**Olive leaf extract concentrated in hydroxytyrosol attenuates protein carbonylation
and the formation of advanced glycation end products in a hepatic cell line (HepG2)**

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Abstract

The glycation takes place both at the cellular level and at the extracellular matrix and generates, consequently, advanced glycation end-products (AGEs) associated with chronic diseases and the aging process. Two olive leaf extracts concentrated in i) oleuropein (OLE-A; 93.9 mg oleuropein g⁻¹) and ii) hydroxytyrosol (OLE-B; 54.5 mg hydroxytyrosol g⁻¹) were evaluated according to their antiglycative and antioxidant capacity *in vitro*. OLE-B exerted the highest anti-AGE effect in different glycation models (IC₅₀: 0.25-0.29 mg mL⁻¹). OLE-B showed the highest antioxidant capacity and methylglyoxal-trapping capacity (IC₅₀ 0.16 mg mL⁻¹). OLE-B showed a significant inhibitory effect against protein carbonylation (21%) and generation of argpyrimidine (26%) in a hepatocyte cellular carbonyl stress model evoked by methylglyoxal (MGO). OLE-B was further fractionated by solid phase-extraction and the protection against protein carbonylation was only exerted by the fraction containing hydroxytyrosol. However, hydroxytyrosol standard, at the same concentration in the extract, inhibited the protein carbonylation below 10% but not significantly. The results point to antiglycative activity of OLE in cells could be due to a synergic effect of hydroxytyrosol and other minor compounds with similar polarity. The research of the antiglycative activity *in vivo* could confirm these promising results and to propose OLE as a natural anti-AGE agent.

Keywords: Olive leaf; hydroxytyrosol; oleuropein; antiglycative activity; advanced glycation end products; dicarbonyl compounds; human hepatic HepG2 cells

INTRODUCTION

Glycation involves a series of complex reactions that are initiated when a carbonyl group of a reducing sugar condenses with an amino group of protein, lipid or nucleic acid. The glycation in living bodies takes place both at the cellular level and at the extracellular matrix and culminates in the formation of advanced glycation end products (AGEs)¹. AGEs have been associated with chronic diseases of high incidence such as Alzheimer, cardiovascular diseases or diabetes mellitus and its complications, as well as with the aging process²⁻⁴. Currently, the antiglycative properties most frequently reported have been the dicarbonyl trapping activity and antioxidant capacity through metal chelation or free radical scavenging⁵.

α -Dicarbonyls compounds are the key precursors of AGEs and are generated *in vivo* during the glycation process, but also from other pathways such as lipid peroxidation, autoxidation of glucose or the glucose metabolism⁶⁻⁷. Reaction of glyoxal (GO) and methylglyoxal (MGO) with lysine and arginine residues in proteins give rise to the formation of AGEs such as carboxymethyllysine (CML), carboxyethyllysine (CEL) and argpyrimidine (ArgP). Therefore, the term “carbonyl stress” has been suggested to describe the unusual accumulation of reactive carbonyl species due to disturbance of their production or cellular metabolism. The accumulation of α -dicarbonyl compounds leading to increased protein modification and formation of AGEs contributes to cell and tissue dysfunction in chronic diseases and aging⁸⁻⁹. The concentrations of MGO in human plasma is 50-150 nmol L⁻¹ increasing until 212 and 312 nmol L⁻¹ in patients with diabetes and with diabetic nephropathy, respectively⁹⁻¹⁰. Cells under normal physiological states are able to detoxify MGO to D-lactate by the glyoxalase system. However, during a situation of oxidative stress, intracellular levels of dicarbonyls can be increased, which could overwhelmed this enzymatic system leading to a cellular damage^{4,6,11}.

Oleuropein (Oleu) and hydroxytyrosol (HT) are the most representative compounds of the phenolic fraction of certain olive by-products, such as olive leaves. Their biological and pharmacological effects have mainly been attributed to their antioxidant activity in several preclinical disease models¹²⁻¹³. Thus, several studies *in vivo* have evidenced a strong relationship between the antioxidant and hypoglycemic

activities of the olive leaf extract (OLE), which have mainly been attributed to its high content of HT and Oleu^{12,14}. In this regard, in diverse cellular models of oxidative stress it has been reported that HT and OLE contribute to maintain the redox homeostasis¹⁵. HT and OLE modulate different signalling pathways related to the cellular redox status, which leads to a cytoprotective effect and improved cell functionality¹⁶.

However, it remains unknown whether constituents of the OLE could exert an antiglycative activity *in vivo*, and subsequently to reduce the reactivity of dicarbonyl compounds involved in the formation of AGEs.

Despite previous investigations have reported the effect of MGO on the protein carbonylation in hepatocytes^{6,11,17}, its implication on the formation of different AGEs has not been studied. Two olive leaf extracts concentrated in Oleu or HT were screened on their capacity to inhibit the formation of fluorescent AGEs and MGO-trapping in simulated physiological conditions. Then, the most active extract was selected to evaluate in a HepG2 cellular carbonyl stress model evoked by MGO the potential inhibitory effect against protein carbonylation and generation of CML, CEL and ArgP.

MATERIALS AND METHODS

Materials and chemicals

Olive leaves (*Olea Europaea* variety Picual, Córdoba, Spain). D(+)-Glucose (GLC), bovine serum albumin (BSA), methylglyoxal (MGO, 40% aqueous solution), glyoxal (GO, 40% aqueous solution), aminoguanidine (AG), Oleuropein (Oleu, purity $\geq$ 98%), 5-methylquinoxaline (5-MQ), o-phenylenediamine (OPD), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), heptafluorobutyric (HFBA), 2,4-dinitrophenylhydrazine, gentamicin, penicillin, streptomycin and crystal violet were provided by Sigma (St Louis, MO, USA). Folin-Ciocalteu reagent, iron (III) chloride and sodium dodecyl sulfate salt were purchased from Panreac (Madrid, Spain). Hydroxytyrosol standard (HT, purity > 99%) was acquired from Seprox Biotech (Madrid, Spain). Pyridoxamine (PM) was acquired from Fluka Chemical (Madrid, Spain). Glacial acetic acid and high performance liquid

chromatography (HPLC)-grade methanol were purchased from Merck (Darmstadt, Germany). Carboxymethyl-L-lysine (CML, $\geq 97\%$), carboxyethyl-L-lysine (CEL, $\geq 97\%$), CML-d2, CEL-d4, and argpyrimidine (ArgP, $\geq 97\%$) were obtained from Laboratories (Strasbourg, France). Bradford reagent was from BioRad Laboratories S.A. (Madrid, Spain). Cell culture dishes and cell culture medium were from Falcon (Cajal, Madrid, Spain) and Lonza (Madrid, Spain), respectively. The Milli-Q water was obtained by an Elix3 water purification system coupled to a Milli-Q Advance 10 module (Millipore, Molsheim, France). All other chemicals and reagents were of analytical grade.

