Olive Leaf Extracts Are a Natural Source of Advanced Glycation End Product Inhibitors

Vassiliki G. Kontogianni,¹ Pantelis Charisiadis,¹ Evangelia Margianni,² Fotini N. Lamari,² Ioannis P. Gerothanassis,¹ and Andreas G. Tzakos¹

¹Department of Chemistry, University of Ioannina, Ioannina, Greece.

²Department of Pharmacy, University of Patras, Patras, Greece.

ABSTRACT Advanced glycation end products (AGEs), which are readily formed and accumulated with sustained hyperglycemia, contribute to the development of diabetic complications. As a consequence, inhibition of AGE formation constitutes an attractive therapeutic/preventive target. In the current study, we explored the phytochemical composition and the *in vitro* effect of two different olive leaf extracts (an aqueous and a methanolic) on AGE formation. The methanolic olive leaf extract inhibited fluorescent AGE formation in a bovine serum albumin (BSA)-ribose system, whereas the aqueous extract had no effect in both BSA-fructose and BSA-ribose systems. The phytochemical profile was investigated with liquid chromatography-ultraviolet-visible (UV-Vis) diode array coupled to electrospray ionization multistage mass spectrometry (LC/DAD/ESI-MSⁿ). Quantification of the major phenolic compounds was performed with high performance liquid chromatography with UV-Vis diode array detection and nuclear magnetic resonance spectroscopy. Among the major phenolic components (luteolin, hydroxytyrosol, luteolin-4'-O- β -D-glucopyranoside, luteolin-7-O- β -D-glucopyranoside, and oleuropein), luteolin and luteolin-4'-O- β -D-glucopyranoside were assigned as potent inhibitors of AGE formation. The extraction procedure greatly affects the composition and therefore the anti-glycation potential of olive leaves.

KEY WORDS: • AGE inhibitors • LC-MS/MS • NMR • olive leaf extracts

INTRODUCTION

GLYCATION, THE NON-ENZYMATIC REACTION of reducing sugars with proteins leads to a variety of fluorescent and non-fluorescent advanced glycation end products (AGEs) involving free radical and carbonyl intermediates. Glycation occurs physiologically in the course of aging and in various pathological processes. AGE accumulation is accelerated in diabetes and is correlated with the severity of its cardiovascular, renal, nervous, and ocular complications.¹ Further, AGEs are also increased and implicated in the pathology of atherosclerosis, neurodegenerative, and autoimmune diseases. AGE formation not only causes protein modification and tissue dysfunction, but also through interaction with specific receptors triggers a cascade of cellular events underlying inflammation, thrombogenesis, and others.²

Inhibition of AGE formation via blocking sugar attachment to proteins, trapping/scavenging the reactive intermediates, or breakdown of established AGE-induced cross-links constitutes an attractive therapeutic/preventive target.^{1,3}

Clinical trials to test the effectiveness of synthetic inhibitors are under way, although the first trials showed serious side effects.⁴ Several *in vitro* and *in vivo* studies have illustrated that natural products, especially those belonging to the polyphenol family, are promising agents for the prevention of AGE formation; their inherent antioxidant capacity reinforce their potential for effective anti-glycation.⁵

Olive leaf extracts have a distinctive composition. The leaves of *Olea europaea* are characterized by unique high oleuropein content, and several other 3,4-dihydroxyphenethyl esters and flavonoids.⁶ One of the traditional medicinal uses ascribed to olive leaves is against diabetes; however, the effect of olive leaf extract and its composition on AGE formation has not yet been investigated. The antioxidant properties of olive leaves have been documented in several *in vitro* and *in vivo* models.^{7,8} However, the lack of antioxidant reinforcement shown in a recent study of olive leaf (extract containing almost exclusively oleuropein) supplementation of healthy human individuals further stresses the importance of meticulous study of extract composition and dosage.⁹

In the current study, we investigated *in vitro* the anti-glycation profile of an aqueous and a methanolic olive leaf extract. The phytochemical profile of the two extracts was determined using liquid chromatography-UV-Vis diode array coupled to electrospray ionization multistage mass

Manuscript received January 23, 2013. Revision accepted May 28, 2013.

