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'In vitro' evaluation of the antioxidant activity and biomembrane interaction of the plant phenols oleuropein and hydroxytyrosol

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Abstract

Oleuropein and hydroxytyrosol, two phenolic compounds contained in olives and olive oil, are known to possess several biological properties, many of which may be related, partially at least, to their antioxidant and free radical-scavenger ability. Hence, together with their scavenging activity against the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH test), we have investigated the antioxidative effect of oleuropein and hydroxytyrosol in a model system consisting of dipalmitoylphosphatidylcholine/linoleic acid unilamellar vesicles (DPPC/LA LUVs) and a water-soluble azo compound as a free radical generator (LP–LUV test). The results obtained were also interpreted in the light of biophenol interactions, studied by differential scanning calorimetry (DSC), with dimyristoylphosphatidylcholine (DMPC) vesicles as a biological membrane model. Our results obtained in the DPPH and LP–LUV tests confirm the good scavenger activity and antioxidant effect of oleuropein and hydroxytyrosol. However, while both compounds exhibit comparable effectiveness in the DPPH test (hydroxytyrosol being slightly more active than oleuropein), oleuropein seems, in the LP–LUV test, a better antioxidant than hydroxytyrosol. Besides oleuropein shows a better antioxidant activity in the membranous system than in homogenous solution. Furthermore, oleuropein, but not hydroxytyrosol, interacts with DMPC vesicles, causing shifts, toward lower values, of the calorimetric peak temperature (T_m), associated to the gel to liquid-crystal phase transition, typical for DMPC multilayers. The hypothesis will be discussed that hydroxytyrosol can serve as scavenger of aqueous peroxyl radicals near the membrane surface, while oleuropein acts also as a scavenger of chain-propagating lipid peroxyl radicals within the membranes.

Keywords: Biophenols; Olive; Antioxidant activity; Model membranes

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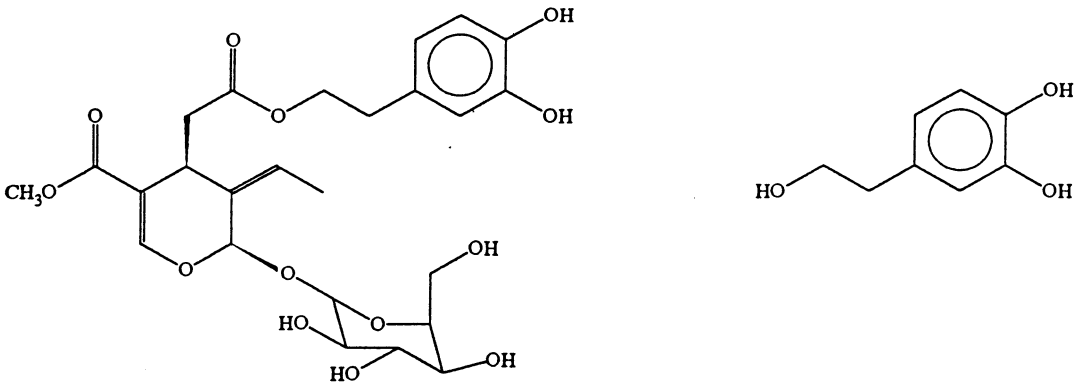

OLEUROPEIN **HYDROXYTYROSOL**

Fig. 1. Structural formulae of oleuropein and hydroxytyrosol.

1. Introduction

One of the major targets for oxygen radicals are undoubtedly phospholipid bilayers of cellular and subcellular membranes. The compounds which inhibit the membranous phospholipid peroxidation seem to exert a pharmacological effect in the prevention of oxygen radical-induced pathological events (Rice-Evans and Diplock, 1993; Roberfroid and Calderon, 1995). However, together with the site of radicals to be generated in the phospholipid bilayers, the localization of antioxidants should be taken into account in understanding the effectiveness of their antioxidant activities (Terao et al., 1994; Saija et al., 1995a; Bonina et al., 1996); in fact the degree of incorporation and the uniform distribution into lipid bilayers and the rate of transport into cells are particular factors influencing the efficiency of antioxidant compounds (Thomas et al., 1992; Kaneko et al., 1994).

