

Free radical scavenging and α -glucosidase inhibition, two potential mechanisms involved in the anti-diabetic activity of oleanolic acid

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SUMMARY: This work investigates the role of oleanolic acid (OA), isolated from the olive (*Olea europaea* L.) leaf, as a radical scavenger and inhibitor of the hydrolyzing enzymes of dietary carbohydrates. New evidence is provided showing that OA may capture 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) and peroxy radicals, and also exert a strong and non-competitive inhibition of α -glucosidase (IC_{50} 10.11 ± 0.30 μ M). The kinetic and spectrometric analyses performed indicate that OA interacts with this enzyme inside a hydrophobic pocket, through an endothermic and non spontaneous process of a hydrophobic nature. These are two possible mechanisms by which OA may facilitate a better control of post-prandial hyperglycaemia and oxidative stress, so contributing to preserving insulin signalling. Obesity, insulin resistance and Type 2 Diabetes Mellitus are considered the first pandemics of the 21st century. In this sense, OA might be used in future preventive and therapeutic strategies, as an ingredient in new drugs and functional foods.

KEYWORDS: Anti-diabetic activity; Antioxidant; α -glucosidase inhibitor; *Olea europaea*; Oleanolic acid

RESUMEN: *La Captación de radicales libres y la inhibición de α -glucosidasa, dos posibles mecanismos involucrados en la actividad antidiabética del ácido oleanólico.* Este trabajo estudia el papel del ácido oleanólico (OA), aislado de la hoja de olivo, como secuestrador de radicales libres e inhibidor de enzimas implicados en la hidrólisis de los carbohidratos de la dieta, dos mecanismos por los que el triterpeno podría mitigar la hiperglicemia postprandial y el estrés oxidativo. Se aportan nuevas evidencias que muestran que el OA puede capturar radicales ácido 2,2'-azino-bis-(3-etilbenzotiazolín)-6-sulfónico y peróxido, y que ejerce una potente inhibición no-competitiva de α -glucosidasa (IC_{50} 10.11 ± 0.30 μ M). El análisis cinético y espectrométrico llevado a cabo indica que OA interacciona con este enzima en el interior de un bolsillo hidrofóbico, mediante un proceso endotérmico no espontáneo, de naturaleza hidrofóbica. Estos son dos posibles mecanismos por los cuales el OA puede facilitar un mejor control de la hiperglucemia postprandial y el estrés oxidativo, lo que contribuye a preservar la señalización de la insulina. La obesidad, la resistencia a la insulina y la diabetes mellitus tipo 2 se consideran la primera pandemia del siglo XXI. En este sentido, el OA podría ser utilizado en futuras estrategias preventivas y terapéuticas, como ingrediente de nuevos fármacos y alimentos funcionales.

PALABRAS CLAVE: Acción antidiabética; Ácido oleanólico; Antioxidante; Inhibidor de α -glucosidasa; *Olea europea*

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1. INTRODUCTION

Type 2 Diabetes Mellitus (T2DM), the most common form of the disease affecting near 90% of diabetic people, is recognized as a redox disorder, in which oxidative stress is a primary event underlying insulin resistance, β -cell failure, and long-term diabetes complications (Watson, 2014). This prompted investigations on the therapeutic use of antioxidants, although none of such studies demonstrated clear effects on the metabolic control of diabetic people (Ceriello and Testa, 2009). Another strategy to minimize oxidative stress is by reducing glycaemia fluctuations during post-prandial periods, since different studies with cells exposed to levels of glucose elevated intermittently have demonstrated the increased expression of biomarkers for oxidative stress (Quagliaro *et al.*, 2003). An interesting approach is the inhibition of enzymes involved in carbohydrate breakdown and the intestinal absorption of glucose. Salivary and pancreatic α -amylases (EC 3.2.1.1) cleaves the α -(1 \rightarrow 4) bonds of starch yielding shorter linear and branched dextrans, which are subsequently hydrolyzed at the non-reducing end into glucose by α -glucosidase (EC 3.2.1.20) in the small-intestinal brush border membrane. Synthetic inhibitors, such as acarbose, voglibose or miglitol, are widely used in the clinic. However, the appearance of adverse effects is frequent, including flatulence, abdominal pain, nausea, vomiting, diarrhea, skin hypersensitivity, an elevation in the hepatic enzymes and even an increased incidence of renal tumors (Fujisawa *et al.*, 2005).

Accordingly, the search for more effective and safer natural bio-molecules, with the dual ability to decrease glucose absorption and reduce oxidative damage, has become an important scientific goal. Oleanolic acid (OA) (3 β -hydroxy-olean-12-en-28-oic acid), a pentacyclic triterpene widely distributed in the plant kingdom, has attracted the attention of researchers for its interesting pharmacological properties (Liu, 2005; Dzubak *et al.*, 2006), including anti-diabetic activity (Castellano *et al.*, 2013).

The data available nowadays concerning the antioxidant activity of OA are somewhat contradictory. Its ability to react with diverse radical species has been evaluated, having obtained an array of varied results (Yin and Chan, 2007; Yang *et al.*, 2007; Wang *et al.*, 2010; Allouche *et al.*, 2011). The OA effect on α -amylase is not completely clear (Khatri *et al.*, 2013; Wang *et al.*, 2011). However, a consensus does exist concerning the efficient inhibition of α -glucosidase by the triterpene, and some IC₅₀ values in the micro-molar range have been reported (Ali *et al.*, 2002; Kang *et al.*, 2012). To our knowledge, studies on the mechanism of this reaction have not been carried out. Consequently, in this work, we investigate the triterpene's ability

to scavenge three radical species (ABTS⁺, DPPH and peroxy), and also to inhibit both α -amylase and α -glucosidase. The paper includes a deeper kinetic study to determine the type of inhibition, as well as fluorescent measurements to obtain new clues about the mechanism of the OA/ α -glucosidase interaction. This research is part of a more general investigation aimed at exploiting the therapeutic potential of OA against diabetes.

