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Research Paper

Antioxidant activity of oleuropein and semisynthetic acetyl-derivatives determined by measuring malondialdehyde in rat brain

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Abstract

Objectives The purpose of this study was the evaluation of the antioxidant activity of natural and semisynthetic polyphenol derivatives from *Olea europea L.*, by assessing malondialdehyde (MDA), an important marker of oxidative stress.

Methods Polyphenol as hydroxytyrosol, oleuropein, oleuropein aglycone as mix of four tautomeric forms and their respective acetyl-derivatives were obtained from olive leaves using semisynthetic protocols. These compounds were administered intraperitoneally to Wistar rats treated with paraquat, an herbicide which is able to cause oxidative stress after central administration. Malondialdehyde was derivatized with 2,4-dinitrophenylhydrazine to produce hydrazone that was purified by solid-phase extraction. Using high-performance liquid chromatography coupled with a diode array, free and total MDA was measured on homogenate rat brain as marker of lipid peroxidation. The analytical method was fully validated and showed linearity in the tested concentration range, with detection limit of 5 ng/ml. Recovery ranged from 94.1 to 105.8%.

Key findings Both natural and semisynthetic polyphenol derivatives from a natural source as olive leaves were able to reduce MDA detection. The more lipophilic acetyl-derivatives showed an antioxidant activity greater than parent compounds. This potency seems to put in evidence a strict correlation between lipophilicity and bioavailability.

Introduction

Epidemiological studies have shown a relationship between the Mediterranean diet and a lowered incidence of pathologies such as cancer, atherosclerosis, cardiovascular and neurodegenerative diseases.[1,2] A key role in this diet's protective action is played by the presence of olive oil.[3–5] Olive extracts are a natural source of polyphenols, widely considered to be potentially beneficial for health. Many studies have revealed their capacity to scavenge reactive species, and human intervention studies have shown that olive polyphenols decrease the levels of oxidized low-density lipoproteins in plasma and positively affect several biomarkers of oxidative damage.[6,7] A more recent study assessed the antioxidant activity of both individual and combined phenolics in *Olea europaea L.* leaf extract, finding that they exhibit good radical scavenging abilities and superoxide dismutase-like activity.[8] Indeed, olive leaves have been used widely in traditional remedies in European and Mediterranean countries; they have been used in the human diet as extracts, herbal teas and powders. In addition, olive leaves (an agricultural waste product) have great potential as a source of a natural antioxidant, *that is* Oleuropein (Ole) that can constitute up to 6–9% of the dry matter derived from olive trees.[9] Simple treatments have been evaluated as procedures to recover natural antioxidants from olive leaves.[10]

Ole belongs to a specific group of coumarin-like compounds, the secoiridoids, which are usually found only in plants belonging to the *Oleaceae* family.

Ole is a glucoside ester of hydroxytyrosol (HT) and elenolic acid. Secoiridoids, in aglyconic forms, arise from

glycosides in olive fruits by hydrolysis of endogenous β-glucosidases during crushing and malaxation. These newly formed substances, which have amphiphilic characteristics, are partitioned between the oily layer and the vegetation water and are more concentrated in the latter fraction because of their polar functional groups.

Carrasco-Pancorbo *et al.* determined the antioxidant activity of several single phenolic compounds in virgin olive oil and showed that HT and Ole aglycone were the strongest in terms of antioxidant capacity.[11] A recent review summarizes the findings on Ole aglycone's beneficial effects against neuro-degeneration and other peripheral inflammatory and degenerative diseases.[12] That activity could result, at least in part, in a remarkable improvement in the pathological signs arising from stress conditions such as oxidative stress. The relative antioxidant potency of individual olive oil phenols may vary depending on their bioavailability. Several studies, carried out with human and animal models, have shown good bioavailability of olive oil phenols.[13,14] Ole is rapidly absorbed after oral administration, with maximum plasma concentration occurring 2 h after administration. Moreover, an important step in the metabolism of olive oil phenolics, *that is* Ole-glycoside, Ole and ligistroside–aglycones, is their formation into HT or tyrosol. These compounds are rapidly distributed and excreted in urine mainly as glucuronides or in very low concentrations as free forms. Indeed, HT is the most important metabolite.

To enhance bioavailability, various lipophilic compounds have been synthesized using a simple and environmentally friendly semisynthetic protocol by chemical manipulation of complex natural substances. Procopio *et al.* reported an international patent for a chemical-catalytic method for the peracetylation of Ole and its derivatives.[15] The aglycone was easily obtained from Ole by acetal linkage hydrolysis mimicking natural glycosidase enzyme action,[16] and it has been studied in different experimental animal models, showing interesting results as an anti-inflammatory agent.[17,18] Synthesis of peracetylated derivatives was performed to test their anti-inflammatory activity, and they were then successfully applied to study breast and thyroid cancer models.[16,19]

The purpose of this study was the evaluation of the antioxidant activity of both natural and semisynthetic polyphenol derived from *Olea europea L.* leaves. Then, the more lipophilic aglycone and acetyl-derivatives were prepared to determine a correlation between lipophilicity and bioavailability. An *in-vivo* rat model was chosen, and malondialdehyde (MDA) was determined as marker of oxidative stress induced by paraquat. A newly developed extraction procedure of hydrazone derivative from homogenate brain and high-performance liquid chromatography (HPLC) was fully validated.