Phenolic compounds (both natural and synthetic) are prototypic chain-breaking antioxidants; their protective effect against lipoperoxidative damage depends on the hydrogen-donating capacity of a hydroxyl group in each molecule (Kahl, 1991). Interest in the biological actions of olive phenols has recently arisen since epidemiological studies have linked high dietary intake of natural antioxidants (such as vitamins and phenolic compounds) with lower incidence of pathological conditions associated to uncontrolled production of free radicals (Martin-Moreno et al., 1994).

The secoiridoids (oleuropein and derivatives) represent one of the major classes of phenolic compounds with antioxidant activity contained in olives and olive oil (Montedoro et al., 1992, 1993). Oleuropein, the bitter principle of olives, and hydroxytyrosol (Fig. 1), derived by hydrolysis from oleuropein and responsible for the high stability of olive oil (Tsimidou et al., 1992), have been previously demonstrated to possess several biological properties, including antimicrobial, vasodilator, hypotensive and hypoglycemic activities, many of which may be related, partially at least, to their antioxidant and free radical-scavenger ability (Visioli and Galli, 1995; Visioli et al., 1995a,b). However, no data are known about the antioxidant activity of these compounds in biomembranes.

Hence, together with their scavenging activity against the stable 1,1-diphenyl-2-picrylhydrazyl radical, we have investigated the antioxidative effect of oleuropein and hydroxytyrosol in a model system consisting of dipalmitoylphosphatidylcholine/linoleic acid unilamellar vesicles and a water-soluble azo compound as a free radical generator. This model system helps in under-

standing the effectiveness of antioxidants against the attack of oxygen radicals on biomembranes from the aqueous phase (Fiorentini et al., 1994; Terao et al., 1994; Castelli et al., 1997). Furthermore, the capability of oleuropein and hydroxytyrosol to interact with dimyristoylphosphatidylcholine multilamellar vesicles, as a biological membrane model, was investigated by means of differential scanning calorimetry, a powerful and non-perturbing thermodynamic technique which allows characterization of the thermotropic phase behaviour of lipid bilayers in liposomal structures, and convenient and sensitive determination of the interaction of drugs with artificial membranes (O'Learly et al., 1986; Jain, 1988; Seydel, 1991; Saija et al., 1995b). The possible relationship between biophenol antioxidant efficiency and membrane interaction was discussed.

2. Materials and methods

2.1. Quenching of DPPH (DPPH test)

The free radical-scavenging capacity of oleuropein and hydroxytyrosol was tested as bleaching of the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH; Nanjo et al., 1996). The reaction mixture (3.5 ml of ethanol) contained 86 μM DPPH and different concentrations of oleuropein, hydroxytyrosol and α-tocopherol (the last one used as a control antioxidant standard); an equal volume (30 μl) of the solvent (ethanol) employed to dissolve the compounds tested was added to control tubes. After 10 min at room temperature, the absorbance was recorded at 517 nm. All experiments were carried out in duplicate and repeated at least three times. Results were expressed as percentage activity and mean scavenging concentrations (SC_{50}) were calculated by using the Litchfield and Wilcoxon test.

2.2. Oxidation of linoleic acid in LA/DPPC LUVS (LP–LUV test)

The method (Castelli et al., 1997) consists in the spectrophotometric determination of the acmulation of products (conjugated dienes) of peroxidation, induced by the water-soluble peroxyl radical generator 2,2′-azobis(2-amidinopropane)-hydrochloride (AAPH), of linoleic acid (LA) in mixed dipalmitoylphosphatidylcholine/linoleic acid (DPPC/LA) unilamellar vesicles (LUVs). Liposomes are generally accepted to be a suitable model to simulate the effect of free radicals on biological structures and evaluate the effectiveness of antioxidant compounds, given that they are surrounded by a lipid bilayer, structurally similar to the lipid matrix of cell membranes (Castelli et al., 1997). The radical initiator AAPH is used since it ensures generation of peroxyl radicals at a constant rate. Furthermore, the peroxyl radical is a common free radical found in the body (Halliwell and Gutteridge, 1990) and has been used in several antioxidant activity assays (Wang et al., 1997); it is slightly less reactive than $OH\cdot$ and thus possesses a half-life of seconds instead of nanoseconds. The following scheme of reactions summarizes this method (Horan et al., 1994):

$$R{-}N{=}N{-}R \rightarrow 2R\cdot + N_2$$

$$R\cdot + O_2 \rightarrow ROO\cdot$$

$$ROO\cdot + LH \rightarrow ROOH + L\cdot$$

$$L\cdot + O_2 \rightarrow LOO\cdot$$

$$LOO\cdot + LH \rightarrow LOOH + L\cdot$$

$$2LOO\cdot \rightarrow \text{non radical products}$$

$$LOO\cdot + InH \rightarrow LOOH + In\cdot$$

$$LOO\cdot + In\cdot \rightarrow \text{non radical products}$$

where R–N=N–R represents the radical initiator, LH linoleic acid, $L\cdot$ a linoleic radical, $LOO\cdot$ a linoleic peroxy radical and InH the inhibitor.