Address correspondence to: Vassiliki G. Kontogianni or Andreas G. Tzakos, Department of Chemistry, University of Ioannina, Ioannina GR-45110, Greece, E-mail: vkontog@cc.uoi.gr or atzakos@cc.uoi.gr

spectrometry (LC/DAD/ESI-MSⁿ). HPLC with UV-Vis diode array detection and NMR spectroscopy were used to quantify the phenolic constituents of the extracts. The *in vitro* anti-glycation properties of the major phenolic components were investigated and a direct correlation of phytochemical composition and bioactivity was provided for the two extracts.

MATERIALS AND METHODS

Plant material, reagents, and standards

Olive leaves were collected from olive trees grown in Northern Greece in November 2005. Reference specimens are retained in the herbarium of the University of Ioannina with voucher accession number UOI051108. The leaves were washed, dried in open air, and stored at -20°C . Acetonitrile and water of HPLC grade were obtained from Scharlau. Acetic acid and methanol of analytical grade were provided by Merck. Oleuropein, hydroxytyrosol, luteolin-4'-*O*- β -D-glucopyranoside, luteolin-7-*O*- β -D-glucopyranoside, and luteolin were obtained from Extrasynthese. Bovine serum albumin (BSA; fatty acid free), ribose, fructose, and aminoguanidine (AG) were supplied from Sigma-Aldrich.

Sample preparation

Fresh leaves (50 g) were boiled in 250 mL distilled water for 1 h and filtered. The water was totally removed with a freeze-dryer to obtain the aqueous olive leaf dried extract (AOLE). To prepare the methanol olive leaf extract (MOLE), 50 g olive leaves were macerated in 250 mL of methanol for 7 days in the dark at room temperature. The extract was separated by filtration and the solvent was evaporated under vacuum.

LC-MS analysis

All LC-MSⁿ experiments were performed on a quadrupole ion trap mass analyzer (Agilent Technologies; model MSD trap SL) retrofitted to a 1100 binary HPLC system equipped with a degasser, autosampler, diode array detector, and electrospray ionization source (Agilent Technologies). All hardware components were controlled by Agilent Chemstation Software.

Both extracts were dissolved in methanol to the concentration of 5 mg dry extract/mL and filtered through 0.45 μm internal diameter (i.d.) membrane filters. A 10 μL aliquot was injected into the LC-MS instrument. Separation was achieved on a 25 cm $\times$ 4.6 mm i.d., 5 μm Altima C18 analytical column (Alltech), at the flow rate of 0.6 mL/min, using a gradient of solvent A (water/acetic acid, 99.9:0.1 v/v) and solvent B (acetonitrile/methanol 50/50% v/v). The gradient used for the analysis of extracts was: 0–25 min, 95%–70% A; 25–35 min, 70%–65% A; 35–40 min, 65%–60% A; 40–50 min, 60%–30% A; 50–55 min, 30%–0% A; and 55–65 min, 0% A. Chromatograms were acquired at 254, 280, and 340 nm.

Both precursor and product (MS²) ion scanning of the phenolic compounds were monitored between *m/z* 50 and

m/z 1000 in negative polarity. The ionization source conditions were as follows: capillary voltage, 3.5 kV; drying gas temperature, 350 $^{\circ}\text{C}$; nitrogen flow 10 L/min; and nitrogen pressure 50 p.s.i. (~ 344.7 kPa). Maximum accumulation time of ion trap and the number of MS repetitions to obtain the MS average spectra were set at 30 ms and 3, respectively.

NMR experiments

NMR experiments were performed on a Bruker AV-500 spectrometer equipped with a TXI cryoprobe (Bruker BioSpin). NMR experiments were used for the quantitative analysis of the extracts, the procedure followed is described in ref.¹⁰

In vitro glycation of BSA

BSA (10 mg/mL, fatty acid-free) was modified *in vitro* at 37 $^{\circ}\text{C}$ by the reducing sugars, ribose or fructose (500 mM). All incubations were carried out in 0.1 M phosphate buffer (pH 7.4) in the absence and presence of different concentrations of extracts (1–100 $\mu\text{g/mL}$), pure compounds (1–100 μM), and AG (1 mM). All solutions contained 3 mM sodium azide to prevent bacterial contamination. After 3 days incubation for ribose-containing samples and 21 days for fructose-containing samples, formation of pentosidine was monitored by measuring its characteristic fluorescence using the excitation and emission maxima of 370 and 440 nm, respectively.^{11,12} Pentosidine is an amino acid adduct, arising by reaction between lysine or arginine residues and sugars. Fluorescence was measured by a Perkin-Elmer LS55 fluorescence spectrometer.