Experimental

Chemicals and standards

Acetonitrile (ACN), acetone (C_6H_3O), benzoic acid, catechol, chloral hydrate ($C_2H_3Cl_3O_2$), dichloromethane (CH_2Cl_2), 2,4-dinitrophenylhydrazine (DNPH), phosphate-buffered saline (PBS), methanol (MeOH), naphthalene, naphthol, paraquat dichloride (PQ), perchloric acid ($HClO_4$), resorcinol, sodium dihydrogen phosphate, sodium chloride (NaCl), sodium hydroxide (NaOH), sulfuric acid (H_2SO_4), trifluoroacetic acid (CF_3COOH), 1,1,3,3-tetraethoxypropane (TEP) were purchased from Sigma-Aldrich Co. (Milan, Italy). All reagents were of analytical grade, unless stated otherwise and the organic solvents were HPLC grade. Water used for the preparation of a mobile phase and chemical reagents was prepared using an Elix TM water purification system (Millipore, Toronto, Canada).

Oleuropein and its semisynthetic derivatives

Ole was extracted from Coratina olive cultivar leaves, using a conventional extraction procedure. The olive leaves were dried for 48 h at 50 °C, milled and refluxed with water (100 g/l) at 100 °C for 8 h. The leaves were filtered, and the solution was dried under reduced pressure. The mixture was treated with C_6H_3O and filtered to remove solids; the solution was evaporated under reduced pressure to obtain the crude product. Ole was separated by means of liquid chromatography on a Supelco Versa–Flash station equipped with a silica cartridge and eluted with a mixture of CH_2Cl_2/MeOH (8 : 2 v/v) as mobile phase. HT, *that is* 2-(3,4-dihydroxyphenyl)ethanol, that occurs as simple phenol or esterified with elenolic acid to form Ole, was synthesized as reported in the literature.[20] Ole aglycone was obtained through controlled hydrolysis as a mixture of four tautomeric forms (OleAa-d): the enolic and dialdehydic forms, OleAa and OleAb, respectively, as well as the ring-closed species OleAc and the hydrated aldehydic derivative OleAd.[16] Then, acetylation of Ole, OleAa-d and HT was performed to obtain more lipophilic acetyl-derivatives, *that is* AcOle, AcOleAa-d and AcHT, respectively (Figure 1a and 1b).[15]

Determination of lipophilicity

The lipophilicity of the studied derivatives was measured by determining the partition coefficient (LogP). The experimental LogP was determined by reversed-phase liquid chromatography (RP-HPLC), as previously described.[16] Stock solutions containing 10 μg/ml of single polyphenols were prepared by dissolving them in mobile-phase solution (30% water/CF_3COOH, pH = 2.4, 70% methanol). Multiple injections of single standards were performed to define

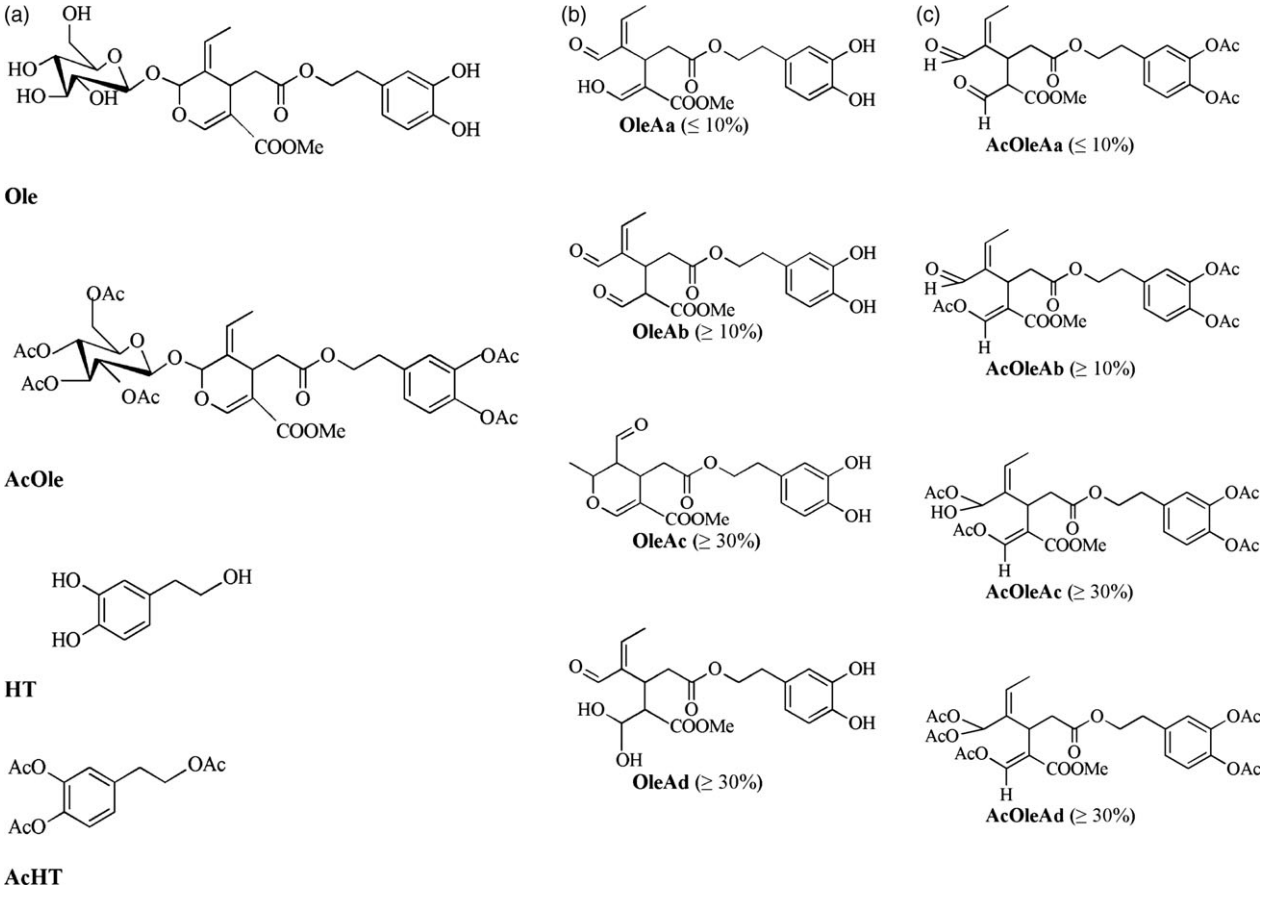

Figure 1 (a) Chemical structures of Ole, HT and their respective peracetylated derivatives AcOle and AcHT. (b) Chemical structures of oleuropein hemiacetal aglycone as a mixture of four tautomeric forms OleAa-d, that is three isomers (a-c), hydrated aldehydic form (d). (c) Chemical structures of acetylated derivatives of oleuropein hemiacetal aglycone as a mix of four tautomeric forms AcOleAa-d.