Multilamellar liposomes (MLVs) were obtained by freshly prepared chloroform-methanol (1:1, v:v) concentrated solutions of DPPC and LA (molar ratio 1:0.125). The solvents were removed under nitrogen in a rotary evaporator and the resulting film was kept overnight under vacuum to remove the residual solvents. Liposomes were prepared by adding 0.9% NaCl aqueous solution to the film, then heating at a temperature above that

of the gel-liquid crystalline phase transition (60°C) and vortexing three times for 1 min, after which the samples were shaken for 1 h in a water bath at 60°C to homogenize the liposomes. LUVs were prepared by submitting the previously prepared MLV dispersion to extrusion through 100 nm polycarbonate membranes (Avestin, Ottawa, Canada) in an extruder system (LiposoFast™ Basic, Avestin), according to the method described by MacDonald and coworkers (MacDonald et al., 1991). Oleuropein, hydroxytyrosol or α-tocopherol (the last one used as a control antioxidant standard) were dissolved in the opportune solvent and an aliquot (8 μl) was added to 1.2 ml of LUV suspension (21 mg DPPC/ml); after which the mixture was incubated for 20 min at 37°C in a shaking water bath. Then, the peroxyl radical generator AAPH was added to the suspension to obtain a final concentration of 10 μM. The oxidation was carried out at 37°C (below the transition temperature of DPPC/LA LUVs) under air. At given time points (5–90 min) 120 μl aliquots of the reaction mixtures were withdrawn and added to 1 ml methanol. The accumulation of LOOH formed from LA was determined spectrophotometrically (Saija et al., 1995a) by measuring the absorbance of the samples at 233 nm.

Since the rate of conjugated diene formation d[LOOH] is determined by measuring the absorbance A (at 233 nm) as a function of time

$$\frac{d[\mathrm{LOOH}]}{dt} = \frac{1}{\epsilon l}\frac{dA}{dt}$$

(where ϵ is the molar extinction coefficient of conjugated dienes and l the width of the cell in cm), the antioxidant efficiency (AE), or dA/dt, is given by the slope (calculated by first-order exponential regression analysis) of the plot of absorbance vs. time (between 5 and 90 min). From the plot dA/dt vs. $[\mathrm{InH}]^{-1}$ the slope S_{InH} was obtained and the relative antioxidant efficiency (defined as the ratio of the AE of the tested compounds to that of α-tocopherol; RAE) was calculated:

$$RAE_{\mathrm{InH}} = \frac{S_{\mathrm{TOC}}}{S_{\mathrm{InH}}} \times 100$$

Furthermore the ratio of oxidation-induced change in absorbance, with and without antioxidant addition, was used to calculate a % inhibition of oxidation (100% corresponding to complete protection and 0% corresponding to no difference from control) by the following equations:

$$[\mathrm{LOOH}]_{\mathrm{R.I.}} = A_{\mathrm{T90}} - A_{\mathrm{T5}}/\epsilon T_{\mathrm{SEC}}$$

$$[\mathrm{LOOH}]_{\mathrm{InH.}} = A'_{\mathrm{T90}} - A'_{\mathrm{T5}}/\epsilon T_{\mathrm{SEC}}$$

$$\%\,\mathrm{inhibition} = ([\mathrm{LOOH}]_{\mathrm{R.I.}} - [\mathrm{LOOH}]_{\mathrm{InH}}/[\mathrm{LOOH}]_{\mathrm{R.I.}}) \times 100$$

where $R.I.$ = radical initiator, A_{T90} = absorbance at the end of the experiment, A_{T5} = absorbance at the beginning of the experiment, A'_{T90} = absorbance at the end of the experiment in the presence of the antioxidant, A'_{T5} = absorbance at the beginning of the experiment in presence of the antioxidant, ϵ (molar extinction coefficient of conjugated dienes) = $26100 \pm 400\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$, T_{SEC} (time, s) = 5100, $[\mathrm{LOOH}]_{\mathrm{I.R.}}$ = hydroperoxide concentration after addition of the radical initiator alone, $[\mathrm{LOOH}]_{\mathrm{InH}}$ = hydroperoxide concentration after addition of the antioxidant.