Equipment

Synergy™ HT-multimode microplate reader with an automatic reagent dispense from Biotek Instruments (Winooski, VT, USA). HPLC (Shimadzu, Kyoto, Japan) equipped with a quaternary pump (LC-20AD), an autosampler (SIL-20AHT), an oven (CTO-10ASVP), a diode-array detector (SPDM20A) and a fluorescence detector (RF-20AXS). LC-MS/MS was performed with a 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a triple quadrupole mass spectrometer (G6410B, Agilent Technologies) via electrospray ionization operating in positive mode.

Preparation of the Olive Leaf Extract (OLE)

An OLE concentrated on phenolic compounds was obtained according to *Lee et al*²⁴¹⁸ with some modifications. Procedure is summarized in **figure 1**. Fresh olive leaves (250 g) were gently washed in water and dried (40°C for 2 days) in an air-forced oven. Then the leaves were ground and 10 g were mixed with 100 mL of ethanol:water (80:20 v/v) by duplicate and kept under shaking for a week in darkness at 37°C. The supernatant was removed by paper filtration to get the olive leaf extract A (OLE-A). Additionally, the pH of half of the supernatant was readjusted to 2.5 with HCl and kept under shaking for another 3 hours under darkness at room temperature to obtain the olive leaf extract B (OLE-B). Both supernatants were dried in a vacuum evaporator (Strike 300, Steroglass, Perugia, Italy) and the dried fraction was extracted with hexane (25 mL, 3 times) and then with ethyl acetate (50 mL, 5 times). The ethyl acetate fraction (250 mL) of OLE-B was again kept in darkness and pH readjusted to 2.5 with HCl. Finally, ethyl acetate fractions were dried under vacuum and subsequently dissolved in

methanol/water solution (60:40, v/v). The final concentration of OLE-A and OLE-B was 12 mg mL⁻¹.

Fractionation of the Olive Leaf Extract

OLE-B was further fractionated by reversed phase solid extraction (C18 SepPak cartridge, 1 mL, Waters Corporation, Milford, MA, USA). Briefly, 1 mL of OLE-B solution (12 mg mL⁻¹ in methanol:water, 60:40, v/v) was directly loaded onto a pre-activated cartridge. The first eluate was collected and named as OLE-BF1. Then 1 mL of methanol was passed through the cartridge and a second fraction was collected (OLE-BF2). The procedure was repeated at least 10 times and the fractions were pooled, vacuum evaporated (Speed Vac, Savant SPD131, Thermo Scientific, Milford, MA, USA), and reconstituted in methanol:water (60:40, v/v).

Determination of phenolic compounds by HPLC-DAD

HT and Oleu in the OLE samples was determined by HPLC-DAD using a Shimadzu HPLC system. The samples were injected (10 µL) onto a Kinetex-C18 column (100 mm x 4.6 mm, 2.6 µm; Kinetex, Phenomenex, Torrance, CA, USA) operating at 0.6 mL min⁻¹. The mobile phases used were acetic acid in water (0.5%, phase A) and methanol (phase B). The running time was 30 min and the gradient method was as follows: 0 min, 5%B; 1min, 5%B; 20min, 60%B; 21min, 60%B; 22min, 5%B; 30min, 5%B. The chromatograms were recorded at 280 nm with a retention time of 5.4 min and 20.8 min for HT and Oleu, respectively. Calibration curves were obtained from pure standards.

Determination of total phenolic content

Total phenolic content (TPC) was measured by the Folin–Ciocalteu assay according to Singleton *et al.*²²¹⁹. Results were expressed as mg gallic acid equivalent (GAE) per g sample and all measurements were performed in quadruplicate. The limit of quantification was set at 0.5 mg GAE per g sample.

Determination of antioxidant capacity according to ABTS assay

The antioxidant capacity of extracts by the ABTS assay was determined as described by Mesias *et al.*²²²⁰. Results were expressed as µmol Trolox equivalent antioxidant capacity (TEAC) per g sample. The limit of quantification was set at 1.1 µmol TEAC g⁻¹ sample. All measurements were carried out in quadruplicate.

Determination of the antioxidant capacity according to FRAP assay

The ability to reduce Fe^{3+} -TPTZ complex to Fe^{2+} -TPTZ complex was determined according to the method described by *Morales et al.*²⁴²¹. Results were expressed as μmol Trolox equivalent antioxidant capacity (TEAC) per g sample. All measurements were carried out in quadruplicate.

***In vitro* glycation assay with bovine serum albumin induced by methylglyoxal, glyoxal and glucose**

The glycation models of BSA with MGO (BSA-MGO assay), GO (BSA-GO assay) and GLC (BSA-GLC assay) were carried out as described by *Mesias et al.*²³²⁰ with slight modifications. The range of concentration of OLE-A and OLE-B in the mixture was 0.14-0.71 mg/mL and the concentrations of reactants were 0.23 mg/mL for MGO and GO, 100 mg/mL for GLC and 10 mg/mL for BSA. The formation of AGEs was characterized by fluorescence with excitation/emission at 340/420 nm for the BSA-MGO or BSA-GO and 360/420 nm for BSA-GLC. Results were expressed as percentage of inhibition of AGEs formation, and it was calculated according to following equation: Inhibition (%) = $1 - \frac{[(\text{fluorescence of solution with inhibitor} - \text{intrinsic fluorescence of sample}) / \text{fluorescence of solution without inhibitor}] \times 100$. The concentration required to inhibit the glycation by 50% (IC_{50}) was calculated from the dose-response curve using the Microsoft-Excel computer software.