2. MATERIALS AND METHODS

2.1. Plant material, reagents and standards

Olive (*Olea europaea* L. cv. Picual) leaves were hand-picked from adult trees (>10 years old) from the experimental olive grove belonging to the Instituto de la Grasa-CSIC in Seville.

All solvents used were of analytical grade. Betulinic acid; hexamethyldisilazane; trimethylchlorosilane; 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS); 2,2-Diphenyl-1-picrylhydrazyl (DPPH); 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH); fluorescein; pancreatic α -amylase; potato starch; 3,5-dinitrosalicylic acid; sodium potassium tartrate; α -glucosidase from *Saccharomyces cerevisiae*; 4-nitrophenyl- α -D-glucopyranoside (*p*-NPG); and acarbose were purchased from Sigma-Aldrich (Sigma-Aldrich Quimica, Madrid, Spain).

2.2. Isolation of oleanolic acid from *O. europaea*

High purity OA was isolated from olive leaves according to the procedure described by us (Guinda *et al.*, Patent 2001/2160553). Briefly, dry leaves were extracted by maceration with 96% ethanol, which was then percolated and subsequently reduced by vacuum. Solid OA was obtained by crystallization and filtration. Its chemical nature was confirmed by GC-MS (P Gutiérrez-Adán, Thesis of Master, University Pablo de Olavide; Seville, 2013), and its purity was determined by differential scanning calorimetry (DSC) and GC-FID (Albi *et al.*, 2001; Guinda *et al.*, 2010).

2.3. Derivatization of oleanolic acid

The low volatility and high molecular weight of OA demands its derivatization prior to the GC analysis. The silylating reagent was prepared by mixing 3 mL of hexamethyldisilazane and 1 mL of trimethylchlorosilane with 9 mL of anhydrous pyridine. Ten milligrams of OA were dissolved in 20 mL absolute ethanol. An aliquot (100 μ L) of this solution and 100 μ L of 0.5 mg/mL betulinic acid (internal standard) were placed in 1 mL gas-tight vials and evaporated to dryness under a N₂ stream. The residue was dissolved in 200 μ L of the silylating reagent and sonicated at 70 °C for 15 min. The solution harboring

the derivatized triterpene was analyzed by GC-FID and GC-MS.

2.4. GC-FID Analysis

The quantification of OA was carried out by a modification of the method described by us in (Guinda *et al.*, 2010). Concisely, 1 μ L of the silylated sample was injected into an Agilent 6890N GC (Agilent Technologies, CA), equipped with a Rtx-65TG Cross-bond capillary column (30 m \times 0.25 mm I.D.; 0.1 mm film thickness) coated with 35% dimethyl-65% diphenyl polysiloxane as stationary phase (Restek, Co., Bellefonte, PA) and a FID detector. The injection was done in the split mode, and hydrogen was used as carrier gas (pressure at column head 140 kPa). The oven temperature was maintained at 260 $^{\circ}$ C, and the injector and detector temperatures at 300 $^{\circ}$ C.

2.5. GC-MS identification of oleanolic acid

The analysis of derivatized OA was performed using a coupled gas chromatograph-mass spectrometry detector (GC-MS) QP2010 Ultra (Shimadzu Europa GmbH) fitted with an AOC-20i auto-sampler, an ion source of electron impact and a quadrupole detector. The splitless mode was used and the injector temperature was set at 290 $^{\circ}$ C. Helium was the carrier gas at a pressure of 53.1 kPa and a flow of 1 mL/min. The oven temperature program was as follow: initial temperature 50 $^{\circ}$ C for 1 min, 50–200 $^{\circ}$ C at 40 $^{\circ}$ C/min, 200–280 $^{\circ}$ C at 10 $^{\circ}$ C/min, held at 280 $^{\circ}$ C for 8.50 min, and finally at 300 $^{\circ}$ C for 1 min. Total run time: 24,25 min. The MS conditions were: interface temperature: 280 $^{\circ}$ C; ion source temperature: 220 $^{\circ}$ C; electron impact: 70 eV; acquisition mode: scan (m/z 50–600). The identification of the OA trimethylsilyl derivative was accomplished by comparing the retention time and abundance ratio of two fragment ions (203 and 189 m/z) with those of the standard compound.

2.6. Purity evaluation by DSC

The OA purity was also assessed by Differential Scanning Calorimetry (DSC), following the method described in (Albi *et al.*, 2001). A TA-Q2000 calorimeter equipped with a cooling unit (Thermal Analysis Instruments; Barcelona, Spain) was used. This instrument was calibrated with indium (99.9 %). The OA samples (10–15 mg) were enclosed in hermetically sealed aluminum pans and analyzed against air (empty pans) as reference. The calorimeter chamber was previously thermostated at 30 $^{\circ}$ C for 10 min. Then, the melting curves from 30 to 350 $^{\circ}$ C (heating rate 10 $^{\circ}$ C/min) were recorded. The data were analyzed using the Peak Program software of the TA-Q2000.

2.7. Radical scavenging

2.7.1. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay

The ABTS+ scavenging capacity was evaluated following a modification of the procedure by Re *et al.* (1999). The radicals were generated by oxidizing its precursor ABTS (7.0 mM in methanol) with potassium persulfate (2.45 mM), maintaining the mixture overnight in the dark at room temperature. The resultant ABTS+ solution was diluted to have an absorbance at 734 nm in the range of 0.7–0.8. An aliquot (10 μ L) of OA samples (0.5, 1.0, 4.0 and 10.0 mM in methanol), or a blank (methanol), was added to 1 mL of the ABTS+ solution. The mixture was stirred and left to react in the dark at room temperature for 5 min, and then the absorbance at 734 nm was recorded in a Beckman DU 640 UV-VIS light spectrophotometer (Beckman Coulter Inc.; Pasadena, CA; USA). The results were expressed as percentage of the initial absorbance of the ABTS+ solution. Butylated hydroxytoluene (BHT) (0.5 mM in methanol) was assayed as reference antioxidant.