individual retention times and their reproducibility, as well as the stability of these selected compounds. Five reference compounds (catechol, resorcinol, benzoic acid, naphthol and naphthalene) with sufficiently different retention times were mixed. Capacity factor k′ was calculated for each compound. LogK′ values were correlated with known LogP values through a linear regression analysis to obtain a correlation curve. Moreover, experimental data were compared to theoretical data to determine how peracetylation improved the lipophilic character of polyphenols. The theoretical LogP values were obtained using two different methods: one based on established chemical interactions (cLogP calculated by Actelion Cheminformatics, Allschwil, Switzerland) and the other based on group contributions (miLogP calculated by Molinspiration Cheminformatics, Bratislava, Slovak Republic).[21,22]

Drugs administration

The antioxidant activity of the studied derivatives was evaluated using the herbicide PQ**,** which is able to cause oxidative stress after central administration (icv) in rats. Male Wistar rats (280–300 g; Charles River, Milan, Italy) were housed three per cage in humidity (60 ± 5%) and temperature (22 ± 2 °C)-controlled environment and were allowed free access to food and water until the time of the experiments. The animals were kept in a 12-h light/12-h dark cycle (lights on 7.00 a.m.–7.00 p.m., off 7.00 p.m.–7.00 a.m.). Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (DM 116192) as well as with EEC regulations (OJ of ECL 358/1 12/18/198 6). Before the experiment, rats were anaesthetized with $C_2H_3Cl_3O_2$ (400 mg/kg), intraperitoneally (ip), and positioned in a stereotaxic frame (D. Kopf Instruments). A stainless steel guide cannula (25 gauges) was then implanted unilaterally into the *Substantia Nigra* under stereotaxic guidance and secured to the skull with dental acrylic. The rats were allowed a one-week recovery period after cannulae implantation. For testing, the animals were placed in individual transparent Perspex cages and allowed to acclimatize to the new environment for 15 min. The rats were randomly divided into eight groups each

comprising six animals treated using saline, PQ alone and PQ plus whichever polyphenol derivative was being studied, *that is* Ole, AcOle, OleAa-d, AcOleAa-d, HT and AcHT, respectively. Infusion of PQ (150 μg/kg, icv) or vehicle (0.9% NaCl) was carried out by means of a 1-ml Hamilton syringe connected by a Teflon tube to an injection cannulae and injected in a total volume of 1 μl/min. The microinjection was considered successful if, after removal of the microinjector, a liquid flow could be induced by pushing the plunger therefore indicating that the injector was not clogged. Polyphenols were given (10 μmol/kg, ip), 15 min before focal injection of PQ. The dose and route of polyphenol administration used here to reduce joint injury were chosen on the basis of previous studies.[17,18] Animals were sacrificed by guillotine 4 h after treatment. Brains were collected, immediately frozen in liquid nitrogen and kept at −80 °C until use. Each of the brains (from both treated and control rats) was accurately weighted and homogenized in PBS added to the brain in a ratio of 1 : 20 p/v (g of biomass/ml of buffer), and the process was carried out in an ice-bath for 3 cycles of 5 min each. The homogenate underwent refrigerated centrifugation (14 000*g* for 15 min); the supernatant was withdrawn, placed in Eppendorf test tubes, each containing 500 μl of extracting phase and then centrifuged at 8000*g* for 10 min. The supernatant was collected and kept for estimation of endogenous MDA.

Validation

The method was validated by multiple analyses of spiked pooled brain homogenate standards, with regard to linearity, limit of detection (LOD) and quantification (LOQ), precision, accuracy and extraction efficiency.

MDA preparation, derivatization and determination

For method validation, MDA was obtained by hydrolysis of TEP, as reported in Figure 2. In detail, 25 μl TEP was dissolved in 100 ml of deionized water to give a 1 mM stock solution. MDA was prepared by hydrolysis of 1 ml TEP stock solution in 50 ml H_2SO_4 1% and stirred for 2 h at room temperature. Then, MDA derivatization reaction was carried out for 30 min at room temperature using DNPH, as shown in Figure 3. In detail, 25 μl of DNPH 5 mM (prepared using 2.9 mg of DNPH in 3 ml of $HClO_4$ 35%) was added to 250 μl of the resulting MDA standard solution (20 nmol/ml). All reactions were protected from exposure to light. The resulting derivatized MDA was a 2,4-dinitrophenylhydrazone (HDZ) insoluble in aqueous medium. It was filtered, dried, weighted and used as a standard to validate the method by determining accuracy, precision, extraction recovery and stability. Standard stock solution of HDZ was prepared in ACN (10 μg/ml). Then, as a control medium, TEP hydrolysis and MDA derivatization reactionμ were performed in PBS alone, whereas for method validation, brain tissue derived from untreated rats was used as homogenate PBS (1 : 20 p/v). In detail, 500 μl of TEP solution was added to 500 μl of buffer or homogenate sample acidified with 500 μl of $HClO_4$ 35%. The samples were vortexed and then centrifuged. The supernatant was collected and added to known amounts of DNPH for 30 min under stirring at 25 °C, to obtain HDZ. Homogenate tissue derived from treated rats underwent different preliminary procedures to determine free and total (free and protein bound) MDA. Free MDA was prepared by acid deproteinization, whereas an alkaline hydrolysis was chosen to determine total MDA.