All experiments were carried out in triplicate and repeated at least three times. Results were calculated as percentage decrease with respect to control values and mean inhibitory concentrations (IC_{50}) were evaluated by using the Litchfield and Wilcoxon test.

2.3. Differential scanning calorimetry (DSC)

Dimyristoylphosphatidylcholine (DMPC) MLVs were prepared in the presence and absence of drugs by the following procedure: chloroform-methanol stock solutions of DMPC and drugs (1:1, v/v) were mixed to obtain the chosen mole fractions (X_{D}) of drugs. The solvents were removed under a nitrogen flow in a rotary evaporator, and the resulting film was freeze-dried under vacuum to remove the residual solvents. Liposomes were obtained by adding to the film 50 mM Tris buffer (pH 7.4), then heating at a temperature above that of the gel-liquid crystalline phase transition (37°C) and vortexing three times for 1

min. The samples were shaken for 1 h in a water bath at 37°C to homogenize the liposomes. This temperature was chosen to avoid a possible degradation of biophenols to be tested.

Aliquots of 120 μl of lipid aqueous dispersion (5 mg of lipid), pure or containing oleuropein or hydroxytyrosol, were transferred to a 150 μl DSC aluminium, hermetically sealed pan (Mettler Toledo Group, Greifensee, Switzerland) and submitted to DSC analysis. DSC was performed by using a Mettler TA 3000 system equipped with a DSC-30 cell and a TC-10 processor (Mettler Toledo Group). The scan rate employed was 2°C/min in a temperature range of 2–37°C. The sensitivity was 1.72 mW, and the reference pan was filled with Tris buffer solution. Indium was employed to calibrate the DSC system for enthalpies and palmitic acid to check the temperature and enthalpy calibrations; after a routine temperature calibration in a large range, indium, palmitic acid and water were employed for a better temperature calibration within a narrow range. Enthalpies were evaluated from the peak areas using the integration program of the TA processor, permitting the choice of different baselines and ranges of integration. For curves showing an ill-defined baseline, a fixed arm planimeter was also employed. The areas calculated with these different methods lie within the experimental error (>5%).

After calorimetric scans, all samples were extracted from the pan and aliquots were used to determine the amount of phospholipid by the phosphorous assay (Bartlett, 1959).

2.4. Log capacity factor (log K′)

Reverse-phase chromatographic retention times can be used to estimate oil/water partition coefficients; a good correlation is found between log octanol/water partition coefficients and log K' using octadecyl silica columns (Saija et al., 1995a).

Log K' values for oleuropein and hydroxytyrosol were determined by HPLC with UV/visible detection. Each drug was dissolved in 100 ml of absolute methanol to give a final concentration of 10 μg/ml; samples were filtered prior to injection using a Millex HV13 filter (Waters-Millipore) and an aliquot (20 μl) was analyzed by HPLC. The HPLC apparatus consisted of a Varian 5000 system (Varian, Walnut Creek, CA, USA) equipped with a 20 μl loop and a Polychrom 3060 UV/VIS detector (Varian). Integration of the chromatographic peaks was achieved with a 4290 integrator (Varian). Chromatography was performed on a Pecosphere HS-5 HC ODS column (particle size: 10 μm; 15 cm × 4.6 mm i.d.; Perkin-Elmer, Norwalk, CT, USA). The mobile phase was 2% CH_3COOH/CH_3CN (78:22). The flow-rate was set at 1.3 ml/min. Detection was effected 278 nm.

Log K' values were calculated from the following relationship:

$$\log K' = \log \frac{T_r - T_0}{T_0}$$

where T_r is the retention time of the biophenol peak and T_0 denotes the retention time of the non retained solvent peak.

2.5. Chemicals

Synthetic 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and L-α-dimyristoylphosphatidylcholine were obtained from Fluka Chemical, Buchs, Switzerland. The lipid solutions were chromatographically pure as assessed by two-dimensional thin-layer chromatography. Lipid concentrations were determined by phosphorous analysis with the method of Bartlett (1959).