2.7.2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging test

The OA ability to capture DPPH radicals was also assessed spectrophotometrically according to the revised protocol by Sharma and Bhat (2009). The reaction mixture (4 mL), containing 50 μ M DPPH alone or in the presence of OA (1, 25, 50, 100 and 1000 μ M), was incubated for 30 min at 30 $^{\circ}$ C in the dark, and then the absorbance at 517 nm was measured using the Beckman DU 640 spectrophotometer. The scavenging activity was expressed as percentage of the initial absorbance of the DPPH solution. BHT (50 and 100 μ M) was also included in the assay as a positive control.

2.7.3. Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay was performed following a modification of the procedure reported by Prior *et al.* (2003), in which fluorescein was the target of the peroxy radicals generated with 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH). An aliquot (20 μ L) of the OA sample [0.1–0.7 mM in 10% DMSO (in 75 mM phosphate buffer, pH 7.4 at 37 $^{\circ}$ C)] or a blank (buffered DMSO) was pre-incubated with 120 μ L of 116.7 nM fluorescein for 10 min at 37 $^{\circ}$ C. Then, the reaction was started by the addition of 60 μ L of 40 mM AAPH. The time course of the fluorescent decay (λ_{ex} = 485 nm, λ_{em} = 525 nm) was recorded for 80 min using a Fluostar Galaxy plate reader (BMG Labtechnologies, Offenburg, Germany). The capacity to scavenge peroxy radicals correlates with the

net area under the fluorescent decay curve (AUC), calculated by subtracting the OA sample AUC from the blank AUC. The AUCs were automatically calculated by the Fluostar software. The ORAC value (expressed as the μmol Trolox equivalent), was determined from the calibration curve obtained with standard Trolox in the concentration range 1–10 μM .

2.7.4. Rancimat method

Rancimat 743 equipment (Metrohm AG; Erisau, Switzerland) was used for evaluating the role of OA against lipid peroxidation. An aliquot (300 μL) of OA solutions (0.5 and 1.0 mM in DMSO) was added to 3.0 g of olive or sunflower oil. The mixture was heated at 100 °C whereas an air flow (20 L/h) bubbled constantly. The effluent gas was collected in de-ionized water, whose electrical conductivity was continuously monitored. In this system, the antioxidant activity correlates with the extension of timing up to the inflection point in the conductivity curve (induction time) (Burkow *et al.*, 1995). The antioxidant activity index (AAI) was calculated from the ratio *induction time of the oil with OA/induction time of oil alone*. BHT (250 and 500 $\mu\text{g/g}$) was likewise included in the assays as a reference antioxidant.

2.8. In vitro inhibition of carbohydrate hydrolyzing enzymes

2.8.1. In vitro assay for α -amylase

α -Amylase was assayed using the chromogenic method adopted by Sigma–Aldrich (www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-a-amylase.html). Porcine pancreatic α -amylase was dissolved in an ice-cold 20 mM sodium phosphate buffer (pH 6.9 at 37 °C) containing 6.7 mM NaCl. The potato starch solution (1% w/v) was prepared in the same buffer.

In a first series of experiments, 40 μL of OA solutions (5–1000 μM in buffered 10% DMSO), 60 μL of phosphate buffer, and 400 μL of the substrate solution were mixed in a screw-top glass tube, and pre-incubated at 37 °C for 5 min. The reaction was subsequently started by the addition of 200 μL of the enzyme solution (1 unit/mL), and the mixture was incubated at the same temperature for 5 min. Then, 350 μL of the DNS color reagent (96 mM 3,5-dinitrosalicylic acid, 5.31 M sodium potassium tartrate in 2 M NaOH) were added to the tube and placed into a 85 °C water bath for 15 min. Then, the mixture was removed and diluted with 3 mL distilled water. The maltose generated in the reaction (absorbance at 540 nm) was quantified from the calibration curve obtained with standard maltose in the range of 50 μM –3.75 mM ($A_{540} = 2.6185 \cdot 10^{-4}$ [maltose] – 0.0279; $r^2 = 0.9972$). Control incubations representing 100% enzyme activity were conducted in an identical

fashion, replacing the OA solution by DMSO. Blank incubations, where the enzyme solution was replaced with phosphate buffer, were also included. In a second series of assays, the enzyme was pre-incubated with OA at 37 °C, and the reaction was started by the addition of the starch solution. In both series, acarbose (20 μM) was introduced as a reference inhibitor.

2.8.2. In vitro assay for α -glucosidase

The α -glucosidase activity was tested by adapting the protocol by Kang *et al.* (2011) to 96-well microplates. To each well, 30 μL of a 100 mM phosphate buffer containing 100 mM NaCl (pH 6.8 at 37 °C), 30 μL of 2.5 mM *p*-NPG in this same buffer, and 30 μL of OA (0.05–50.0 μM in buffered 10% DMSO), were added. The plate was pre-incubated at 37 °C for 5 min, and the reaction was started by adding 30 μL of the α -glucosidase solution (0.2 U/mL α -glucosidase in phosphate buffer). After 20 min at the same temperature, 120 μL of 100 mM sodium carbonate were added to stop the reaction. The release of 4-nitrophenol ($\epsilon = 18.3 \text{ mM}^{-1} \text{ cm}^{-1}$) was determined by measuring the absorbance at 405 nm in a Multiskan Spectrum (Thermo Labsystems; Milford, MA, USA) micro-plate reader. One enzyme activity unit is defined by the release of 1.0 μmol α -D-glucopyranoside per minute at 37 °C. Control incubations representing 100% enzyme activity were conducted, replacing OA solutions with DMSO. Likewise, blank incubations were carried out where the enzyme solution was replaced by the phosphate buffer. The inhibitory activity of OA was assayed in the range of 0.05–50.0 μM , and 20 μM acarbose was introduced as reference inhibitor.