Free MDA

Homogenate rat brain samples (500 μl) and $HClO_4$ 35% (500 μl) were mixed and the mixture vortexed to precipitate protein. After centrifugation at 14 000*g* for 10 min, clear supernatant was transferred to a 10-ml glass tube with a conical glass stopper and added to DNPH solution (100 μl, 5 mM) then mixed and incubated for 30 min at 25 °C in the dark.

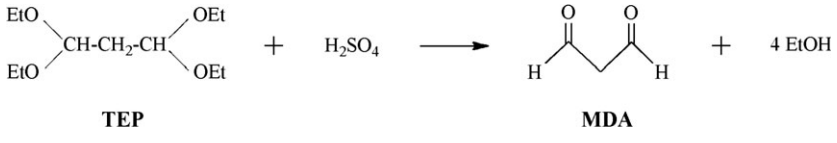

Figure 2 MDA obtained by acidic hydrolysis of TEP.

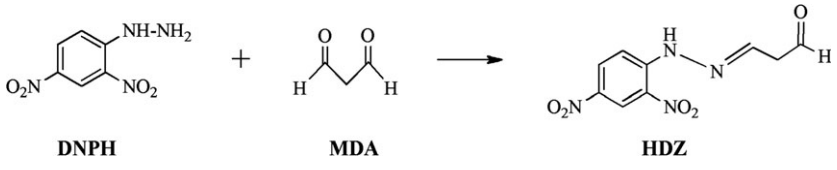

Figure 3 Derivatization process of endogenous and exogenous MDA with DNPH to obtain 2,4-dinitrophenylhydrazone(HDZ).

Total MDA

To determine total MDA, an alkaline hydrolysis of the protein binding was performed on the bound MDA fraction: 50 μl of 2 M aqueous NaOH was added to 500 μl of homogenate brain rat sample and incubated in a 60 °C water bath for 30 min., and then, the hydrolysed samples were acidified with 100 μl of $HClO_4$ 35%. After centrifugation at 14 000*g* for 10 min, the supernatant was transferred to a 10-ml glass tube with a conical glass stopper and added to DNPH solution (100 μl, 5 mM), then mixed and incubated for 30 min at 25 °C in the dark.

Extraction

Brains derived from untreated and treated rats underwent the same extraction procedures using solid-phase extraction (SPE). Conditioned cartridges, based on a polymeric matrix with a high hydrophilic–lipophilic balance (HLB Oasis SPE 1 ml, 30 mg; Waters-Milford, MA, U.S.A.), and a LiChrolut extraction unit (Merck) were chosen to isolate HDZ from biological samples. Samples (1 ml) were applied to conditioned cartridges and washed with 1 ml of water containing MeOH 5%; then, analytes were eluted twice using 1 ml of MeOH. For all dry steps, pressure was maintained at 39.9 kPa for 3 min. The eluate was dried under a nitrogen stream at 45 °C, and the residue was dissolved in 200 μl of mobile phase. The extract underwent chromatographic separation. The spiked brain homogenate samples were prepared to build standard curves and were refrigerated at −20 °C until assay.

Chromatography

A Jasco PU 1580 pump and LG 1580-02 ternary unit (Tokyo, Japan) with a 100-μl loop injection valve were used. The chromatographic system was used with a Jasco MD-1510 diode-array detector (DAD) (Tokyo, Japan), working at 310 nm (the maximum absorption wavelength of the HDZ). Data were processed using Borwin chromatography software (version 1.21) from Jasco (Tokyo, Japan). A mixture of acidified water and ACN (45 : 55 v/v) was used as mobile phase, and CF_3COOH was chosen to reach pH = 2.2. It was delivered at a flow rate of 1 ml/min through a Nucleosil 100-5 RP/18 (25 cm × 4.6 cm, 4 μm) reverse-phase column (Agilent, USA), with a C18 guard column (4.5 cm × 0.46 cm). A Gastorr GF 103 (Jones Chromatography, Colorado, USA) block heater was utilized to maintain the analytical column at 25 °C.

Statistical analysis

Results are presented as the mean ± standard deviation (SD) and coefficient of variation percentage (CV %) within each group of three rats. Comparisons of free and total MDA content between the control group, the group treated with paraquat and the groups treated with paraquat plus each of the polyphenol derivatives studied were made using one-way analysis of variance (ANOVA) with Tukey's test for post-hoc comparison. Differences were considered significant at $P < 0.05$. Correlation analysis was performed using Pearson's test. All computations were performed using Statgraphic Plus version 2.1 (Statistical Graphics Corp. Rockville, MD).

Results

Experimental and calculated LogP determination

Experimental LogP values were determined by RP-HPLC. To calibrate the system, a suite of well-defined organic compounds with known LogK' values were used as standards. Then, LogP values of each studied polyphenol were found by correlation with LogK′ through a linear regression (LogP= a LogK' + b) with a regression coefficient of 0.99. Moreover, a comparison between the experimental and calculated LogP was performed, as reported in Table 1.

All of the acetylated entities proved to be much more lipophilic than the corresponding starting species, and a good correlation was registered between the experimental and calculated LogP values.