Linoleic acid, 2,2′-azobis(2-amidinopropane)hydrochloride, 1,1-diphenyl-2-picrylhydrazyl radical, acetic acid and acetonitrile were supplied by Sigma-Aldrich Italia. Oleuropein was obtained from Extrasynthese, France.

Hydroxytyrosol was synthetized according to the method described by Le Tutour and Guedon (1991).

3. Results

3.1. DPPH test

Oleuropein and, especially, hydroxytyrosol elicited a good concentration-dependent scavenging effect, allowing the calculation of the half-scavenging concentrations (SC_{50}) reported in Table 1.

Table 1
Scavenging effect on DPPH radicals by oleuropein and hydroxytyrosol

Drugs	SC_{50} (μM)	95% Confidence limits (μM)
α-Tocopherol	18.59	16.10–21.47
Oleuropein	25.22	22.85–27.82
Hydroxytyrosol	20.51	16.76–25.10

Experiments were carried out as described in Section 2.

Table 2
Antioxidant activity against peroxidation of linoleic acid in DPPC/LA LUVs by oleuropein and hydroxytyrosol

Drugs	IC_{50} (μM)	95% Confidence limits (μM)	RAE
α-Tocopherol	0.96	0.66–1.40	100.0
Oleuropein	15.15	11.94–19.19	29.16
Hydroxytyrosol	18.18	15.65–21.12	16.69

Experiments were carried out as described in Section 2.

3.2. LP–LUV test

Incubation of DPPC/LA LUVs in presence of AAPH induced a large increase in the accumulation of LOOH formed from LA peroxidation. The addition of the two biophenols tested reduced the amount of LOOH formed in a concentration-dependent manner, oleuropein being more effective than hydroxytyrosol (Fig. 2). The RAE values and half-inhibition concentrations (IC_{50}) have been calculated from the concentration-activity curves and are reported in Table 2.

3.3. DSC and log K′

Table 3 shows the calorimetric peak temperature (T_m), associated with the gel to liquid-crystal phase transition, of DMPC dispersion in the presence of different mole fractions (X_D) of the two drugs tested. Oleuropein interacts with DMPC vesicles, causing shifts, toward lower values, of the calorimetric peak temperature (T_m) associated to the gel to liquid-crystal phase transition, typical for DMPC multilayers (Table 3). The enthalpy changes (ΔH), related to the calorimetric peak area, remained nearly constant. On the contrary, no detectable change in T_m of DMPC dispersions was observed in presence of hydroxytyrosol, for all the molar fractions investigated.

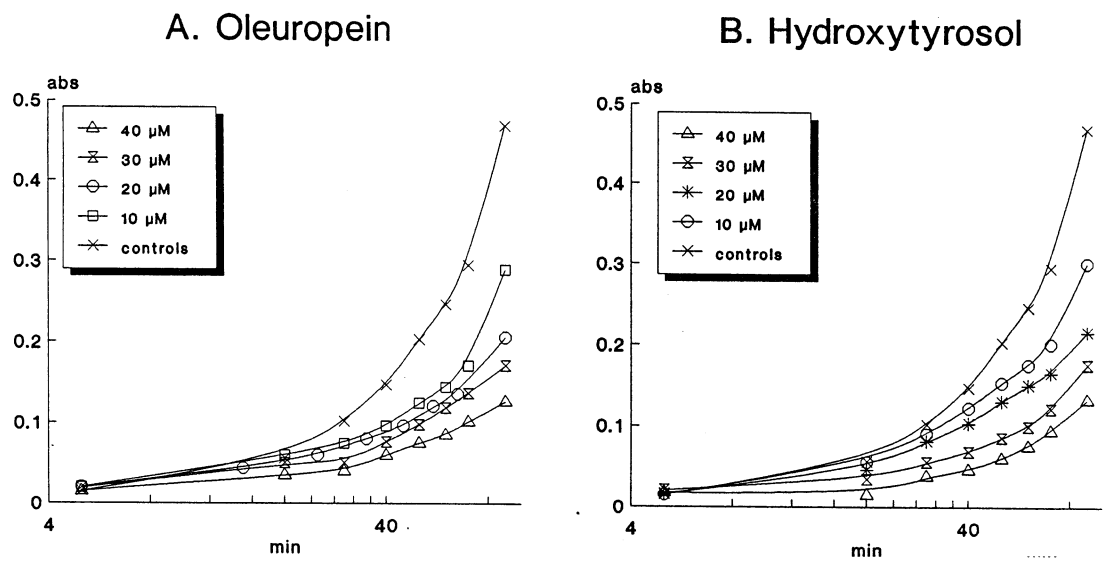

Fig. 2. Antioxidant activity against LA peroxidation in DPPC/LA LUVs by increasing concentrations of oleuropein (A) and hydroxytyrosol (B). Experiments were carried out as described in Section 2.