2.8.3. Fluorescence quenching measurements

In these experiments, α -glucosidase was assayed at 1 μM in the presence of OA (0–25 μM). The reaction mixture (whose final volume was brought to 4.0 mL with phosphate buffer) was incubated for 10 min at 20, 30 and 37 °C. The fluorescent spectra of α -glucosidase were obtained on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies; Santa Clara, CA, USA) equipped with a 10.0 mm quartz cell and a thermo-stated bath. The excitation wavelength was 280 nm, and the emission spectra were recorded from 300 to 500 nm. The excitation and emission bandwidths were both set at 5 nm. Appropriate blanks corresponding to the phosphate buffer alone were assayed and subtracted to correct background fluorescence.

2.9. Statistical analysis

All data are presented as the means \pm standard deviation (SD) from a series of three independent experiments carried out with five replicates. The

data were evaluated by a one-way ANOVA with the SigmaPlot 12.5 software (SPSS, Inc., Chicago, IL), and the differences between means were assessed using the Duncan's multiple-range test. Statistical significance was considered at $p < 0.05$.

3. RESULTS

3.1. Obtaining pure oleanolic acid from olive leaf title One

Eighteen grams and six hundred thirty two milligrams of crystallized OA were obtained from one kilogram of dried olive leaves. Its purity (98.6%) was determined by both GC-FID and DSC (data not shown).

3.2. Free radical scavenging

3.2.1. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS+) assay

The stable radical ABTS+ is commonly used for the estimation of total antioxidant capacity. The simpler and more frequently applied approach of this assay consists of analyzing the activity of a sample against pre-formed radicals. With this scheme, we have found that OA is a moderate scavenger of ABTS+, and its action is dose-dependent (Table 1). Thus, 10 mM OA removed 24% of the initially present ABTS+. In comparison, 0.5 mM BHT abolished these radicals almost completely. On the other hand, we have tested two other solvents in addition to methanol, for preparing the OA solutions: carboxymethylcellulose (CMC) and DMSO, but they did not improve the scavenging activity of the triterpene.

3.2.2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging test

DPPH is also very common for the study of natural antioxidants. Its solutions have a characteristic deep purple color (λ_{\max} 515–517 nm) that decolorize by the action of radical scavengers. Performing this procedure, it became evident that OA does not capture DPPH radicals, at least at concentrations lower than 1 mM (data not shown). The use of DMSO substituting for methanol as the triterpene solvent did not provide better results.

3.2.3. Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay determines the ability of a compound to preserve fluorescein from the attack of peroxy radicals generated with AAPH. Using this method, OA showed a low and dose-dependent capability to react with these radicals (Figure 1). The net areas

TABLE 1. Scavenging of the ABTS+ radical by oleanolic acid. Results are expressed as the means \pm SD ($n = 15$). CMC = carboxymethylcellulose [2.5% (w/w)]. BHT was included in the assay as positive control. Different letters within a column indicate that values are different at $p < 0.05$

	absorbance (λ_{734})	reduction (%)
ABTS+	0.779 \pm 0.041 ^a	
+ OA (mM) in methanol		
0.5	0.682 \pm 0.035 ^{b,d}	12.5 \pm 0.6
1.0	0.676 \pm 0.038 ^{b,d}	13.2 \pm 0.7
4.0	0.645 \pm 0.047 ^{b,e}	17.2 \pm 1.2
10.0	0.586 \pm 0.030 ^{c,h}	24.8 \pm 1.3
+ OA (mM) in CMC		
0.5	0.691 \pm 0.054 ^d	11.2 \pm 0.9
1.0	0.673 \pm 0.034 ^{b,d,f}	13.5 \pm 0.7
4.0	0.636 \pm 0.032 ^{e,f,h}	18.4 \pm 0.9
+ OA (mM) in DMSO		
4.0	0.631 \pm 0.028 ^{e,g,h}	18.9 \pm 0.9
10.0	0.599 \pm 0.031 ^h	23.2 \pm 1.2
+ BHT 0.5 mM	0.026 \pm 0.002 ⁱ	96.7 \pm 3.5

under the fluorescent decay curves were linearly fitted to the OA concentrations in the range of 0.1–0.7 mM. An ORAC value of 1.628 nmol Trolox equiv/ μ mol OA was determined from the calibration curve obtained with standard Trolox (Net AUC = 19.904 nmol Trolox + 6.335; $r^2 = 0.9899$).

3.2.4. Rancimat method

The Rancimat method, based on the induction of lipid oxidation by exposure to airflow at high temperatures, is likely the most commonly used procedure for measuring the oxidative stability of oils and fats. The process occurs via a chain reaction (with participation of hydroxyl and peroxy radicals), in which the double bonds of unsaturated fatty acids undergo cleavage, releasing volatile short-chain organic acids, aldehydes and ketones. The reaction is characterized by a relatively slow initial period before it suddenly speeds up. The time for this to happen, called induction time, is taken as an indication of the oxidative stability of the lipid sample. The Rancimat procedure can be also applied for assessing the efficiency of antioxidants, by appraising how long they are able to prolong the initial induction step. This paper reports the use of such equipment for evaluating the antioxidant potential of OA for the first time.