Chromatography

Representative chromatograms of homogenate rat brain samples after SPE extraction are reported. An acid solution of derivatizing agent DNPH and an organic solution of derivatization product HDZ were injected into the column and identified by their relative retention times:

Table 1 Comparison between the experimental and calculated LogP of oleuropein (Ole), acetylated oleuropein (AcOle), oleuropein aglycone as a mixture of tautomeric forms (OleAa-d), acetylated oleuropein aglycone as a mixture of tautomeric forms (AcOleAa-d), hydroxytyrosol (HT), acetylated hydroxytyrosol (AcHT)

Compounds	Experimental LogP	Calculated LogP	
	LogK'	cLogP	miLogP
Ole	0.351	−0.37	−0.365
AcOle	2.611	2.050	1.672
OleAa-d	1.770[a]	1.430	1.520
AcOleAa-d	2.397[a]	2.854	1.620
HT	0.890	1.100	0.516
AcHT	1.704	2.050	0.826

[a]The average value of the set of peaks corresponding to the complex mixture was considered.

3.37 ± 0.35 min for DNPH and 5.64 ± 0.42 for HDZ, respectively. The analytes were determined using a **DAD** set at 310 nm. Figure 4 reports chromatograms of drug-free rat samples. In detail, Figure 4a shows the chromatogram without interfering peaks corresponding to the retention times of the studied compounds, which could have affected the precision and accuracy of measurements at the lowest calibration standard; Figure 4b shows the chromatogram derived from a derivatization reaction performed in separate batches of drug-free rat brain homogenate, reporting two peaks: Peak 1 represents DNPH (Rt = 3.43), whereas Peak 2 represents HDZ (Rt = 5.64) formed by derivatization of free MDA. Figure 5 hows chromatograms of homogenate brain samples of treated rats. In detail, Figure 5a shows the measured concentration of HDZ (6.261 μg/ml) corresponding to free MDA determined in rats treated with PQ alone; Figure 5b shows the measured concentration of HDZ (4.582 μg/ml) corresponding to free MDA determined in rats treated with PQ and pretreated with Ole; Figure 5c shows the measured concentration of HDZ (1.796 μg/ml) corresponding to free MDA determined in rats treated with PQ and pretreated with AcHT. All of the chromatograms reported a peak corresponding to DNPH; in fact, it was always added in excess to guarantee the complete reaction of the unknown concentration of endogenous MDA, formed *in vivo* in response to induced oxidative stress.

Validation of analytical method

Calibration curves were obtained by plotting the peak area of the unknown concentration versus that of the known concentration of HDZ added to drug-free rat brain homogenate. The curves were constructed from four replicate

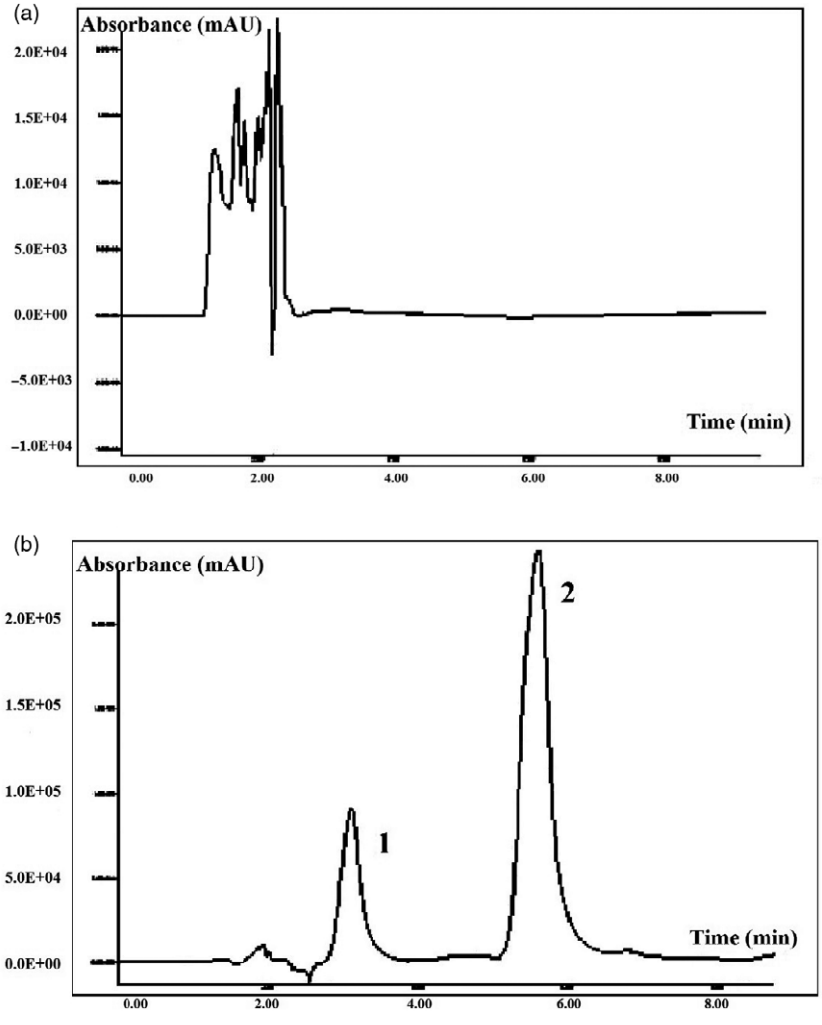

Figure 4 Representative chromatograms of homogenate rat brain sample (λ = 310 nm): (a) sample of untreated rat; (b) derivatization reaction performed in drug-free rat brain homogenate, reporting two peaks referred to DNPH and HDZ. Peak: 1 DNPH, Rt = 3.43; Peak 2 HDZ, Rt = 5.64.