As shown in Fig. 3 (reporting the calorimetric heating curves of DMPC liposomes in the presence of different drug X_D), oleuropein induced a concentration dependent perturbation of the ordered lipid structure until $X_D = 0.12$; then a phase separation between two different domains was observable.

Finally the values of log K' calculated for oleuropein and hydroxytyrosol were, respectively, 1.048 and 0.625.

4. Discussion

A brief comment regarding the interaction of olive phenols with model membranes is needed as a prelude to the discussion of results. Between the two compounds tested, oleuropein, but not hydroxytyrosol, appears to interact with DMPC membranes; this also occurs despite the presence, in oleuropein backbone structure, of a sugar moiety, generally claimed to prevent drug access to lipid membranes (Ratty and Das, 1988). Lipophilicity of drugs has been clearly demonstrated to modulate their incorporation or interaction with lipids in the model membranes (O'Learly et al., 1986; Jain, 1988). Oleuropein is more lipophilic than hydroxytyrosol, as shown by their log K' values (respectively 1.048 and 0.625). Thus, one can suppose the existence of a lipophilicity-dependent relationship in the different effect of oleuropein and hydroxytyrosol on the thermotropic behaviour of lipid membranes.

Table 3
Main transition peak temperature (T_m, °C) of DMPC dispersions at different molar fractions of oleuropein and hydroxytyrosol

Molar fractions	T_m (°C)	
	Oleuropein	Hydroxytyrosol
0.000	24.0	24.0
0.030	23.7	23.9
0.045	23.4	23.8
0.060	23.7	23.9
0.090	22.8	24.1
0.120	21.7	24.0
0.165	19.1	23.9
0.240	19.7	23.9

Experiments were carried out as described in Section 2.

The interaction observed between oleuropein and DMPC liposomes may be largely explained in terms of a fluidifying effect due to the introduction of lipophilic molecules into the ordered structure of the lipid bilayer (Jain, 1988; Castelli et al., 1989; Castelli and Valencia, 1989; Saija et al., 1995a,b). In fact drug molecules act as a spacer in such a structure causing a destabilization of the lipid mosaic with a decrease in the T_m of the gel to liquid crystal phase transition. The negligible variation in the ΔH can be explained as a superficial interaction between oleuropein molecules and lipids, since molecules can interact with lipids in liposomes as 'interstitial impurities' (intercalating between the flexible acyl chains of lipids and causing T_m variations but no ΔH changes), according to the temperature depression of melting point for ideal solutions (Guggenheim, 1952; Lee, 1977; Jorgensen et al., 1991).

The particular calorimetric pattern observed following oleuropein interaction with DMPC vesicles leads us to make some considerations about the distribution of this biophenol in membranes. Given that hydrophobic molecules better dissolve in the liquid-crystalline phase (where lipid chains are in a disordered conformation), at temperatures over T_m, drug molecules are well dispersed in the lipid sea; instead at temperatures below T_m, drug molecules can aggregate, being less soluble in the rigid structure of the gel phase (Lee, 1977). We can suppose that, by reaching a determinate molar fraction, drug molecules dissolved into lipidic membranes start to aggregate with each other and therefore to segregate into isle-like clusters, segregation increased by increasing the X_D (Jain, 1988). The results of such a process is the subtraction of drug molecules from the homogeneous lipid-drug dispersion, causing the shift of T_m to lower values for the fraction of lipids rich in the drug, while the lipid fraction free of drug is not perturbed in its ordered lipid lamellar structure and so shows a shift to higher values like that shown by the pure DMPC.