We first ran experiments with an olive oil, chosen by its low content in minor components. In this lipid system, 0.5 mM OA did not prevent oil oxidation, but instead it exerted a slight pro-oxidant activity, as

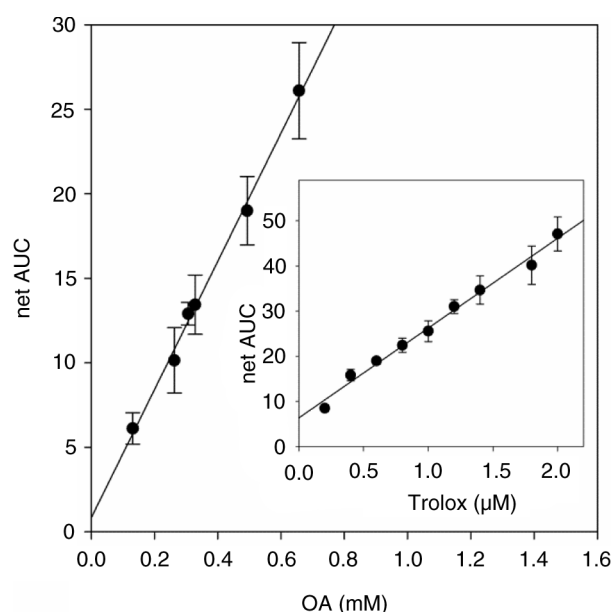


FIGURE 1. Oxygen radical absorbance capacity (ORAC) assay for OA. Net area under the fluorescent decay curve in the presence of increasing concentrations of OA. The inset is the calibration curve obtained with standard Trolox. Results are expressed as mean \pm SD of three independent experiments carried out with five replicates.

evidenced by the shortening of the induction period (Figure 2). By doubling the OA concentration in the medium no significant changes were observed. A second sequence of trials was performed with refined sunflower oil, whose triglycerides are richer in polyunsaturated fatty acids than those of olive oil. In that lipid matrix, the triterpene showed a clear and dose-dependent pro-oxidant action. So, in the presence of 1.0 mM OA the oxidative stability of the sunflower oil fell by 50%. In both experimental series, BHT was able to remarkably delay oil oxidation. To the best of our knowledge, this is the first time a pro-oxidant action of OA is reported.

3.3. Inhibition of carbohydrate hydrolyzing enzymes

3.3.1. Effect of OA on the pancreatic α -amylase

α -Amylase activity was assayed using potato starch as substrate to determine the maltose produced in the reaction. We performed two series of experiments, considering whether starch and OA were first pre-incubated and the reaction was started by the addition of the enzyme solution; or whether the enzyme was first pre-incubated with the triterpene and then the reaction initiated by addition of the substrate. With both approaches, no enzyme inhibition was observed in the OA concentration range 5.0 μ M–1.0 mM. However, 20 μ M acarbose (the reference inhibitor) was able to repress the

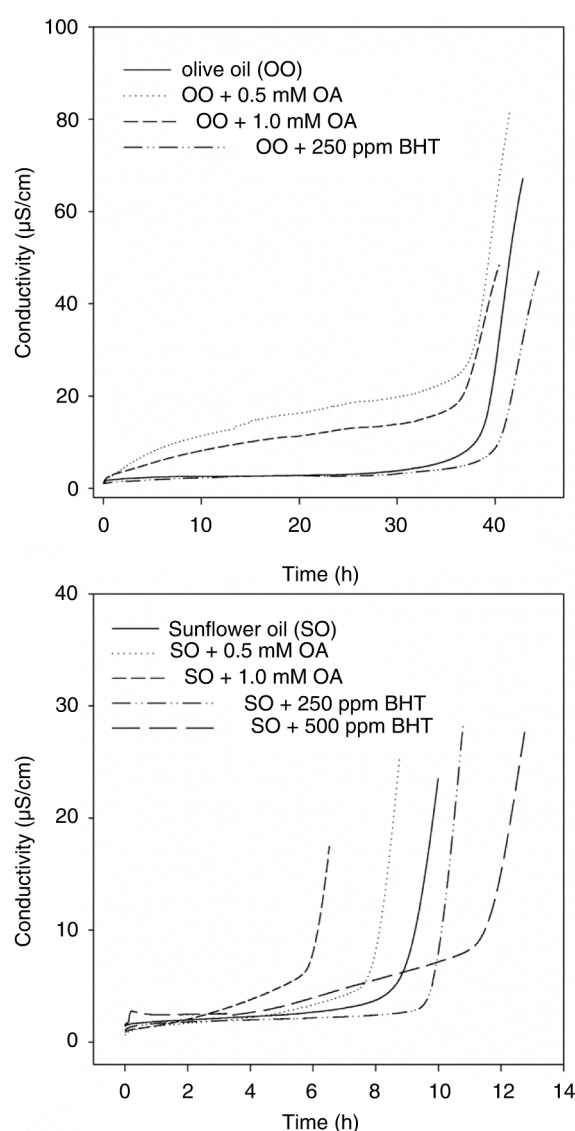


FIGURE 2. Effect of OA on the oxidative stability of olive and sunflower oils (Rancimat method). Typical conductivity vs time curves representative of three independent experiments performed with five replicates.

enzyme activity by 27.2 and 30.1%, respectively (data not shown).

3.3.2. Inhibition of the α -glucosidase activity by OA

Unlike α -amylase, OA caused a strong inhibition of the α -glucosidase activity. This effect was dose-dependent, with the enzyme activity decreasing remarkably when the OA dose augmented (Figure 3). In fact, the α -glucosidase action was virtually abolished at 20 μ M OA and above. The dose resulting in 50% inhibition (IC_{50}) was estimated to be $10.11 \pm 0.30 \mu$ M.

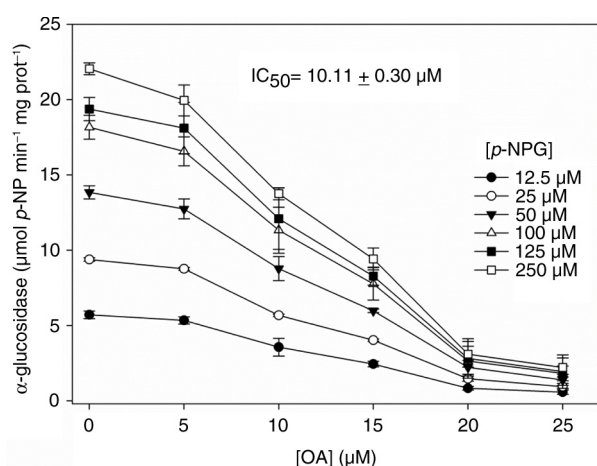


FIGURE 3. Inhibitory effect of OA on the α -glucosidase activity. In the inset the OA dose resulting in 50% inhibition (IC_{50}) is expressed. Results are expressed as mean \pm SD of three independent experiments carried out with five replicates.