Evaluation of direct MGO trapping capacity by HPLC-DAD

Direct MGO trapping capacity was determined as described by *Mesias et al.*²³²⁰ after MGO conversion into the respective quinoxaline derivative (2-MQ). The range of concentration of OLE-A and OLE-B in the mixture was 0.05-0.25 mg mL⁻¹ for HT, and 0.01-0.1 mg mL⁻¹ for Oleu. The incubation was carried out at 37°C for 168 h in PBS (100 mM, pH 7.4). The amount of unreacted MGO was calculated from the ratio of 2-MQ and 5-MQ (internal standard) as compared with the control. The percentage of inhibition of MGO was calculated with the next formula: MGO decrease (%) = $\frac{[(\text{amount of MGO in control} - \text{amount of MGO in sample with OLE or phenol compounds standard}) / \text{amount of MGO in control}] \times 100$. The concentration required to trap MGO

by 50% (IC₅₀) was obtained from the dose-response curves using Microsoft-Excel computer software.

Cell culture and treatments

Human HepG2 cells were grown in DMEM containing 5.5 mM D-glucose and 2 mM glutamine and supplemented with 2.5% fetal bovine serum (FBS) and 50 mg/L antibiotics (gentamicin, penicillin and streptomycin). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. Subsequently, the experimental treatment was carried out for the indicated period and compounds in serum-free media.

To evaluate the protective effect of the OLE-B extract (13 µg/mL in methanol:water, 60:40 v/v), its fractions and HT (5 µM in methanol:water, 60:40 v/v) against MGO challenge (2 mM, 6 h), the mentioned substances were added to the cells for 20 h. To the control cells the methanol:water solution was added in the same proportion that was used to dissolve the tested sample. Then, the medium was discarded and fresh medium containing 2 mM MGO was added for additional 6 h. Later, cells were harvested and carbonyls were analysed.

Cell viability

The viability of HepG2 cells was determined using the crystal violet assay²⁵²². Cells were seeded in 96-well plates at low density (10⁴ cells per well), grown for 20 h with different concentrations of olive leaf extract (0.3-264 µg mL⁻¹) and incubated with crystal violet (0.2% in ethanol) for 20 min. Plates were rinsed with distilled water and 1% sodium dodecyl sulphate was added. The absorbance was measured at 570 nm using a SynergyTM HT-multimode microplate spectrophotometer. Results were expressed as relative percentage of crystal violet stained control cells.

Determination of protein carbonylation

The content of protein carbonyl products generated by glycoxidation of hepatic cells was measured as previously described²⁶²³. Briefly, cells were lysed in PBS (pH 7.4) with an ultrasonic Processor (Vibra-Cell, Connecticut, USA). Then, the extracts were centrifuged (10000g, 15 min) and supernatants were collected. Absorbance was measured at 360 nm and carbonyl content was expressed as nmol mg⁻¹ protein using

an extinction coefficient of 22000 nmol/L/cm. Protein concentration by using the Bradford reagent.

Determination of CML and CEL by LC-ESI-MS-QQQ

The formation of CML and CEL in cells was determined as described by *Navarro & Morales*²⁷²⁴. Samples (10 µL) were separated on a porous graphitic carbon column (Hypercarb, 100 mm x 2.1 µm, 5 µm, ThermoFisher Scientific) at a flow rate of 0.5 mL min⁻¹. The isocratic elution was applied using a mobile phase of 5 mM NFPA / acetonitrile (95:5 v/v). The product ion at *m/z* 84 was used for quantification of CML (*m/z* 205), CML-d2 (*m/z* 207) and CEL (*m/z* 219) while *m/z* 88 was used for CEL-d4 (*m/z* 223). The ratio of response factor for CML or CEL to that of their respective labelled internal standards was used for quantitation. CML and CEL calibration was carried out in the range of 0.01-1 µg mL⁻¹ and data were processed using MassHunter Data Acquisition and MassHunter Qualitative Analysis (Agilent Technologies). Results were expressed as µmol CML or CEL g⁻¹ protein.

Determination of argpyrimidine by HPLC-fluorescence

The determination of ArgP formation in cells was carried out as described by *Navarro & Morales*²⁷²⁴. Samples (10 µL) were eluted onto a Mediterranean-Sea-ODS2 column (250 mm x 4 mm, 5 µm; Teknokroma, Barcelona, Spain) at a flow rate of 0.8 mL min⁻¹. The mobile phase was performed with HFBA (1 mL L⁻¹) (solvent A) and ACN (500mL L⁻¹) containing HFBA (1 mL L⁻¹) (solvent B) under the following gradient elution: 0 min, 20% B; 25 min, 100% B; 26 min, 100% B; 27-37 min, 20% B. ArgP was detected at 335 nm and 385 nm for excitation and emission wavelength, respectively, and eluted at 15.1 min. Calibration was carried out in the range 0.01-0.5 µg mL⁻¹ with pure standard. Results were expressed as µmol ArgP g⁻¹ protein.

Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics program (version 21.0). Data were expressed as the mean value ± standard deviation. One-way analysis of variance and the Bonferroni test were applied to determine differences between means. Differences were considered to be significant at *P* < 0.05.

RESULTS

Quantification of main phenolic compounds in two olive leaf extracts and evaluation of their antioxidant activity

Olea europaea L. leaf is considered a by-product of olive with a high content of phenolic compounds whose profile has been studied for their potential health benefits. Olive leaf is rich in secoiridoids, especially in oleuropein that constitute the main compound of interest. Other components may occur in appreciable amount such as hydroxytyrosol, oleoside, apigenin and luteolin derivatives as luteolin-7-*O*-glucoside or luteolin-4-*O*-glucoside^{28-29,25-26}. On the basis of the above considerations two extracts of olive leaf with different content in Oleu and HT were obtained. The contents in Oleu, HT and an approximate composition of total phenolic (TPC), are summarized in **table 1** for OLE-A and OLE-B. TPC in the OLE-B extract was 3.4-fold higher than OLE-A (320.8 ± 46.0 versus 93.8 ± 27.1 mg GAE g⁻¹). As expected, the OLE-A extract contained 93.9 mg of Oleu per gram of OLE while the OLE-B, which was additionally macerated with diluted HCl, included a high amount of HT (54.5 mg HT g⁻¹ OLE) to the detriment of Oleu, which was reduced until 1.82 mg Oleu g⁻¹ OLE (**table 1**).

Since there is an established relationship between the health beneficial effects and the antioxidant capacity of phenolic constituents^{12,27}, the antioxidant capacity of OLE was measured by ABTS and FRAP assays. The OLE-B showed an antioxidant capacity significantly higher than OLE-A for both *in vitro* assays.