However, the present results cannot permit us to exclude the possibility that, at high concentrations, the T_m shift might be influenced by a cer-

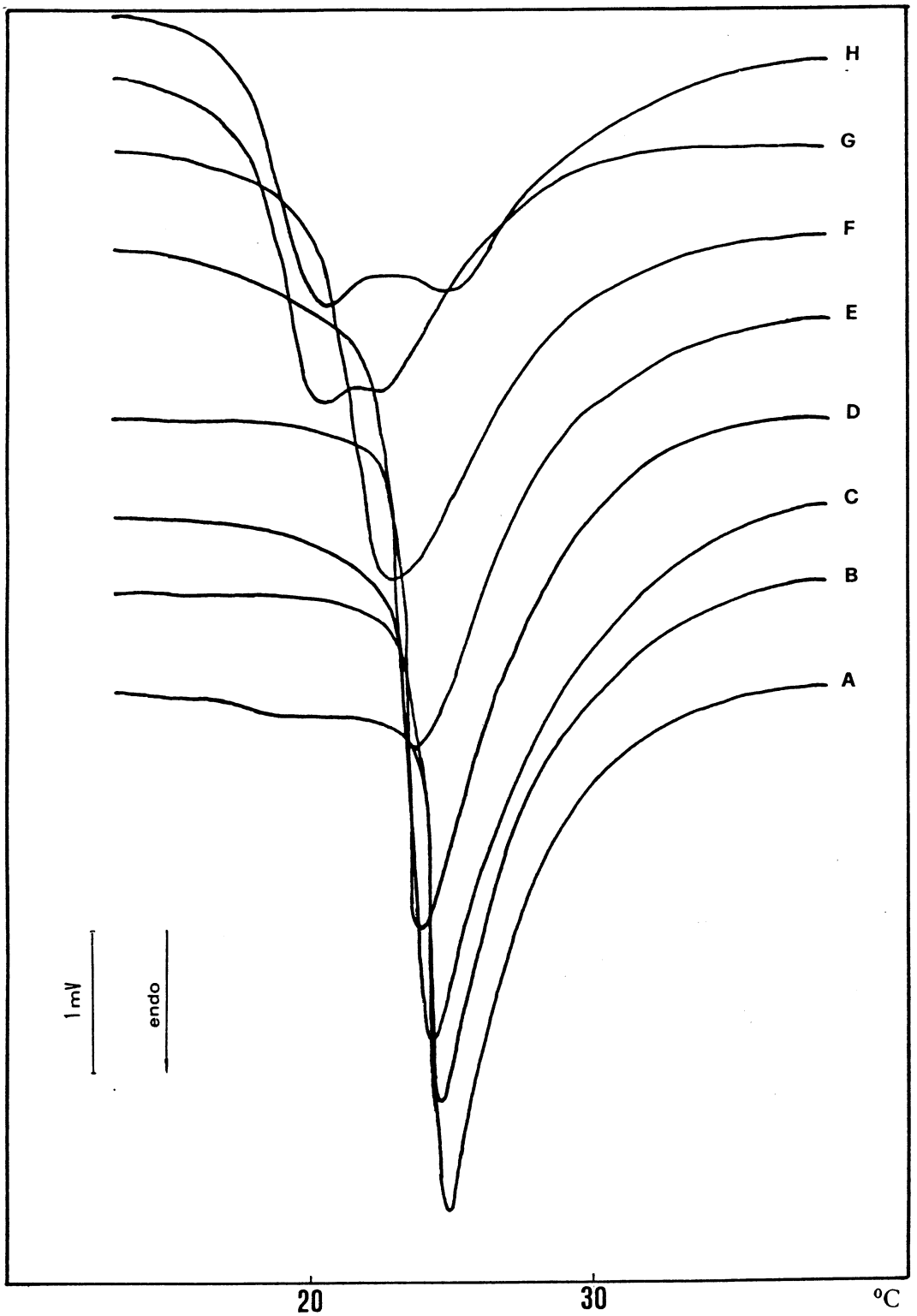

Fig. 3. DSC heating curves of hydrated DMPC containing oleuropein at the following molar fractions: A = 0.000, B = 0.030, C = 0.045, D = 0.060, E = 0.090, F = 0.120, G = 0.165, H = 0.240. Experiments were carried out as described in Section 2.

tain difficulty of the drug to be introduced into vesicles, as well as by the capacity of oleuropein to leave the lipidic membranes, transferring in the aqueous medium. Similarly, catechins (Ikigai et al., 1993) and quercetin and hesperetin (Saija et al., 1995b, 1996) are reported to alter the barrier function of lipid bilayers.