For a deeper study of the inhibition mechanism we first corroborated that α -glucosidase catalysis followed a Michaelis-Menten kinetic. The reaction proceeded at its maximum rate ($26.84 \mu\text{mol } p\text{-NP min}^{-1} \text{ mg prot}^{-1}$) with substrate concentrations greater than $150 \mu\text{M}$, and a K_m value of $51.34 \mu\text{M}$ was determined for p -NPG. We also studied the time-course of the reaction in the absence and presence of OA (5 , 15 and $25 \mu\text{M}$), finding that the activity *vs* time relationship progressed linearly for at least 25 min (data not shown). Then, we evaluated the enzyme activity at increasing concentrations of p -NPG, in the presence of different doses of OA. The Lineweaver-Burk's kinetic analysis of results indicated that OA was a non-competitive inhibitor of α -glucosidase (Figure 4). The inhibition constant (K_i) of $9.12 \pm 0.24 \mu\text{M}$ was calculated by both the Dixon and Cornish-Bowden analyses.

3.3.3. Fluorescence quenching of α -glucosidase by oleanolic acid

The powerful inhibition that OA exerts on α -glucosidase suggests that the triterpene directly binds to the protein. To achieve new evidence for this interaction, we conducted, for the first time to our knowledge, binding studies employing fluorescent spectroscopy. The spectra of α -glucosidase in the presence of different OA concentrations were recorded at 20 , 30 and 37°C (Figure 5). Under the same experimental conditions, OA did not display intrinsic fluorescence or UV-VIS absorption in the wavelength range 300 – 500 nm, not interfering with the α -glucosidase spectra (data not shown). α -Glucosidase exhibited a strong fluorescent emission peak at 333 nm after excitation at 280 nm, indicating that tryptophans located in inner regions

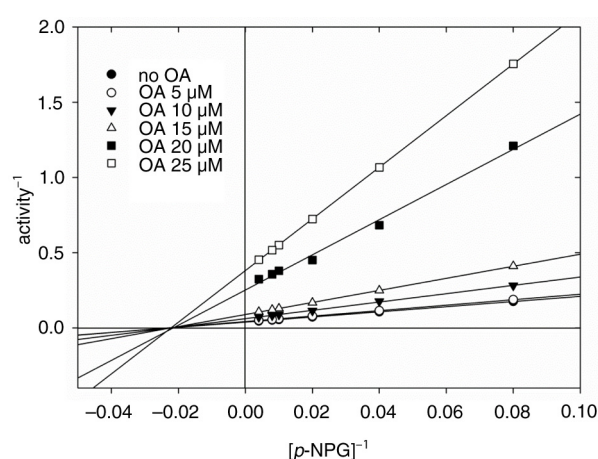


FIGURE 4. Lineweaver-Burk plots for the α -glucosidase inhibition by OA.

of the protein are practically the only ones emitting fluorescence. In the presence of OA, significant and dose-dependent decreases in the fluorescent intensity were observed. This quenching effect did not involve hypsochromic or bathochromic shifts, which reveals that the OA/ α -glucosidase interaction only affected those tryptophyls positioned within a cleft of the enzyme.

Figure 6 shows the Stern-Volmer plots ($F_0/F = 1 + K_{SV}[OA]$) for the quenching of α -glucosidase fluorescence by OA. In this equation, F_0 and F denote the steady-state fluorescent intensities in the absence and presence of the triterpene, respectively; and K_{SV} is the Stern-Volmer quenching constant, a measure of the interaction strength between the protein and its ligand. The plot's linearity illustrates that within the assayed concentration range, a single class of enzyme fluorophores is affected by OA. The results also show that the K_{SV} values increased with increasing temperature, suggesting that quenching proceeded mainly through a dynamic mechanism of collisions between OA and tryptophans. This relationship may also be described by the equation $\log [(F_0 - F)/F] = \log K_A + n \log [Q]$ (Yan *et al.*, 2014), where K_A is the binding constant, and n the number of binding sites on the protein (Figure 6). It can be observed that both K_A and n rose with the increase in temperature, showing again that the OA/ α -glucosidase interaction was favored under warmer conditions. Moreover, the values of n show a trend to one, supporting the existence of a single class of binding site for OA.

More information about this interaction may be inferred from the signs of the thermodynamic parameters in the process. The changes in enthalpy (ΔH) and entropy (ΔS) can be determined from the Van't Hoff equation [$\ln K_{SV} = -(\Delta H/RT) + (\Delta S/R)$; where R is the gas constant], and the free energy changes (ΔG) can be estimated from the

