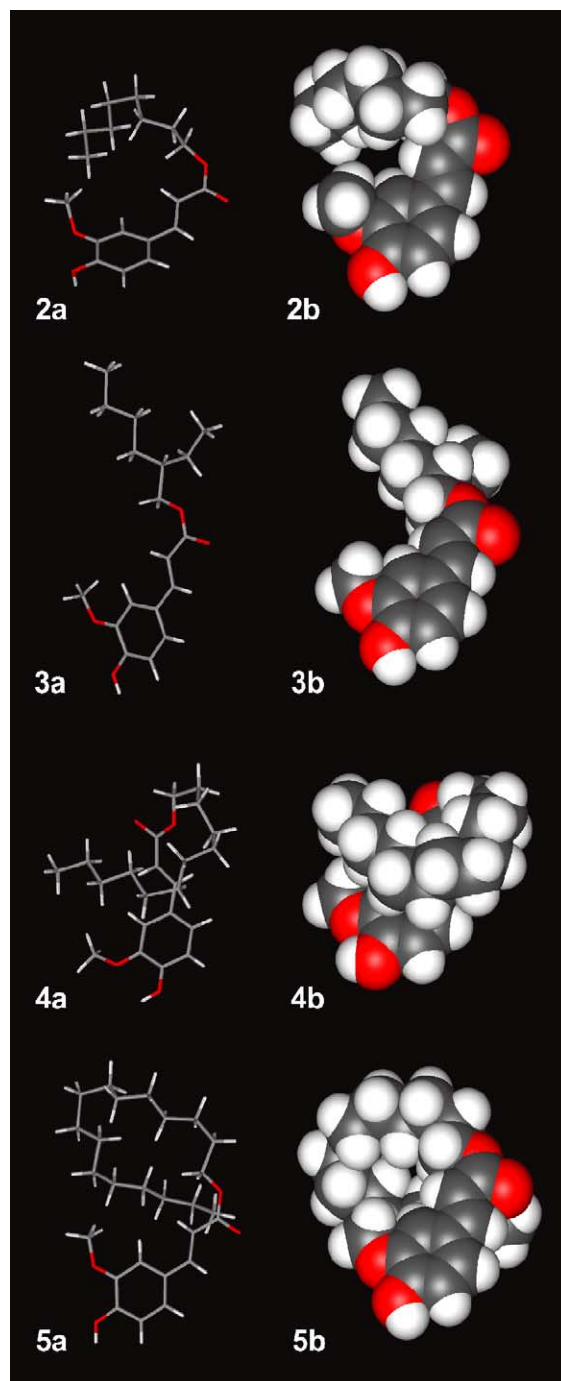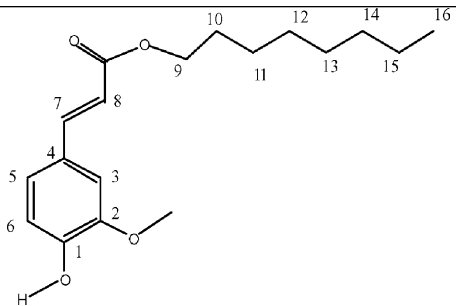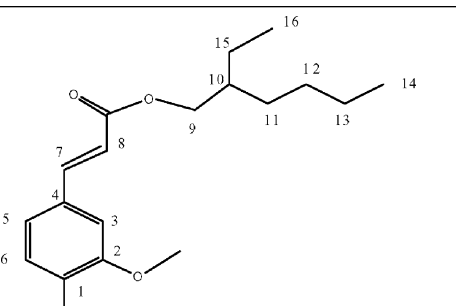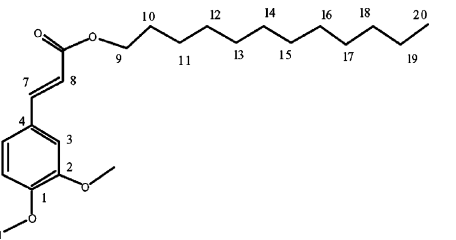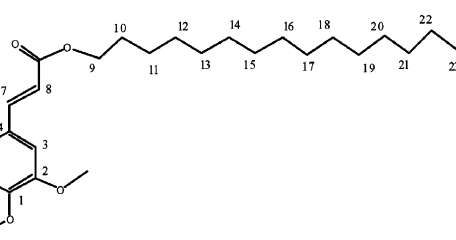

Fig. 5. The most probable conformations of ferulic acid derivatives in CDCl$_3$ solution as inferred from NMR experiments and molecular modelling. The compounds **2**–**5** are reported as stick (a) and space (b) representation.