The different modification of the fluidity of the model membrane may be a critical factor in determining many functions of cell membranes and the activities of molecules embedded in them. Olive phenolic compounds are reported to interfere with various biological processes (lipoprotein oxidation, platelet aggregation, platelet and leukocyte eicosanoid production, cardiovascular control, etc.). However, some dissociation in the pharmacological profile of oleuropein and hydroxytyrosol is quite evident; for example, hydroxytyrosol, but not oleuropein, significantly affects platelet aggregation (Galli et al., 1994). One could speculate that such a dissociation might be, partially at least, related to the observed different capability of oleuropein and hydroxytyrosol to interact with lipid bilayers and to modulate membrane fluidity.

Our results obtained in the DPPH and LP–LUV tests confirm the good scavenger activity and antioxidant effect of oleuropein and hydroxytyrosol, both molecules possessing the structural requirement (a catechol group) needed for optimal antioxidant and/or scavenging activity. In agreement with these findings, oleuropein and hydroxytyrosol are reported to prevent thermally initiated autoxidation of methyl linoleate in homogenous solution (Le Tutour and Guedon, 1991), protect low density lipoproteins (LDL) from oxidation (Visioli and Galli, 1994, 1995; Visioli et al., 1995a,b) and inhibit production of isoprostanes and other markers of lipid peroxidation occurring during LDL oxidation (Salami et al., 1995).

The results obtained in the LP–LUV test (that is a membranous system) did not reveal the same order of effectiveness shown in the homogenous solution of DPPH test and, at least for oleuropein, the half-effective concentrations calculated in these two tests are far different. In fact, while both compounds exhibit comparable effectiveness in the DPPH test (hydroxytyrosol being slightly more active than oleuropein), oleuropein seems, in the LP–LUV test, a better antioxidant than hydroxytyrosol (see RAE values reported in Table 2). Furthermore, as shown by SC_{50} and IC_{50} values reported in Table 1 and Table 2, oleuropein shows a better antioxidant activity in the membranous system than in homogenous solution. Consistent with these observations, it is known that given the reactivity of an antioxidant in homogenous solution, the actual antioxidant effect in membranes can either decrease or increase due to several factors which are not considered in chemical tests. In fact, liposomal systems allow researchers not only to determine the intrinsic reactivity of a potential antioxidant, but also to evaluate its potency in a membranous system (structurally similar to the lipid matrix of cell membranes) where the contribution of the physical properties of the antioxidant (drug partitioning between the aqueous phase and the lipid phase, drug-induced membrane stabilizing or destabilizing effects, rate of drug transport across membranes, segregation of drug molecules into clusters, poor drug accessibility at the water/lipid interface, etc.) toward inhibition of lipid peroxidation is important.

In AAPH-induced peroxidation of LUVs, the chain-initiating radical is generated in aqueous phase and chain-propagating lipid peroxyl radicals are located within membranes. Scavenging of aqueous peroxyl radicals at the surface of membranes, as well as scavenging of lipid peroxyl radicals within the membranes, seems to play a considerable part in antioxidant activity of lipophilic antioxidants. Hydroxytyrosol is more hydrophilic than oleuropein; besides, unlike hydroxytyrosol, oleuropein seems to be located within biomembranes. One suggestive hypothesis is that hydroxytyrosol can serve as scavenger of aqueous peroxyl radicals near the membrane surface, while oleuropein acts also as a scavenger of chain-propagating lipid peroxyl radicals within the membranes. In agreement with our hypothesis, flavonoids have been suggested to be located at the cellular membrane-lipid/water interface (Parasassi et al., 1984; Saija et al., 1995a,b) and can serve as scavengers of aqueous peroxyl radicals near the membrane surface (Terao et al.,

1994), while α-tocopherol, which is fully localized in the hydrophobic zone of the lipid bilayer (Bonina et al., 1996), acts as a scavenger of chain-propagating lipid peroxyl radicals within the membranes (Terao et al., 1994).

In conclusion, olive phenols oleuropein and hydroxytyrosol act as powerful antioxidants against lipid peroxidation in phospholipid bilayers induced by aqueous oxygen radicals. Since oxidative attack from the aqueous phase seems to be an important reaction for initiating membrane lipid peroxidation, these biophenols could have important applications in human diseases accompanied by free radical injury; their different capability to interact with biological membranes should be taken into account to further clarify their 'in vivo' antioxidative activity.

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