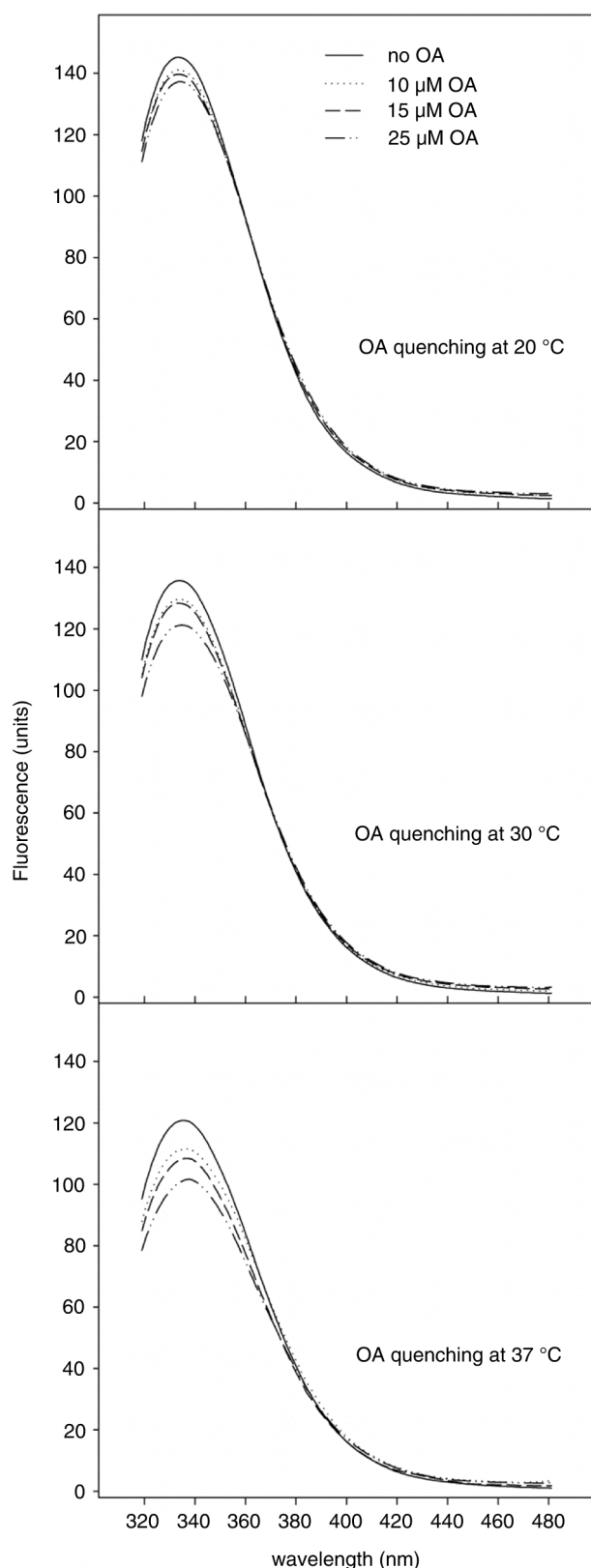


FIGURE 5. Fluorescent emission spectra (300–500 nm) of α -glucosidase at 20, 30 and 37 °C, in the presence of increasing concentrations of OA ($\lambda_{\text{ex}} = 280$ nm).

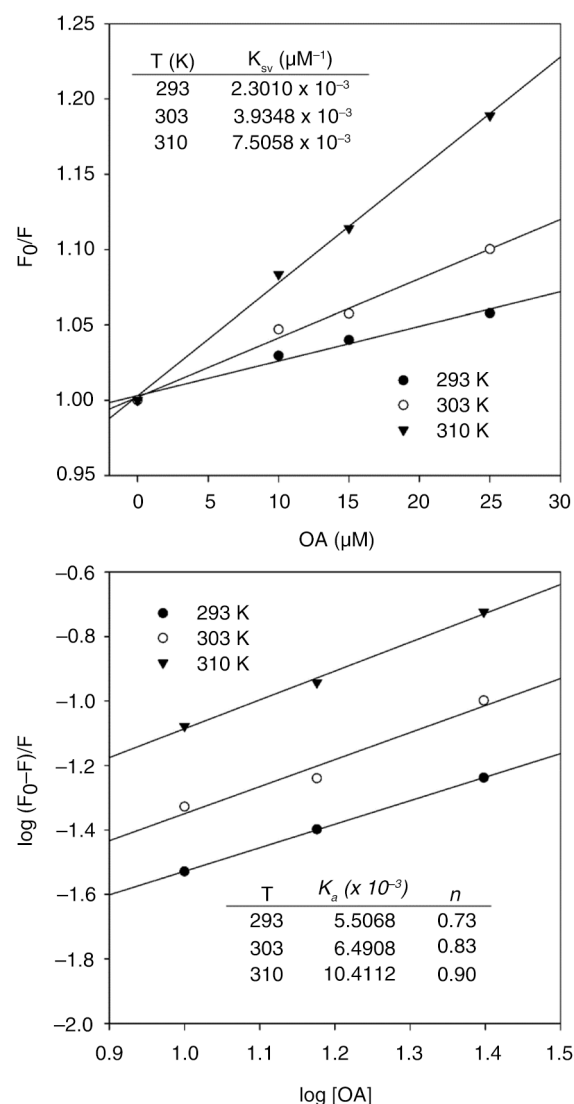


FIGURE 6. Stern-Volmer plots for the fluorescence quenching of α -glucosidase by OA.

$\Delta G = \Delta H - T\Delta S$ relationship. Both ΔH and ΔS were positive, proposing that the OA/ α -glucosidase association has a hydrophobic nature. Likewise, ΔG values were positive, signifying that the triterpene/protein association is endergonic in the assayed temperature range (20–37 °C).

4. DISCUSSION

The experimental and clinical evidence indicates an inverse association between insulin sensitivity and oxidative stress, up to the point that diabetes is acknowledged as a phenotype of dysfunctions in mitochondria and endoplasmic reticulum, associated with an excessive ROS production or an impairment of the endogenous antioxidant system (Lowell and Shulman, 2005). Because post-prandial

hyperglycaemia induces oxidative stress, the search for natural bio-molecules with the dual ability to decrease glucose absorption and oxidative damage has become an important research topic. Within that scenario, this paper reports new findings about OA as a radical scavenger and inhibitor of the α -glucosidase activity.

The antioxidant power of OA is subject to controversy since it is markedly influenced by the singularities of the experimental systems employed in its evaluation. We have evaluated the OA ability to scavenge three radical species (ABTS⁺, DPPH and peroxy), finding that it can capture ABTS⁺ radicals in a moderate and dose-dependent manner, but it lacks the ability to scavenge DPPH species. These results are in agreement with those previously provided by other authors (Yang *et al.*, 2007; Wang *et al.*, 2010; Allouche *et al.*, 2011).