The fluorescence intensity of BSA incubated either alone (blank for positive control) or only in the presence of the extracts at the same conditions (blank for samples) was subtracted from that of BSA incubated only in the presence of fructose or ribose (positive controls) or from those of the samples (BSA in the presence of sugars and extracts), respectively, to eliminate interferences from possible intrinsic fluorescence of the extracts. Each experiment was performed twice in triplicates.

RESULTS

In vitro effect of AOLE and MOLE on AGE formation

AG (positive control) at the concentration of 1 mM inhibited fluorescent AGE formation in BSA incubated in the presence of fructose for 21 days by 65.47% and in BSA incubated in the presence of ribose for 3 days by 67.95%, in accordance to literature.¹¹ The aqueous AOLE at the final concentrations of 10 and 100 $\mu\text{g/mL}$ did not significantly affect pentosidine formation in both BSA-fructose and BSA-ribose systems. However, the methanolic (MOLE) extract inhibited ($P < .05$) fluorescent AGE formation in BSA incubated with ribose by 43.07% at the concentration of 100 $\mu\text{g/mL}$, although it did not significantly affect pentosidine formation in the BSA-fructose system. To identify the compounds responsible for the different bioactive

profiles of the AOLE and MOLE we explored the phytochemical constituents of the two extracts via LC-MS and NMR analysis.

LC-MS analysis

The LC/DAD/ESI-MSⁿ analysis of the olive leaf extracts led to the separation and identification of the majority of the constituents. Overall, 15 compounds were identified in AOLE and 16 compounds in MOLE belonging to two natural product classes: flavonoids and secoiridoids. Identification of the compounds was carried out by comparing retention times and masses with those of the five authentic standards. For the remaining compounds for which no standards were available, identification was based on accurate mass measurements of the pseudomolecular [M-H]⁻ ions and their fragmentation pattern as documented in the literature.^{13,14} The total ion current (TIC) chromatograms of MOLE and AOLE are presented in Figure 1. Data obtained from the ESI-MSⁿ analysis of the extracts are summarized in Table 1.

In detail, in the TIC chromatogram of both extracts, peak 1 eluting at 12.6 min displayed a precursor ion at m/z 315 attributed to hydroxytyrosol glucoside. Its MS² spectrum is characterized by the fragments at m/z 153 arising from the cleavage of the glycosyl bond and the ion at m/z 123 corresponding to loss of the CH₂OH group. Peaks O2, M3, and O4 eluting at 13.1 and 19.1 min gave a [M-H]⁻ ion at m/z 389 and showed the same fragments (m/z 345, 227, 183) obtained by ESI-MS². The fragment at m/z 345 can be justified by the elimination of a CO₂ molecule from a carboxylic group (44 Da). The fragment at m/z 227 can be attributed to loss of a hexose residue (162 Da) and the fragment at m/z 183 to a substituent loss of CO₂. This fragmentation pattern is consistent with the presence of oleoside, having two carboxylic groups and a hexose in its structure. The later-eluting peak was identified as the

isomeric secologanoside, which elutes after oleoside under reverse-phase conditions.¹⁴

The MS of the peaks arising at 30.6 min (Peak O5) and 34.6 min (Peak O8) displayed a precursor ion at m/z 555; the MS² spectrum obtained by fragmentation of this ion presented the following m/z values: 537, 393, and 323, described in literature for 10-hydroxy oleuropein and maybe one of its isomers.¹⁴ Peak O6 (33.3 min) gave the deprotonated molecule [M-H]⁻ at m/z 195 and the fragment at m/z 59 obtained by ESI-MS² corresponding to a COOCH₃ moiety, suggesting the presence of hydroxytyrosol acetate. Peaks M6 and O7 (33.6 min) displayed a precursor ion at m/z 593 and presented a strong fragment (m/z 285) obtained by ESI-MS², attributable to the elimination of a rutinoyl (rhamnoglucosyl) moiety-residue (308 Da) from the precursor ion, indicating the presence of luteolin-7-*O*-rutinoside detected in the past in olive leaf extracts.¹³

Peak O10 arising at 40.7 min displayed a precursor ion at m/z 319 and its dimeric form at m/z 639. Its MS² spectrum is characterized by the fragments at m/z 319, 195, and 165. The ion at m/z 319 may be tentatively characterized as oleuropein aglycon decarboxymethyl dialdehyde form (3,4 DHPEA-DEDA).¹³ Peak arising at 46.9 (M13 and O13) min gave a pseudomolecular ion at m/z 523 and its MS² spectrum is characterized by the fragments at m/z 361, 291, and 259 corresponding to a deoxy analogue of oleuropein named ligstroside.