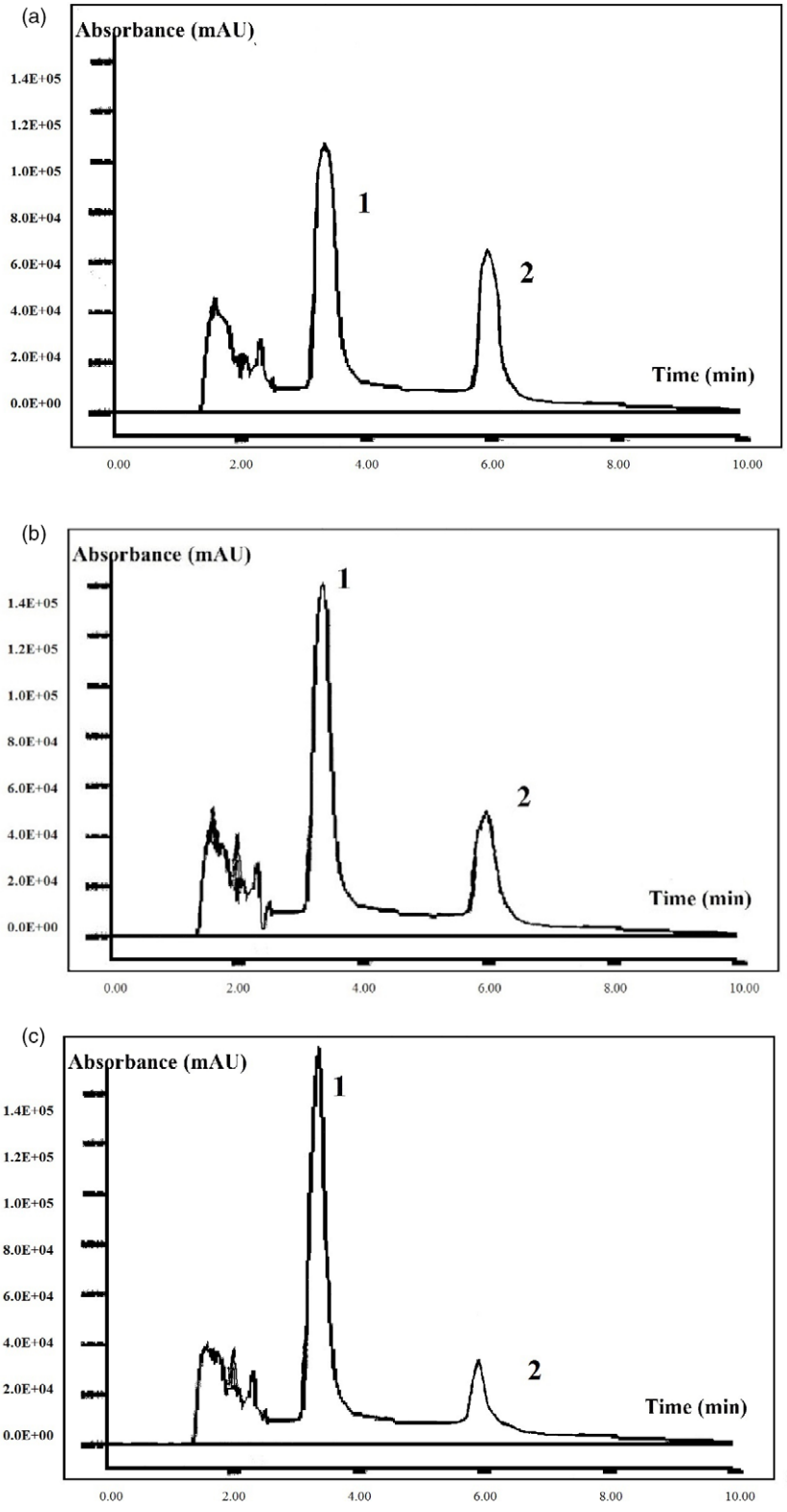

Figure 5 Representative chromatograms of homogenate brain sample of rats treated with PQ (150 μg/kg, icv) and polyphenol derivatives (10 μmol/kg, ip) (λ = 310 nm), as follows: (a) rat treated with PQ alone; peak 1 DNPH Rt = 3.30; peak 2 HDZ Rt = 5.52; (b) rat treated with PQ and Ole; peak 1 DNPH Rt = 3.38; peak 2 HDZ Rt = 5.48; (c) rat treated with PQ and AcHT; peak 1 DNPH Rt = 3.25; peak 2 HDZ Rt = 5.65.

measurements of nine concentrations (0.05-0.1-0.2-0.5-1-2-4-6-8 μg/ml). A linear response was observed over the examined concentration range. The square of the correlation coefficient of HDZ was 0.99953. Data of curve equation $Y = A\ x + B$ are reported as follows: A = 80.074; B = −2.380; standard error V y = 4946; mean % error = 5.927. The method allowed the determination of 50 ng/ml of HDZ in brain homogenate samples with a signal-to-noise ratio of 10 (LOQ) and LOD of 5 ng/ml with a signal-to-noise ratio of 3.

Precision and accuracy for within-day assay and between-day assay were reported in Tables 2 and 3, respectively. Data were determined on homogenate brain samples, derived from non-treated rats, by adding 0.1-0.2-0.5-1-2-4 and 6 μg/ml of HDZ in replicate (n = 3). In the within-day assay, CV ranged from 0.48 to 8.33%, where the relative mean error (RME) ranged from 0.02% to 0.11%. In the between-day assay, the highest CV% was 9.95 and calculated using 1 μg/ml of HDZ, where the highest RME% was 0.16 and calculated using 0.1 μg/ml of HDZ.

Extraction efficiency was measured as recovery of HDZ from aqueous standards and pooled brain homogenate standards by adding amounts of HDZ 0.1-0.2-0.5-1-2-4-6 and 8 μg/ml in replicate (n = 3). Recovery values ranged from 94.1 to 105.8%, measured spiking 4 and 1 μg/ml, respectively, as reported in Table 4.