Inhibition of the formation of fluorescent AGEs *in vitro*

Previous studies have proved that several phenolic compounds present in olive leaves could exert a strong *in vitro* antiglycative capacity^{24,28-29}. To gain further insight into the influence of the Oleu and HT concentration as the major phenolic compounds in the two extracts obtained (OLE-A, OLE-B) on their antiglycative capacity, a first screening was carried out through the formation of fluorescent AGEs in different models of glycation (BSA-GLU, BSA-MGO, BSA-GO) under simulated physiological conditions. The **table 2** displays the concentration necessary of extract to inhibit the 50% of the formation of fluorescent AGEs (IC₅₀). OLE-B reached an IC₅₀ from 0.248 to 0.294 mg mL⁻¹ in all the glycation model systems, whereas OLE-A reached a concentration from 0.425 to 0.462 mg mL⁻¹ for the BSA-MGO and BSA-GO respectively, and 0.716 mg mL⁻¹ for BSA-GLC. Antiglycative capacity of OLE-B was significantly higher than OLE-A for the

three models of glycation. Additionally, the antiglycative activities of OLE-A and OLE-B kept a similar trend when compared to their antioxidant activity; therefore, it is likely that the antiglycative capacities of OLE-A and OLE-B extracts could be related to their antioxidant capacity.

α -dicarbonyl-trapping capacity of olive leaf extract

MGO is a key promoter of the glycation process and it could be formed from oxidation of glucose and lipids, as well as intermediates of the glycation process to finally generate AGEs. **Figure 2** depicts the measurement of the MGO-trapping capacity of the extracts. OLE-A and OLE-B extracts showed a dose-dependent response to MGO-trapping. The IC_{50} were 0.238 and 0.159 mg mL⁻¹ for OLE-A and OLE-B, respectively. MGO-trapping capacity of OLE-B was significantly higher than OLE-A. Further, the α -dicarbonyl-trapping capacity of the major phenolic compounds in OLE-A and OLE-B was also evaluated with standards. The Oleu and HT standard displayed an IC_{50} value of 0.085 mg mL⁻¹ and 0.028 mg mL⁻¹ respectively. The MGO-trapping activities of pure compounds were significantly more efficient than OLE-B and OLE-A at the same concentration although the Oleu and HT content in OLE-A and OLE-B extracts reached the IC_{50} value at 0.025 and 0.008 mg mL⁻¹, respectively. Therefore, the relative concentrations of Oleu and HT in the extracts were significantly lower than the IC_{50} value reached by their respective standards.

Treatment of HepG2 cells with olive leaf extract

Cell viability

In view that OLE-B extract showed the highest antiglycative, carbonyl-trapping and antioxidant capacities in the *in vitro* models at simulated physiological conditions, OLE-B extract was selected in order to investigate the antiglycative activity in a hepatocyte cellular model. The human HepG2 cells have been used previously by other authors for the study of protein carbonylation and this cell line has been widely used for biochemical studies to be considered a well characterized model^{6,11,23,30}, as retains the hepatocytes morphology and most of their function in culture.

After a 24 h-treatment with different concentrations of OLE-B (0.3-264 μ g mL⁻¹) cell viability was evaluated by the crystal violet assay. As shown in **figure 3**, OLE-B did

not provoke cell injury at the range of concentration from 0.3 to 66 $\mu\text{g mL}^{-1}$, which corresponds to 0.1-25 μM HT. However, a significant decrease in the number of viable cells was observed at higher concentrations of OLE-B. An intermediate concentration of 13 $\mu\text{g mL}^{-1}$ was selected for further experiments. These results are in line with those of Goya *et al.*³³³⁰ reported for pure HT which did not produce cell injury in HepG2 below 50 μM .

Inhibition of protein carbonylation in HepG2 cells induced by methylglyoxal

To elucidate the potential inhibitory effect of HT on the protein carbonylation a hepatocyte cellular model of carbonyl stress induced with a MGO (2 mM) was assessed. As shown in **figure 4a**, MGO evoked a protein carbonylation nearly 2-fold higher than in HepG2 control cells. The incubation of cells with 13 $\mu\text{g mL}^{-1}$ of OLE-B showed no significant differences with respect to the controls, but the protein carbonylation was significantly decreased (21.15%) when cells were previously treated with OLE-B and later submitted to MGO challenge. OLE-B showed a protective effect on the protein carbonylation induced by MGO in HepG2 cells.

OLE-B was further fractionated under solid-phase extraction into fraction-1 (OLE-BF1) and fraction-2 (OLE-BF2). OLE-BF1 contained the higher fraction of HT (41.42 mg g^{-1} OLE) and other minor constituent with similar polarity. OLE-BF2 stands out for its content in compounds less polar and therefore low HT content (7.63 mg g^{-1} OLE). Then the potential inhibition of the carbonyl stress evoked by MGO in HepG2 cells treated with OLE-BF1 and OLE-BF2 was assessed (**figure 4b**). As shown for the OLE-B, OLE-BF1 and OLE-BF2 alone did not affect to the carbonyl protein content when compared to untreated cells. After incubating the cells with MGO, OLE-BF1 was able to reduce significantly the protein carbonylation similarly to the unfractionated extract. However, OLE-BF2 was not able to protect the cells against the protein carbonylation. The result confirms that the fraction concentrated in HT, OLE-BF1, was responsible of the protection at the cellular level. In order to gain more insight, the effect of pure HT at the same concentration in which is present in the OLE-B was evaluated. The results showed an inhibition below 10% being not statistically significant (**figure 4b**).

Inhibition of CML, CEL and ArgP formation in HepG2 cells

To further elucidate the antiglycative properties of OLE in HepG2 cells the levels of specific AGEs were assessed. As shown, MGO provoked carbonyl stress in HepG2 cells; thus, the AGEs formed from MGO, such as CEL and ArgP were the most representative in this model. CEL, is a non-fluorescent AGE generated from lysine and MGO, whereas ArgP is a fluorescent AGE originated from the reaction between two molecules of MGO and arginine. On the other hand, CML formed by condensation of a lysine with GO, was also investigated to be considered as marker of glycation in many biological systems and food. The formation of the mentioned AGEs in cells under physiological conditions (control cells) was lower than their respective values of LOQ ($0.79 \mu\text{g mL}^{-1}$, $0.24 \mu\text{g mL}^{-1}$ and $0.38 \mu\text{g mL}^{-1}$ for CEL, ArgP and CML, respectively). After the incubation of HepG2 cells with MGO, CEL values were $278 \mu\text{g CEL g}^{-1}$ protein, and the formation of ArgP was slightly lower ($213 \mu\text{g ArgP g}^{-1}$ protein). OLE-B ($13 \mu\text{g mL}^{-1}$) was able to prevent the ArgP formation in 26% but it did not inhibit CEL formation (**figure 5**). As expected, the formation of CML was lower than its LOQ value (data not shown).