Quantitative measurements with HPLC and NMR spectroscopy

Quantitative analysis was performed by means of an HPLC/diode array method using the authentic standards that are commercially available. Flavonoids were quantitated at 340 nm and oleuropein and hydroxytyrosol at 280 nm. The calibration curves were proved to be linear in the ranges of 0.5–50 mg/L for hydroxytyrosol, luteolin-4'-*O*-β-D-

FIG. 1. Total ion chromatogram of methanol olive leaf extract (MOLE) (A) and aqueous olive leaf extract (AOLE) (B).

TABLE 1. PHENOLIC COMPOUNDS IDENTIFIED IN OLIVE LEAF EXTRACTS

R_t (min)	$[M-H]^-$ (m/z)	MS^2 $[M-H]^-$ (m/z)	Compounds	Detected in ^a
12.6	315	153, 123	Hydroxytyrosol glucoside	M1, O1
13.1	389	345, 227, 183	Oleoside	O2
13.7	153	123	Hydroxytyrosol ^b	M2, O3
19.1	389	345	Secologanoside	M3, O4
24.8	377	307, 275	Oleuropein aglycon	M4
27.7	415	149, 221, 251	Unknown	M1', O1'
30.6	555	537, 323	10-Hydroxyoleuropein	O5
32.8	623	461, 315	Verbascoside	M5
33.1	195	59	Hydroxytyrosol acetate	O6
33.6	593	285	Luteolin-7- <i>O</i> -rutinoside	M6, O7
34.6	555	393, 323	10-Hydroxyoleuropein isomer	O8
35.2	447	285	Luteolin-7- <i>O</i> - β -D-glucopyranoside ^b	M7, O9
36.7	565	505	Unknown	O2'
38.1	577	415, 373	Unknown	M2', O3'
40.7	639, 319	319, 195, 165	Oleuropein aglycon decarboxymethyl dialdehyde form (3,4 DHPEA-DEDA)	O10
41.9	447	285	Luteolin-4'- <i>O</i> - β -D-glucopyranoside ^b	M8, O11
42.6	539	377, 345, 307, 275	Oleuropein ^b	M9, O12
44.1	377	307, 275	Oleuropein aglucon	M10
45.1	539	377, 307, 275	Oleuropein isomer	M11
46.1	539	377, 307, 275	Oleurosides	M12, O13
46.9	523	361, 291, 259	Ligstrosides	M13, O14
48.7	285	—	Luteolin	M14
49.5	377	307, 275	Oleuropein aglycon	M15
50.7	377	307, 275	Oleuropein aglycon	M16, O15

^aM, MOLE; O, AOLE; number indicates the peak in TIC chromatogram; the symbol ' is used for nonidentified compounds.

^bIdentification confirmed using commercial standards.

MOLE, methanol olive leaf extract; AOLE, aqueous olive leaf extract; TIC, total ion current.

glucopyranoside, luteolin-7-*O*- β -D-glucopyranoside, and luteolin with R^2 0.998, 0.996, 0.994, and 0.991, respectively. The calibration curve for oleuropein was also proved to be linear in the range 20–200 mg/L with R^2 0.995. Using NMR spectroscopy the concentration levels (mg/g) of the constituents of the two extracts were calculated with the method of spiking with the standard compounds at known concentration.^{10,15} The quantitative results are summarized in Table 2. The quantitative results provided by NMR data are in good agreement with those obtained by HPLC. The AOLE had a lower concentration of the above phenolic compounds in comparison with the MOLE, while luteolin was completely absent from AOLE.

In vitro effect of phytochemicals of AOLE and MOLE on AGE formation

The inhibitory potential of the major phenolic components (luteolin, hydroxytyrosol, luteolin-4'-*O*- β -D-glucopyranoside, luteolin-7-*O*- β -D-glucopyranoside, and oleuropein) on AGE formation was explored in BSA incubated with fructose (Fig. 2A) and in BSA incubated with ribose (Fig. 2B). In both systems, the inhibitory effect of luteolin and luteolin-4'-*O*- β -D-glucopyranoside was dose dependent, whereas that of luteolin-7-*O*- β -D-glucopyranoside, hydroxytyrosol, and oleuropein was very small or nonsignificant. Experiments at the concentration of 1 μ M of tested compounds showed no effect of fluorescent pentosidine formation.