Table 2

Numbering system of compounds **2–5** used throughout the text and 2D-NOE (NOESY) results

NOE effect	Strength		NOE effect	Strength

Ferulic acid derivative: linear C8 (**2**)

OCH$_3$ ↔ H9	Strong
OCH$_3$ ↔ H3	Strong
H3 ↔ H8	Strong
H8 ↔ H16	Medium/strong
H9 ↔ H16	Medium
OCH$_3$ ↔ H16	Medium

Ferulic acid derivative: branched C8 (**3**)

OCH$_3$ ↔ H9	Strong
H3 ↔ H8	Strong
H3 ↔ OCH$_3$	Strong

Ferulic acid derivative: C12 (**4**)

OCH$_3$ ↔ H3	Strong
H3 ↔ H8	Strong
H7 ↔ H20	Medium/strong
H10 ↔ H20	Medium/strong

Ferulic acid derivative: C16 (**5**)

OCH$_3$ ↔ H3	Strong
H3 ↔ H8	Strong
H7 ↔ H24	Medium/strong
H10 ↔ H24	Medium/strong

the presence of NOE interactions among the methyl protons of the chain H16 and methylenic protons H9 (H9 ↔ H16), OCH$_3$ (OCH$_3$ ↔ H16) and H8 (H8 ↔ H16) protons, respectively, clearly suggest a folding of the chain towards the area delimitated by the latter as clearly depicted in Fig. 5, compound **2**(a, b).

In the dodecyl (**4**) and hexadecyl (**5**) derivatives the presence of NOE interactions between the methyl protons (H20 and H24 for *n*-C$_{12}$ and *n*-C$_{16}$, respectively) of the alkyl chain, the ethylenic proton H7 (H7 ↔ H20 and H7 ↔ H24) and the methylenic protons H10 (H10 ↔ H20 and H10 ↔ H24), clearly suggest a folding of the alkyl chains towards the corresponding ethylenic moiety. Moreover, these experimental results together with the absence of the OCH$_3$ ↔ H9 NOE interaction are consistent with a different folding for these derivatives in respect to the *n*-C$_8$ ester. Actually, if we consider as "clockwise" the folding of the alkyl chain in the *n*-C$_8$ derivative, then the folding in the *n*-C$_{12}$ and *n*-C$_{16}$ esters is "anticlockwise" **4**, **5** (a, b).

3.4.2. Molecular modelling

The dynamics of all compounds have to be considered in the light of NOE results. The main mean conformation in solution of all the compounds is reported in Fig. 5.

The alkyl chain in linear *n*-C$_8$ and *n*-C$_{12}$ derivatives is oriented in an opposite way. In *n*-C$_8$ derivative, the alkyl chain is folded towards the aromatic ring (loop conformation) in clockwise fashion. In *n*-C$_{12}$ derivative, the alkyl chain is folded not only towards the aromatic ring, but also on itself. In the *n*-C$_{16}$ derivative, the alkyl chain is folded on itself (anticlockwise) and towards the aromatic ring. In the branched derivative, the NOE effects do not involve the protons in the branched chain, and these groups can freely rotate.

4. Conclusions

The antioxidant activity of a molecule is currently investigated in "in vitro" systems which make use of acellular and cellular models: the first furnish a preliminary evidence of the capacity to quench free radicals species (OH$^\bullet$, ROO$^\bullet$, RO$^\bullet$, R$^\bullet$, etc.), the second (phosphatidylcholine liposomes, liver microsomes, erythrocytes, cell cultures) give deeper insight into the protective action of the molecule (more close

to an in vivo situation) since provide a biological information that takes into account also its interaction with specific receptor/s on membrane surface.

Until now this point has been rather disregarded since for the elucidation of the antioxidant/biological action, only the lipophilic character of the molecule is considered, and few attention has been paid to its ability/modality to bind to the phospholipid bilayer and to dispose itself in a suitable position over/inside the membrane surface.

The results relative to the C$_8$ derivatives (**2**, **3**) show that folding increases the capacity of the molecule to anchor, through van der Waals forces, to the phospholipid pocket located on membrane/cell surface, while the straight conformation of the branched C$_8$ derivative, together with the presence of an hindering ethyl group, hampers the access of the molecule to the lipid binding sites.

For what concerns the spatial behaviour of *n*-C$_{12}$ (**4**) and *n*-C$_{16}$ (**5**) derivatives, from the stick and space representations (Fig. 5: a, b), **4** has the same conformation of **2**, with a more expanded and flexible lipophilic anchor chain. For compound **5** (a, b) the anchor chain is completely folded towards the aromatic ring and hence less available for interaction. Consequently **4** is more prone to interact with the phospholipid binding sites and this explains its greater activity in respect to **2** and **5**.

Hence the indication that comes from the results of our study demonstrates that:

1. anchoring of the side chain of the ferulates is the prerequisite for exhibiting biological activity
2. it is the modality of anchoring to the phospholipid bilayer which affects the orientation of phenol nucleus, over/inside the membrane, against the flux of oxy radicals, generated by the two promoters, CuOOH in the erythrocytes, Fe^{3+}/ascorbic acid in liver microsomes (see the perfect match of the antioxidant profiles in both the membrane models (Fig. 4)) and consequently dictates the antioxidant properties of ferulates.

Finally if we consider that all the ferulates (**2**–**5**) possess in an octanol/buffer system the same degree of lipophilicity [9], we can conclude that for understanding the antioxidant behaviour of a molecule in a membrane model, antioxidant testings by themselves can sound meaningless, when not properly

correlated to conformational studies on spatial arrangement/disposition of the molecule, a major issue which awaits further confirmation by studies on three-dimensional interactions with biological membrane.

Acknowledgements

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