DICUSSION

A considerable number of studies have evidenced the relationship between phenolic compounds derived from the olive leaves and the health beneficial effects such as antihypertensive, anticarcinogenic, anti-inflammatory, hypoglycaemic, antimicrobial and hypocholesterolaemic properties^{34-36,31-33}. Recently, it has been reported that several phenolic compounds naturally present in this by-product, such as luteolin, rutin, HT, verbascoside, and Oleu display antiglycative activity^{28,34}.

In the present study, two olive leave extracts with different content in the major phenolic compounds were prepared. An extract was concentrated in Oleu (OLE-A) and the second extract was concentrated in HT (OLE-B). It is known that Oleu and HT exert hypoglycaemic, hypolipidaemic and antiatherogenic effects, which have been attributed to its antioxidant activity^{14,35}. However, few studies have established a relationship between health beneficial properties and both antioxidant and antiglycative abilities through the reduction of the reactive dicarbonyl formed by the oxidative stress²⁷. In a previous study, Navarro & Morales²⁴ reported that HT exerted an antiglycative activity in several *in vitro* models and specifically an inhibition of CML, CEL and ArgP formation through the dicarbonyls-trapping. In addition, it should be

taken into account that Oleu is an ester of elenolic acid with HT so that the differences in Oleu and HT content between extracts could be due to the breakdown of Oleu into HT under mild acidic conditions used for the preparation of the OLE-B. On the basis of the above considerations, the extract concentrated in HT displayed the highest antioxidant, antiglycative and carbonyl-trapping capacity. These results are in line with those reported by *Navarro & Morales*³⁶ in whose study the olive mill wastewater exerted an antiglycative capacity by direct α -dicarbonyls-trapping and was mostly attributed to the high content of hydroxytyrosol and secondly to the presence of verbascoside among others.

This investigation demonstrated by first time the antiglycative activity of an olive leave extract in a cellular model under a situation of carbonyl stress. The carbonylation of proteins, as well as the ArgP formation evoked by MGO were significantly reduced by preincubating the cells with OLE-B. In previous studies, where GO and MGO triggered hepatocyte protein carbonylation, several dicarbonyl scavengers such as aminoguanidine and penicillamine were assayed in order to prevent the protein carbonylation and cytotoxicity¹¹. However, studies with natural extracts carried out to elucidate a similar antiglycative effect in *in vitro* and *in vivo* assays have acquired special relevance to avoid the side effects of synthetic drugs such as aminoguanidine. Recent studies suggested that olive oil administration reduced the inflammatory response, peroxidation of lipids and carbonylated proteins in mice that have been chronically stressed and also stimulated the wound healing of pressure ulcers^{40-41,37-38}. In the OLEA project, the olive leaf extract has been the target of several investigations where it has been reported that olive leaf extract exerted cytoprotective and anti-inflammatory effects. In addition, this extract demonstrated a better protection against β -cell toxicity at a lower concentration than the doses tested for each isolated phenolic compound, such as Oleu or HT, pointing to the potential synergic effects of phenolic compounds³⁹.

In accordance to literature, olive mill waste polyphenols and especially HT significantly reduced hyperglycaemia and oxidative stress produced by diabetes in rats^{43,40}. In the same way, the administration of olive leaf extract rich in HT and Oleu (8 and 16 mg kg⁻¹ body weight for 4 weeks) to alloxan-diabetic rats showed an

antidiabetic activity attributed to its antioxidant effects, since it decreased the glucose and cholesterol levels and was able to restore the antioxidant defence system^{18,14}. OLE has also demonstrated to avert oxidative stress-induced cell damage by modulating the activity of key antioxidant enzymes, as well as improved the insulin secretion, mitigated necrosis, apoptosis (diminished caspase 3/7) and ROS generation in cultured beta cells¹⁵⁻¹⁶. *Perez-Herrera et al.*⁴¹ described in obese individuals that oils rich in phenols (0.45 mL kg⁻¹ body weight) inhibited of nuclear factor-kappa B (NFkB) in the postprandial state and, consequently, reduced the postprandial inflammation. Additionally, their beneficial effects were attributed mainly to the HT content, major compound in the hydrolysate olive leaf extract tested. Similarly, OLE has shown an anti-inflammatory effect in beta pancreatic cells through the inhibition of stress activated protein kinase (SAPK/JNK) cascade¹⁵.

The HT was tested at realistic concentrations which are within the range recommended for *in vitro* studies (0.1-10 µM)⁴². At the concentration of 5 µM, a pure standard of HT did not inhibit the carbonylation of proteins induced by MGO. However, OLE-B at a concentration that corresponds to the dose of HT (13 µg mL⁻¹ OLE-B contains 5 µM HT) exerted a slight, yet significant, decrease in the levels of protein carbonylation probably due to synergisms of HT with other minor phenol compounds present in the extract. In agreement to literature, other minor phenolic compounds present in the olive leaves such as luteolin, apigenin or gallic acid have been described as scavengers of reactive dicarbonyl species⁷. In this line, it is reasonable to assume that HT is just one of the many bioactive substances present in the extract and that the synergic effects of phenolic compounds in foodstuffs should be taken into account. At this point it should be noted that the possible synergism established between the more polar phenolic compounds as HT present in OLE-BF1 could exert the inhibitory activity of HepG2 protein carbonylation not being the constituents of OLE-BF2 relevant for this activity. However, to elucidate whether the health beneficial properties of OLE-B were exerted by HT or the compounds derived from its metabolism require further studies in which both chemical and molecular mechanisms of action should be considered.