DISCUSSION

The methanolic olive leaf extract inhibited fluorescent AGE formation in BSA-ribose system, whereas the aqueous extract had no effect in both BSA-fructose and BSA-ribose systems. To determine the phytochemical profile of the extracts, liquid chromatography-UV-Vis diode array coupled to electrospray ionization multistage mass spectrometry (LC/DAD/ESI-MSⁿ) was used. The MOLE had many common constituents (Table 1) with AOLE. Their main difference was the existence of more oleuropein aglycons in MOLE. Oleuropein is present in the form of aglycon due to the action

TABLE 2. CONCENTRATIONS OF COMPOUNDS IN AQUEOUS AND METHANOLIC OLIVE LEAF EXTRACTS

Compound	MOLE		AOLE	
	HPLC	NMR	HPLC	NMR
Hydroxytyrosol	8.5 $\pm$ 0.7	7.0 $\pm$ 0.4	1.1 $\pm$ 0.2	1.3 $\pm$ 0.1
Luteolin-4'- <i>O</i> - β -D-glucopyranoside	3.3 $\pm$ 0.4	2.9 $\pm$ 0.3	0.3 $\pm$ 0.06	0.2 $\pm$ 0.05
Luteolin-7- <i>O</i> - β -D-glucopyranoside	4.1 $\pm$ 0.5	3.4 $\pm$ 0.2	0.3 $\pm$ 0.04	0.4 $\pm$ 0.05
Luteolin	1.9 $\pm$ 0.3	1.6 $\pm$ 0.1		
Oleuropein	40.7 $\pm$ 5.0	36.9 $\pm$ 3.0	11.2 $\pm$ 0.9	9.2 $\pm$ 1.1

Values are presented as mean $\pm$ standard deviation in units of mg/g dry extract. HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance spectroscopy.

FIG. 2. Effect of the main olive leaf components on fluorescent advanced glycation end product formation in bovine serum albumin incubated with fructose for 21 days (A) or with ribose for 3 days (B). The final concentration values of hydroxytyrosol (HT), oleuropein (OLE), luteolin (LUT), luteolin-7-*O*- β -D-glucopyranoside (LUT-7G), and luteolin-4'-*O*- β -D-glucopyranoside (LUT-4G) in the test mixtures were 10 and 100 μ M. The fluorescence intensity of positive control values was set at 100%.

of hydrolytic enzymes. This hydrolysis also causes partial modification of the aglycon due to a keto-enolic tautomeric equilibrium that involves the ring opening of secoiridoids. Whenever we found two peaks in the LC/DAD/ESI-MSⁿ with the same mass spectra, we presumed that the first peak corresponds to the dialdehydic form and the other to the aldehydic form (closed-ring structure) of the compound. Caruso *et al.*¹⁶ observed that four peaks were present in the ion chromatogram of ions at *m/z* 377 in olive oil. MOLE and AOLE had 10 common constituents. In MOLE four oleuropein aglycon isomers were identified, while in AOLE only one isomer was identified.

It is the first time that the *in vitro* antiglycation properties of oleuropein and hydroxytyrosol are investigated and the lack of significant effects at the concentration range of 1–100 μ M is presented. In agreement with previous studies, we found that AGE inhibitory potential is correlated to the flavones present in the extracts.¹⁷ Previous structure–activity studies have shown that luteolin, among 10 other flavonoids, was the most potent inhibitor on each stage of protein glycation; flavones exhibit stronger inhibitory effects when compared with flavonols, flavanones, and isoflavones.³ In our

study, luteolin-7-*O*- β -D-glucopyranoside despite having a catechol group is not such an efficient inhibitor as luteolin-4'-*O*- β -D-glucopyranoside (not having vicinal hydroxyl groups). This confirms a previous study showing that glycosylation of the hydroxyl group at the C-7 position, and methylation or glycosylation of the 4'-hydroxyl group of flavonoids reduces anti-glycation activity.¹⁸ Interestingly, Fujiwara *et al.*¹⁹ have shown that natural products containing the catechol moiety may even enhance non-fluorescent AGE formation at high concentrations.