Table 2 Precision and accuracy for within-day assay of the studied compound performed on homogenate brain samples by adding 0.1-0.2-0.5-1-2 and 4 μg/ml of 2,4-dinitrophenylhydrazone (HDZ) in replicate (n = 3)

Spiked concentration HDZ μg/ml	Found concentration HDZ μg/ml	SD	CV (%)	RME %
0.1	0.095	0.001	1.05	0.11
0.2	0.189	0.004	1.86	0.09
0.5	0.526	0.003	0.48	0.01
1	1.058	0.088	8.33	0.07
2	1.935	0.110	5.70	0.03
4	4.019	0.287	7.14	0.02

SD, Standard Deviation; CV, Coefficient of variation; RME, Relative mean error.

Table 3 Precision and accuracy for between-day assay of the studied compound performed on homogenate brain samples by adding 0.1-0.2-0.5-1-2 and 4 μg/ml of 2,4-dinitrophenylhydrazone (HDZ) in replicate (n = 3)

Spiked concentration HDZ μg/ml	Found concentration HDZ μg/ml	SD	CV (%)	RME %
0.1	0.096	0.002	1.50	0.16
0.2	0.189	0.006	2.75	0.15
0.5	0.530	0.013	2.50	0.05
1	1.069	0.105	9.95	0.09
2	1.930	0.109	5.40	0.03
4	3.989	0.343	8.25	0.02

SD, Standard Deviation; CV, Coefficient of variation; RME, Relative mean error.

MDA determination

Table 5 shows the results of free and total MDA determination measured as HDZ in homogenate brain samples. Rats were randomly divided into eight groups, each comprising six animals, and were treated as follows: group 1-vehicle (0.9% NaCl, icv); group 2-PQ alone (150 μg/kg, icv); groups 3-8-PQ (150 μg/kg, icv) plus each of the six

Table 4 Determination of extraction recovery of 2,4-dinitrophenylhydrazone (HDZ) (0.1-0.2-0.5-1-2-4-6 and 8 μg/ml) from homogenate brain samples derived from untreated rats (n = 3)

Spiked concentration HDZ μg/ml	Recovered concentration HDZ μg/ml	SD	CV (%)	RME %	Recovery %
0.1	0.095	0.001	1.05	0.11	95.0
0.2	0.189	0.004	1.86	0.09	94.3
0.5	0.526	0.003	0.48	0.01	105.1
1	1.058	0.088	8.33	0.07	105.8
2	1.935	0.110	5.70	0.03	96.8
4	3.762	0.116	3.09	001	94.1
6	6.072	0.203	334	0.01	101.2
8	7.644	0.159	2.09	0.00	95.6

SD, Standard deviation; CV, Coefficient of variation; RME, Relative mean error.

Table 5 Determination of free and total malondialdehyde (MDA) measured as 2,4-dinitrophenylhydrazone (HDZ) in homogenate brain samples obtained from rats divided into eight groups (n = 6) and treated with the tested compounds, as follows: group 1 with vehicle (0.9% NaCl, icv); group 2 with paraquat (PQ) (150 μg/kg, icv); groups 3–8 with PQ plus polyphenol derivatives given intraperitoneally (10 μmol/kg), *that is*: oleuropein (Ole), acetylated oleuropein (AcOle), oleuropein aglycone as a mixture of tautomeric forms (OleAa-d), acetylated oleuropein aglycone as a mixture of tautomeric forms (AcOleAa-d), hydroxytyrosol (HT), acetylated hydroxytyrosol (AcHT). Bound MDA is expressed in percentage

Group of rats	Tested compounds	Free MDA as HDZ μg/ml ± SD	Total MDA as HDZ μg/ml ± SD	Bound MDA %
1	Vehicle	0.107 ± 0.010	0.233 ± 0.013	33
2	PQ	6.261 ± 0.094	7.654 ± 0.061	28
3	Ole + PQ	4.582 ± 0.065	6.620 ± 0.079	31
4	AcOle + PQ	4.219 ± 0.047	6.226 ± 0.071	32
5	OleAa-d + PQ	4.127 ± 0.044	6.027 ± 0.088	32
6	AcOleAa-d + PQ	2.413 ± 0.025	3.814 ± 0.041	32
7	HT + PQ	1.913 ± 0.021	2.509 ± 0.028	24
8	AcHT + PQ	1.796 ± 0.010	2.362 ± 0.063	24

SD, standard deviation.

polyphenol derivatives, *that is* Ole, AcOle, OleAa-d, AcOleAa-d, HT and AcHT (10 μmol/kg, ip). A central inoculation of herbicide PQ resulted in a strong enhancement of MDA levels, in comparison with control rats. The intraperitoneal polyphenol administrations significantly decreased free MDA levels ($F = 1008E04$; $P < 0.001$) and total MDA levels ($F = 1185E04$; $P < 0.001$) in brain tissue, compared to MDA *levels* determined in rats treated with PQ alone. Bound MDA is expressed as a percentage. It was evident that acetylated derivatives were more active than the respective parent compounds ($P < 0.001$). Moreover, acetylated hemiacetal aglycone product used as a mixture of four tautomeric forms (AcOleAa-d) resulted in a reduction of MDA concentration. In particular, rats treated with acetylated oleuropein aglycone showed a decrease in total MDA of 70% versus a decrease in 24% for the corresponding non-acetylated derivative.

The antioxidant activity of analysed polyphenols was as follows: Ole<AcOle<OleAa-d<AcOleAa-d<HT<AcHT.

Discussion

The analytical method here described proved to be highly sensitive and specific to determine the hydrazone derivative obtained after the MDA derivatization process; it was fully validated and provided good accuracy, precision and recovery.

For the first time, we developed an original SPE extraction procedure applied to homogenate rat brain to determine HDZ as marker of induced oxidative stress.