In vivo and *in vitro* studies have demonstrated that the HT and related compounds are readily bioavailable. It has been shown that HT is absorbed in a dose-dependent manner after ingestion and its absorption depends on the administration matrix^{46,13}. In this line, studies in HepG2 and Caco-2 cells, as well as clinical trials in humans and animals, have demonstrated that HT is metabolized and absorbed⁴³⁻⁴⁴. In addition, *Visoli et al.*³⁵ reported a HT excretion in humans of 30-60% of HT administered. Further, it is known that HT can be excreted unchanged or as metabolite. Indeed, the metabolites generated, such as its sulfate or glucuronide conjugate, homovanillic acid, homovanillic alcohol, 3,4-dihydroxyphenylacetic acid (DOPAC) and 3,4-dihydroxyphenylacetaldehyde have been described. Homovanillic acid and homovanillic alcohol demonstrated to be strong radical scavengers while DOPAC displayed a boosted antiglycative capacity in physiological conditions *in vitro*.

CONCLUSION

The olive leaf extract concentrated in HT (OLE-B) demonstrated a boosted antiglycative and antioxidant capacity with respect to olive leaf extract concentrated in Oleu (OLE-A). Additionally, the antiglycative activity of OLE-B was evaluated in a HepG2 cell model not showing cytotoxicity within a realistic range of concentration and exhibiting a slight inhibition of protein carbonylation, and a selective inhibition of the formation of ArgP under a carbonyl stress condition. The fractionation of the OLE-B evidenced that OLE-BF1, fraction containing HT among other minor constituents, presented a reduction of protein carbonylation similar to that of OLE-B. This inhibitory effect was not observed in OLE-BF2 or HT standard at the same concentration. It is plausible that the antiglycative activity of OLE in cells could be due to a synergic effect of HT and other minor phenolic compounds. However, further research is necessary to confirm the antiglycative activity *in vivo* and to obtain a deeper understanding of its mechanism of action, including the balance in the cellular redox status and close-related signalling approaches, before being proposed as a natural AGE inhibitor.

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Conflict of Interest

The authors declare no conflict of interest.

Abbreviations

AGEs	Advanced glycation end-products
AG	Aminoguanidine
ArgP	Argpyrimidine
BSA	Bovine serum albumin
CEL	Carboxyethyllysine
CML	Carboxymethyllysine
DOPAC	3,4-dihydroxyphenylacetic acid
GAE	Gallic acid equivalent
GLC	Glucose
GO	Glyoxal
HT	Hydroxytyrosol
LOQ	Limit of quantitation
MGO	Methylglyoxal
OLE	Olive leaf extract
Oleu	Oleuropein
PM	Pyridoxamine
TPC	Total phenolic content

REFERENCES

1. P. J. Thornalley, S. Battah, N. Ahmed, N. Karachalias, S. Agalou, R. Babaei-Jadidi and A. Dawnay, Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry, *Biochem. J.*, 2003, **375**, 581-592.
2. G. Münch, J. Thome, P. Foley, R. Schinzel and P. Riederer, Advanced glycation endproducts in ageing and Alzheimer's disease, *Brain Res. Rev.*, 1997, **23**, 134-143.
3. K. Nowotny, T. Jung, A. Hohn, D. Weber and T. Grune, Advanced glycation end products and oxidative stress in type 2 diabetes mellitus, *Biomolecules*, 2015, **5**, 194-222.
4. J. W. Baynes, The role of AGEs in aging: causation or correlation, *Exp Gerontol*, 2001, **36**, 1527-1537.
5. X. Peng, J. Ma, F. Chen and M. Wang, Naturally occurring inhibitors against the formation of advanced glycation end-products, *Food Funct.*, 2011, **2**, 289-301.
6. N. Shangari and P. J. O'Brien, The cytotoxic mechanism of glyoxal involves oxidative stress, *Biochem. Pharmacol.*, 2004, **68**, 1433-1442.
7. X. Shao, H. Chen, Y. Zhu, R. Sedighi, C. T. Ho and S. Sang, Essential Structural Requirements and Additive Effects for Flavonoids to Scavenge Methylglyoxal, *J. Agric. Food Chem.*, 2014, **62**, 3202–3210.
8. T. Miyata, Y. Izuhara, H. Sakai and K. Kurokawa, Carbonyl stress: increased carbonyl modification of tissue and cellular proteins in uremia, *Perit. Dial. Int.*, 1999, **19 Suppl 2**, S58-61.
9. N. Rabbani and P. J. Thornalley, Dicarbonyl stress in cell and tissue dysfunction contributing to ageing and disease, *Biochem. Biophys. Res. Commun.*, 2015, **458**, 221-226.
10. M. Handl, E. Filova, M. Kubala, Z. Lansky, L. Kolacna, J. Vorlicek, T. Trc, M. Pach and E. Amler, Fluorescent advanced glycation end products in the detection of factual stages of cartilage degeneration, *Physiol. Res.*, 2007, **56**, 235-242.
11. K. Yang, D. Qiang, S. Delaney, R. Mehta, W. R. Bruce and P. J. O'Brien, Differences in glyoxal and methylglyoxal metabolism determine cellular susceptibility to protein carbonylation and cytotoxicity, *Chem.-Biol. Interact.*, 2011, **191**, 322-329.
12. S. Bulotta, M. Celano, S. M. Lepore, T. Montalcini, A. Pujia and D. Russo, Beneficial effects of the olive oil phenolic components oleuropein and hydroxytyrosol: focus on protection against cardiovascular and metabolic diseases, *J. Translation. Med.*, 2014, **12**, 219.