Our results demonstrate that the methanolic olive leaf extract inhibited fluorescent AGE formation in BSA-ribose system, whereas the AOLE had no effect in both BSA-fructose and BSA-ribose systems. The inhibitory potential of MOLE on AGE formation is attributed to the presence of luteolin and luteolin-4'-*O*- β -D-glucopyranoside in high concentrations. Although oleuropein was the major constituent of MOLE, it had no significant effect on AGE formation at the concentration range tested; however at higher concentrations it reduces AGE formation, that is, at 1000 μ M it inhibits AGE formation in BSA-ribose by 50% (our data). The inhibitory potential of MOLE flavone constituents on AGE formation coupled with the potent inhibitory action of luteolin and luteolin-7-*O*- β -D-glucoside on α -glucosidase,^{20,21} the inhibitory effect on GLUT2 mediated glucose and fructose efflux,²² and the potent inhibition of insulin-induced glucose uptake by adipose cells²³ encourage further research on the antidiabetic potential of olive leaf extracts. Our study clearly shows that the extraction procedure greatly affects the composition and therefore the anti-diabetic potential of olive leaves.

However, the *in vivo* efficacy of an extract greatly depends on the bioavailability of its constituents. It has been shown that after an acute ingestion of olive phenolic compounds, they are absorbed, metabolized, and distributed through the blood stream to practically all parts of the body, even across the blood–brain barrier.²⁴ Free forms of certain phenolic compounds were determined in certain tissues, that is, oleuropein in the plasma and brain, luteolin in the kidney, testicle, brain, and heart, or hydroxytyrosol in the plasma, kidney, and testicle, but plasma mainly contained the metabolites. Using the Caco-2/TC7 cells as a model of the human intestinal epithelium, limited metabolism of olive oil phenolics was observed; the methylated conjugates were the major metabolites detected.²⁵ Extensive transport of the parent aglycones and their conjugates to the basolateral side was also shown.²⁵ A previous study on the uptake and metabolism of the phenolic compounds from an olive leaf extract in SKBR3 cells has shown important oleuropein and luteolin-7-*O*- β -D-glucoside uptake in the cytoplasm and further metabolism; luteolin aglycone was found among other metabolites.²⁶ Thus, although luteolin is found in tissues after ingestion despite the metabolism and is transferred to the cytoplasm of cells, further research is necessary on the AGE inhibitory properties of the olive phenol metabolites.

Thus, our study contributes to the ongoing search for new anti-diabetic agents among the natural polyphenols,

which are characterized by their antioxidant and non-toxic properties. This report draws attention on the extraction procedure of olive leaves and on the potential of the constituent flavones as multifunctional anti-diabetic agents.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Esthir Gani Foundation (award to A.G.T.) and the Regional Operational Program of Thessaly-Mainland Greece-Epirus Research and Technological Development in the Region of Epirus Research Program “New Knowledge” (award to A.G.T.). We are grateful to the Greek Community Support Framework III, Regional Operational Program of Epirus 2000–2006 (MIS 91629), for supporting the purchase of an LC-NMR cryo instrument.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