Different assays have been developed to evaluate the antioxidant activity of plants and food constituents: The use of an adequate assay is a critical point. There is a need to agree governance on *in-vitro* antioxidant methods based on an understanding of the mechanisms involved, because some of the assays are done at non-physiological pH values. In brief, the most widely used assay is MDA assay, which is associated with lipid peroxidation. In biological matrices, MDA exists both free and bound to -SH and -NH_2 groups of macromolecules, *that is*, proteins and nucleic acids and only low amounts of free MDA were detected in biological samples.[23] In the past, MDA was mostly determined after its reaction with thiobarbituric acid (TBA) in spectrophotometry and measuring absorbance at 532 nm.[24] Although it has been considered a favourable assay because of its ease of use, it is lacking in specificity. TBA reacts not only with MDA but with many other compounds of biological origin; identical MDA-TBA adducts were yielded from non-lipid-related materials. An important review identified gas and liquid chromatography-based analyses as the most widely used techniques for MDA determination in different matrices, as well as several derivatization-based strategies.[25] Even now, the use of MDA as a biomarker for assessing oxidative stress *in-vitro* and *in-vivo* model of different diseases is considered to be best practice,[26,27] despite the differences between the analytical and validation methods.[28,29]

Our fully validated study demonstrated the reliability of MDA assessment using a HPLC associated with DAD. Derivatization process was shown to be highly specific and presented clear advantages. In our experience, hydrazone derivative formation proceeds readily at room temperature under mildly acidic pH conditions; moreover, the formed HDZ is not unique to a given aldehyde, and therefore, a solid-phase extraction procedure was established to improve selectivity towards MDA-hydrazone derivative. In fact, chromatographic separation of other DNPH derivatives as short-chain water-soluble carbonylic compounds, namely formaldehyde, acetaldehyde and acetone, was not seen.

Studying the stability of HDZ, we determined a significant sensitivity to light; in fact, a reduction of up to 18% of the initial MDA peak signal was observed within 1 h of exposure to light. Subsequently, all of the samples were protected from exposure to light during the derivatization with DNPH and subsequent steps. Then, HDZ solutions were stable for more than 12 h at room temperature.

Because MDA may originate from different sources that are mainly free and protein bound MDA, with our method we could quantify total MDA using an alkaline hydrolysis with 2 M NaOH (for 30 min under heating to 60 °C) which was found to be optimal. Then, a determination of bound MDA in rat brains was successfully ameliorated.

To stimulate MDA formation in animal brains, an experimental model described by Mollace *et al.* was employed.[30] They demonstrated that a microinfusion of PQ into the *Substantia Nigra* produces brain damage via the abnormal formation of oxygen free radicals.

Because the lipophilicity of a substance is one of the parameters which influence its biological activity, natural and semisynthetic derivatives of *Olea europaea L.* were studied to describe their antioxidant properties and correlate their activity with lipophilicity. Then, a comparison of experimental LogP values and theoretical partition coefficients calculated by two different procedures was performed. All of the acetylated entities were reported to be much more lipophilic than the corresponding starting species. Moreover, we showed that all of the acetyl-derivatives of phenolic compounds possessed an antioxidant activity higher than their parent compounds. In particular, a mixture of tautomeric forms of acetylated Ole aglycone had an enhanced capacity to antagonize MDA formation, compared to deacetylated derivative. AcHT had more effective antioxidant activity than deacetylated form HT. The results obtained using aglycone forms suggest the presence of an active substance that can be naturally produced *in vivo* by enzymatic Ole hydrolysis. Otherwise, Ole showed only a

reduced protective action towards PQ-induced oxidative stress.

As shown in Table 5, a small amount of MDA was detected in the control rats treated only with vehicle. The concentrations 0.157 and 0.233 μg/ml of HDZ of free and total MDA, respectively, were considered to be physiological and were significantly different to the PQ-treated levels (6.261 and 8.654 μg/ml of HDZ of free and total MDA, respectively). Both natural and semisynthetic derivatives of *Olea europaea L.* showed to possess an antioxidant activity.

HT was able to reduce both free MDA (1.913 μg/ml) and total MDA (2.509 μg/ml). This result could be related to catecholic moiety. It acts by breaking the radical chain and forming a phenoxyl radical, stabilized by an intramolecular hydrogen bond with the free hydrogen of the ortho-hydroxyl group. Nevertheless, the antioxidant properties of *o*-diphenolic moiety seem to be insufficient to ensure adequate protection against PQ-induced stress, pointing to the decisive role played by the aglycone tautomeric mixture's dialdehydic function.

Moreover, a lowest concentration of free MDA (1.796 μg/ml) and total MDA (2.362 μg/ml) was determined in rat treated with AcHT. Interestingly, the reported data confirm the hypothesis that a major lipophilic character can help to ensure improved biological availability of the molecules.

Indeed, all the acetylated compounds (AcOle, AcOleAa-d and AcHT) exerted a better protective action than their hydrophilic analogues. These species are supposed to better cross the blood–brain barrier and biochemically be converted *in vivo* into their original active forms.

Conclusion

This study was performed to assess how the neurotoxic effect induced by central infusion of PQ in rats is antagonized by a pretreatment with antioxidant compounds derived from *Olea europaea L.*, given intraperitoneally as natural and semisynthetic polyphenols with different lipophilicity. The lipophilic character of the starting species was significantly improved by their acetylation, as proved by Log *P* calculations.

Pretreatment with oleuropein, oleuropein aglycone, hydroxytyrosol and related acetyl-derivatives, able to cross the brain–blood barrier even given systemically, antagonized an exaggerated formation of reactive oxygen species. Most importantly, all of the peracetylated derivatives exerted a higher antioxidant activity than the parent compounds.

The results confirm the hypothesis that a major lipophilic character, improving biological availability, can help to ensure enhanced activity of the molecules.

Declaration

All animal experiments were carried out in accordance with the EU Directive 2010/63/EU for animal.experiments. (Http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.Htm). All experimental protocols were carried out the University of Magna Graecia's (Catanzaro, Italy) animal testing facility after approval by the Local Ethics Committee.

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