- 526 13. S. Cicerale, L. Lucas and R. Keast, Biological activities of phenolic compounds present in
527 virgin olive oil, *Int.J. Mol. Sci.*, 2010, **11**, 458-479.
- 528 14. H. Jemai, A. El Feki and S. Sayadi, Antidiabetic and antioxidant effects of
529 hydroxytyrosol and oleuropein from olive leaves in alloxan-diabetic rats, *J. Agric. Food*
530 *Chem.*, 2009, **57**, 8798-8804.
- 531 15. V. Ergin, Hariry R. E. and Ç. Karasu, Carbonyl Stress in Aging Process: Role of Vitamins
532 and Phytochemicals as Redox Regulators. *Aging and Disease*, 2013, **4**(5), 276-294.
- 533 16. A. Cumaoglu, L. Rackova, M. Stefek, M. Kartal, P. Maechler and C. Karasu, Effects of
534 olive leaf polyphenols against H₂O₂ toxicity in insulin secreting beta-cells. *Acta*
535 *Biochim. Pol.*, 2011, **58**(1), 45-50.
- 536 17. Q. Dong, M. S. Banaich and P. J. O'Brien, Cytoprotection by almond skin extracts or
537 catechins of hepatocyte cytotoxicity induced by hydroperoxide (oxidative stress
538 model) versus glyoxal or methylglyoxal (carbonylation model), *Chem.-Biol. Interact.*,
539 2010, **185**, 101-109.
- 540 18. O. H. Lee, B. Y. Lee, J. Lee, H. B. Lee, J. Y. Son, C. S. Park, K. Shetty and Y. C. Kim,
541 Assessment of phenolics-enriched extract and fractions of olive leaves and their
542 antioxidant activities, *Bioresource Technol.*, 2009, **100**, 6107-6113.
- 543 19. V. L. Singleton, R. Orthofer and R. M. Lamuela-Raventós, in *Methods in Enzymology*,
544 Academic Press, 1999, vol. Volume 299, pp. 152-178.
- 545 20. M. Mesias, M. Navarro, V. Gokmen and F. J. Morales, Antiglycative effect of fruit and
546 vegetable seed extracts: inhibition of AGE formation and carbonyl-trapping abilities, *J.*
547 *Sci. Food Agric.*, 2013, **93**, 2037-2044.
- 548 21. F. J. Morales, S. Martin, Ö. Ç. Açar, G. Arribas-Lorenzo and V. Gökmen, Antioxidant
549 activity of cookies and its relationship with heat-processing contaminants: a
550 risk/benefit approach, *Eur. Food Res. Technol.*, 2008, **228**, 345-354.
- 551 22. A. B. Granado-Serrano, M. A. Martin, M. Izquierdo-Pulido, L. Goya, L. Bravo and S.
552 Ramos, Molecular mechanisms of (-)-epicatechin and chlorogenic acid on the
553 regulation of the apoptotic and survival/proliferation pathways in a human hepatoma
554 cell line, *J. Agric. Food Chem.*, 2007, **55**, 2020-2027.
- 555 23. I. Cordero-Herrera, M. A. Martin, L. Goya and S. Ramos, Cocoa flavonoids protect
556 hepatic cells against high-glucose-induced oxidative stress: relevance of MAPKs, *Mol.*
557 *Nutr. Food Res.*, 2015, **59**, 597-609.
- 558 24. M. Navarro and F. J. Morales, In vitro investigation on the antiglycative and carbonyl
559 trapping activities of hydroxytyrosol, *Eur. Food Res. Technol.*, 2016, **242**, 1101-1110.

- 560 25. Á. Peralbo-Molina, F. Priego-Capote and M. D. Luque de Castro, Tentative
561 Identification of Phenolic Compounds in Olive Pomace Extracts Using Liquid
562 Chromatography–Tandem Mass Spectrometry with a Quadrupole–Quadrupole–Time-
563 of-Flight Mass Detector, *J. Agric. Food Chem.*, 2012, **60**, 11542-11550.
- 564 26. R. Quirantes-Pine, J. Lozano-Sanchez, M. Herrero, E. Ibanez, A. Segura-Carretero and A.
565 Fernandez-Gutierrez, HPLC-ESI-QTOF-MS as a powerful analytical tool for
566 characterising phenolic compounds in olive-leaf extracts, *Phytochem. Analysis : PCA*,
567 2013, **24**, 213-223.
- 568 27. M. W. Poulsen, R. V. Hedegaard, J. M. Andersen, B. de Courten, S. Bugel, J. Nielsen, L.
569 H. Skibsted and L. O. Dragsted, Advanced glycation endproducts in food and their
570 effects on health, *Food Chem. Toxicol.*, 2013, **60**, 10-37.
- 571 28. V. G. Kontogianni, P. Charisiadis, E. Margianni, F. N. Lamari, I. P. Gerothanassis and A.
572 G. Tzakos, Olive leaf extracts are a natural source of advanced glycation end product
573 inhibitors, *J. Med. Food*, 2013, **16**, 817-822.
- 574 29. M. Navarro, A. Fiore, V. Fogliano and F. J. Morales, Carbonyl trapping and antiglycative
575 activities of olive oil mill wastewater, *Food Funct.*, 2015, **6**, 574-583.
- 576 30. L. Goya, R. Mateos and L. Bravo, Effect of the olive oil phenol hydroxytyrosol on human
577 hepatoma HepG2 cells, *Eur. J. Nutr.*, 2007, **46**, 70-78.
- 578 31. K. Kawaguchi, T. Matsumoto and Y. Kumazawa, Effects of antioxidant polyphenols on
579 TNF-alpha-related diseases, *Curr. Topics Med. Chem*, 2011, **11**, 1767-1779.
- 580 32. J. E. Hayes, P. Allen, N. Brunton, M. N. O'Grady and J. P. Kerry, Phenolic composition
581 and in vitro antioxidant capacity of four commercial phytochemical products: Olive leaf
582 extract (*Olea europaea* L.), lutein, sesamol and ellagic acid, *Food Chem.*, 2011, **126**,
583 948-955.
- 584 33. M. de Bock, E. B. Thorstensen, J. G. Derraik, H. V. Henderson, P. L. Hofman and W. S.
585 Cutfield, Human absorption and metabolism of oleuropein and hydroxytyrosol
586 ingested as olive (*Olea europaea* L.) leaf extract, *Mol.r Nutr. Food Res.*, 2013, **57**, 2079-
587 2085.
- 588 34. S. Pashikanti, D. R. de Alba, G. A. Boissonneault and D. Cervantes-Laurean, Rutin
589 metabolites: novel inhibitors of nonoxidative advanced glycation end products, *Free*
590 *Rad. Biol. Med.*, 2010, **48**, 656-663.
- 591 35. F. Visioli, C. Galli, F. Bornet, A. Mattei, R. Patelli, G. Galli and D. Caruso, Olive oil
592 phenolics are dose-dependently absorbed in humans, *FEBS letters*, 2000, **468**, 159-160.