1. Sourris KC, Harcourt BE, Forbes JM: A new perspective on therapeutic inhibition of advanced glycation in diabetic microvascular complications: common downstream endpoints achieved through disparate therapeutic approaches? *Am J Nephrol* 2009;30:323–335.
2. Yamagishi SI, Maeda S, Matsui T, Ueda S, Fukami K, Okuda S: Role of advanced glycation end products (AGEs) and oxidative stress in vascular complications in diabetes. *BBA-Gen Subjects* 2012;1820:663–671.
3. Wu CH, Yen GC: Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. *J Agric Food Chem* 2005;53:3167–3173.
4. Freedman BI, Wuerth JP, Cartwright K, et al.: Design and baseline characteristics for the aminoguanidine clinical trial in overt type 2 diabetic nephropathy (ACTION II). *Control Clin Trials* 1999;20:493–510.
5. Peng X, Ma J, Chen F, Wang M: Naturally occurring inhibitors against the formation of advanced glycation end-products. *Food Funct* 2011;2:289–301.
6. Benavente-Garcia O, Castillo J, Lorente J, Ortuno A, Del Rio JA: Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves. *Food Chem* 2000;68:457–462.
7. Kontogianni VG, Gerothanassis IP: Phenolic compounds and antioxidant activity of olive leaf extracts. *Nat Prod Res* 2012;26:186–189.
8. Cumaoglu A, Ari N, Kartal M, Karasu C: Polyphenolic extracts from *Olea europea* L. protect against cytokine-induced β -cell damage through maintenance of redox homeostasis. *Rejuvenation Res* 2011;14:325–334.
9. Kendall M, Batterham M, Obied H, Prenzler PD, Ryan D, Robards K: Zero effect of multiple dosage of olive leaf supplements on urinary biomarkers of oxidative stress in healthy humans. *Nutrition* 2009;25:270–280.
10. Charisiadis P, Primikyri A, Exarchou V, Tzakos A, Gerothanassis IP: Unprecedented ultra-high-resolution hydroxy group ^1H NMR spectroscopic analysis of plant extracts. *J Nat Prod* 2011;74:2462–2466.
11. Sharma SD, Pandey BN, Mishra KP, Sivakami S: Amadori product and age formation during nonenzymatic glycosylation of bovine serum albumin *in vitro*. *J Biochem Mol Biol Biophys* 2002;6:233–242.
12. Ardestani A, Yazdanparast R: *Cyperus rotundus* suppresses AGE formation and protein oxidation in a model of fructose-mediated protein glycoxidation. *Int J Biol Macromol* 2007;41:572–578.
13. Ryan D, Antolovich M, Herlt T, Prenzler PD, Lavee S, Robards K: Identification of phenolic compounds in tissues of the novel olive cultivar Hardy's Mammoth. *J Agric Food Chem* 2002;50:6716–6724.
14. Fu S, Arráez-Roman D, Segura-Carretero A, et al.: Qualitative screening of phenolic compounds in olive leaf extracts by hyphenated liquid chromatography and preliminary evaluation of cytotoxic activity against human breast cancer cells. *Anal Bioanal Chem* 2010;397:643–654.
15. Charisiadis P, Exarchou V, Troganis AN, Gerothanassis IP: Exploring the “forgotten” -OH NMR spectral region in natural products. *Chem Commun* 2010;46:3589–3591.
16. Caruso D, Colombo R, Patelli R, Giavarini F, Galli G: Rapid evaluation of phenolic component profile and analysis of oleuropein aglycone in olive oil by atmospheric pressure chemical ionization-mass spectrometry (APCI-MS). *J Agric Food Chem* 2000;48:1182–1185.
17. Suzuki R, Okada Y, Okuyama T: Two flavone C-glycosides from the style of *Zea mays* with glycation inhibitory activity. *J Nat Prod* 2003;66:564–565.
18. Matsuda H, Wang T, Managi H, Yoshikawa M: Structural requirements of flavonoids for inhibition of protein glycation and radical scavenging activities. *Bioorg Med Chem* 2003;11:5317–5323.
19. Fujiwara Y, Kiyota N, Tsurushima K, et al.: Natural compounds containing a catechol group enhance the formation of N ϵ -(carboxymethyl)lysine of the Maillard reaction. *Free Radic Biol Med* 2011;50:883–891.
20. Kim JS, Kwon CS, Son KH: Inhibition of α -glucosidase and amylase by luteolin, a flavonoid. *Biosci Biotechnol Biochem* 2010;64:2458–2461.
21. Nicole E, Souard F, Faure P, Boumendjel A: Flavonoids as promising lead compounds in type 2 diabetes mellitus: molecules of interest and structure-activity relationship. *Curr Med Chem* 2011;18:2661–2672.
22. Kwon O, Eck P, Chen S, et al.: Inhibition of the intestinal glucose transporter GLUT2 by flavonoids. *FASEB J* 2007;21:366–377.
23. Nomura M, Takahashi T, Nagata N, et al.: Inhibitory mechanisms of flavonoids on insulin-stimulated glucose uptake in MC3T3-G21PA6 adipose cells. *Biol Pharm Bull* 2008;31:1403–1409.
24. Serra A, Rubió L, Borràs X, Macià A, Romero MP, Motilva MJ: Distribution of olive oil phenolic compounds in rat tissues after administration of a phenolic extract from olive cake. *Mol Nutr Food Res* 2012;56:486–496.
25. Soler A, Romero MP, Macià A, et al.: Digestion stability and evaluation of the metabolism and transport of olive oil phenols in the human small-intestinal epithelial Caco-2/TC7 cell line. *Food Chem* 2010;119:703–714.
26. Quirantes-Piné R, Zurek G, Barrajón-Catalán E, et al.: A metabolic-profiling approach to assess the uptake and metabolism of phenolic compounds from olive leaves in SKBR3 cells by HPLC-ESI-QTOF-MS. *J Pharm Biomed Anal* 2013;72:121–126.