- 593 36. M. Navarro and F. J. Morales, Mechanism of reactive carbonyl species trapping by
594 hydroxytyrosol under simulated physiological conditions, *Food Chem.*, 2015, **175**, 92-
595 99.
- 596 37. S. Rosa Ados, L. G. Bandeira, A. Monte-Alto-Costa and B. Romana-Souza,
597 Supplementation with olive oil, but not fish oil, improves cutaneous wound healing in
598 stressed mice, *Wound Repair Regeneration*, 2014, **22**, 537-547.
- 599 38. A. Donato-Trancoso, A. Monte-Alto-Costa and B. Romana-Souza, Olive oil-induced
600 reduction of oxidative damage and inflammation promotes wound healing of pressure
601 ulcers in mice, *J. Dermatol. Sci.*, 2016, **83**, 60-69.
- 602 39. V. Ergin, R. E. Hariry and C. Karasu, Carbonyl stress in aging process: role of vitamins
603 and phytochemicals as redox regulators, *Aging and Disease*, 2013, **4**, 276-294.
- 604 40. K. Hamden, N. Allouche, M. Damak and A. Elfeki, Hypoglycemic and antioxidant effects
605 of phenolic extracts and purified hydroxytyrosol from olive mill waste in vitro and in
606 rats, *Chem.-Biol. Interact.*, 2009, **180**, 421-432.
- 607 41. A. Perez-Herrera, J. Delgado-Lista, L. A. Torres-Sanchez, O. A. Rangel-Zuniga, A.
608 Camargo, J. M. Moreno-Navarrete, B. Garcia-Olid, G. M. Quintana-Navarro, J. F. Alcala-
609 Diaz, C. Munoz-Lopez, F. Lopez-Segura, J. M. Fernandez-Real, M. D. Luque de Castro, J.
610 Lopez-Miranda and F. Perez-Jimenez, The postprandial inflammatory response after
611 ingestion of heated oils in obese persons is reduced by the presence of phenol
612 compounds, *Mol. Nutr. Food Res.*, 2012, **56**, 510-514.
- 613 42. P. A. Kroon, M. N. Clifford, A. Crozier, A. J. Day, J. L. Donovan, C. Manach and G.
614 Williamson, How should we assess the effects of exposure to dietary polyphenols in
615 vitro?, *Am. J. Clin. Nutr.*, 2004, **80**, 15-21.
- 616 43. R. Mateos, L. Goya and L. Bravo, Metabolism of the olive oil phenols hydroxytyrosol,
617 tyrosol, and hydroxytyrosyl acetate by human hepatoma HepG2 cells, *J. Agric. Food*
618 *Chem.*, 2005.
- 619 44. K. L. Tuck and P. J. Hayball, Major phenolic compounds in olive oil: metabolism and
620 health effects, *J. Nutr. Biochem.*, 2002, **13**, 636-644.

Table 1: Hydroxytyrosol (HT), oleuropein (Oleu), total phenolic content (TPC), and antioxidant capacity of olive leaf extract-A (OLE-A) and olive leaf extract-B (OLE-B). Results are expressed as mean $\pm$ standard deviation for $n = 4$. Different letters in the same row denote significant differences $P < 0.05$. LOQ < 0.5 GAE (mg g^{-1}) or <1.1 TEAC ($\mu\text{mol g}^{-1}$).

	OLE-A	OLE-B
HT (mg g^{-1} OLE)	3.6 ± 0.2	54.5 ± 5.0
Oleu (mg g^{-1} OLE)	93.9 ± 12.8	1.8 ± 0.1
TPC (mg GAE/g^{-1})	93.8 ± 27.1^a	320.8 ± 46.0^b
ABTS (TEAC $\mu\text{mol L}^{-1}$)	1409.8 ± 46.4^a	2061.1 ± 23.3^b
FRAP (TEAC $\mu\text{mol g}^{-1}$)	2409.0 ± 55.1^a	3603.6 ± 28.6^b

Table 2: Antiglycative capacity of olive leaf extract-A (OLE-A) and olive leaf extract-B (OLE-B) against the formation of fluorescent AGEs in BSA-MGO, BSA-GO and BSA-GLC models. Results are expressed as concentration of sample that exerts the 50% inhibition of the glycation. Different letters in the same column denote significant differences $P < 0.05$. Aminoguanidine (0.57 mg/mL) was used as a positive control and presented an inhibition of 98.4%, 93.9% and 88.5% for BSA-MGO, BSA-GO and BSA-GLC models, respectively.

sample	BSA-MGO IC_{50} (mg mL ⁻¹)	BSA-GO IC_{50} (mg mL ⁻¹)	BSA-GLC IC_{50} (mg mL ⁻¹)
OLE-A	0,425 ± 0,008 ^a	0,462 ± 0,039 ^a	0,716 ± 0,012 ^a
OLE-B	0,259 ± 0,002 ^b	0,248 ± 0,011 ^b	0,294 ± 0,003 ^b

Figure 1. A schematic diagram of fragmentation and subsequent obtaining the olive leaf extract concentrated in oleuropein (OLE-A) or hydroxytyrosol (OLE-B). Dotted line represents additional treatment for OLE-B.

Figure 2. MGO-trapping capacity of hydroxytyrosol (HT) and oleuropein (Oleu) standards (0.01-0.1 mg mL⁻¹) and olive leaf extracts (OLE-A and OLE-B) (0.05-0.25 mg mL⁻¹) after incubation at 37°C for 168 h. Results are expressed as mean ± SD for n = 4. Pyridoxamine (0.1 mg/mL) was used as a positive control and presented MGO-trapping capacity of 99.5%. HT (solid circle), Oleu (solid triangle), OLE-A (open box), OLE-B (open diamond).

Figure 3. Effect of olive leaf extract-B (OLE-B) on HepG2 cell viability after a 24 h-treatment with different concentrations (0.3-264 mg mL⁻¹). Cell viability is expressed as relative percentage of control cells stained with crystal violet. Data represent means ± SD of eight to ten samples. Different letters denote statistically significant differences (P<0.05).

Figure 4: Protective effect of olive leaf extract-B at 13 µg mL⁻¹ (OLE-B) [A]; and OLE-B fraction-1 (OLE-BF1), OLE-B fraction-2 (OLE-BF2) and hydroxytyrosol (HT, 5 µM) [B] on the hepatocyte protein carbonylation induced by methylglyoxal (MGO) in HepG2 cells. Results are expressed as protein carbonyl content per mg of protein. Data represent means ± SD of six to nine samples. Different letters denote statistically significant differences (P<0.05).

Figure 5. Antiglycative effect of olive leaf extract-B (OLE-B, 13 µg mL⁻¹) on the formation of carboxyethyllysine (CEL) and argpyrimidine (ArgP) induced by MGO in HepG2 cells. Results are expressed as mean ± standard deviation for n=3. Different letters denote significant differences (P < 0.05).

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

A.

B.

FIGURE 5