The role of triterpene against peroxy radicals deserves a more detailed discussion. The ORAC assay has demonstrated that OA moderately captures the radicals generated from AAPH, although it failed with those formed from complex lipid matrixes in the Rancimat procedure. In fact, this last protocol has revealed that OA might exert a pro-oxidant action. These results do not coincide with those of Yin and Chan (2007), who have studied the triterpene potential for reducing peroxy radicals using a modified TBARS assay on liposomes. They determined that OA had a high and dose-dependent activity when liposome peroxidation was induced by AAPH and 2,2'-azobis-(2,4-dimethylvaleronitrile). Closer to the Rancimat method, Assimopoulou *et al.* (2005) investigated the OA effect on the oxidation of lard and sunflower oil in an oven at 65 °C. They monitored the evolution of peroxide value, reporting that OA did not exhibit a clear antioxidant activity. Wang *et al.* (2010) examined the effect of OA in liver microsomes of Sprague-Dawley rats, comparing the triterpene action with those of well-known antioxidants, and found that the protection offered by OA against peroxy radicals depended on the substance used for inducing lipid peroxidation. This study also evidenced that some molecules recognized as antioxidants (vitamin C or α -lipoic acid) could act as pro-oxidants depending of the nature of the lipid oxidizing agent. Once again, different experimental approaches lead to different conclusions, many of which are conflicting.

Another mechanism by which OA may contribute to controlling oxidative stress is by decreasing post-prandial hyperglycaemia, through the inhibition of carbohydrate-hydrolyzing enzymes. In this paper, we present results proving that OA is not an α -amylase inhibitor. This finding is in agreement with other published data (Hou *et al.*, 2009; Wang *et al.*, 2011) although there are also contradictory results supporting that OA might effectively repress this activity (Khatri *et al.*, 2013; Ali *et al.*, 2006;

Komaki *et al.*, 2003), with quite variable IC₅₀ values in the range of 10 μ M–2.6 mM.

Unlike α -amylase, OA exerts a strong and dose-dependent inhibition of α -glucosidase. We have determined an IC₅₀ 10.11 \pm 0.30 μ M, a very similar value to those reported by other authors (Ali *et al.*, 2002; Hou *et al.*, 2009; Kang *et al.*, 2012). In addition, we present here new contributions to the understanding of the OA/ α -glucosidase interaction, performing a deeper kinetic study and fluorescent measurements. We found that OA is a non-competitive inhibitor of α -glucosidase (K_i = 9.12 \pm 0.24 μ M), which binds the enzyme in a neighboring region of the active site. The comparison of the inhibitory efficiency of OA with those of other natural and synthetic structural analogs allows us to outline some molecular features with impact on inhibition: a) the relevancy of the hydrophobic skeleton with a hydroxyl group in C3 and a carboxyl group in C28 is acknowledged; b) the introduction of a second phenolic hydroxyl at C2, as in maslinic or corosolic acids, might increase the inhibitory power of the triterpene (Ali *et al.*, 2006); c) the substitution of the methyl at C23 by a more bulky ligand (an acetyl group, for example) would diminish this ability (Ali *et al.*, 2002); and d) the β -orientation of the phenolic hydroxyl in C3 seems to be clearly influential for the inhibition, since corosolic acid with a β -oriented hydroxyl such as OA exhibits an IC₅₀ value of 7.47 μ M, whereas 3-epi-corosolic acid, its α -stereoisomer, displays a IC₅₀ 63.9 μ M (Chowdhury *et al.*, 2014).

The fluorescent measurements of the OA/ α -glucosidase complex provide useful information about the binding mechanism and binding site. The intrinsic fluorescence of a folded protein is the result of that emitted by its individual aromatic residues, with most of the emissions due to the excitation of tryptophans and few due to tyrosines and phenylalanines. On the other hand, tryptophans located in inner regions of proteins with hydrophobic micro-environments emit maximum fluorescence near 331 nm, whereas those residues located on the protein surface exposed to a high-polarity aqueous media emit at longer wavelengths (\approx 350 nm) (Lacovicz, 2006). Therefore, the wavelength of maximum emission can be interpreted in terms of the ratio between these two classes of tryptophans. Moreover, intrinsic fluorescence often decreases when a protein interacts with another compound. The process (quenching) happens during the excited state lifetime (collisional or dynamic quenching) or due to the formation of a complex in the ground state (static quenching).

In our case, the maximum fluorescence emitted by α -glucosidase as well as quenching by interaction with OA are detected at 333 nm, indicating that the tryptophyls involved in the triterpene binding are almost exclusively located inside a hydrophobic pocket. The Stern-Volmer plots determined a single class of binding site on α -glucosidase (n tending

towards 1), with all the participating tryptophans identically accessible (linearity of the plots within the experimental range of OA concentrations). We have also found that the α -glucosidase/triterpene interaction responds to a collisional mechanism which is favored at warmer temperatures, such as that operating in the human body (rising values of quenching constant at increasing temperature). The study of thermodynamic parameters corroborated the idea that inhibition of α -glucosidase by OA is an endothermic and entropy-driven, a non-spontaneous process of predominant hydrophobic nature. However, complementary contribution of hydrogen bridges cannot be ruled out because the reaction medium is aqueous and both OA and α -glucosidase molecules include prominent hydroxyl groups. These outcomes are coherent with reported data postulating the existence of tryptophans neighboring the α -glucosidase active site, which are likely to be more relevant for maintaining an efficient substrate accommodation rather than for the catalysis itself (Faridmoayer and Scaman, 2005). So, the OA binding to these tryptophyls would alter the folding of the protein, hindering the access of the substrate to the inner cleft with the active site.

In addition to studies on the inhibition of amylase and glucosidase activities, in recent years new data have been reported showing that OA can also repress the expression of these proteins in the small intestine (Gabás-Rivera *et al.*, 2013; Khathi *et al.*, 2013), in addition to the glucose transporters SGLT1 and GLUT2, involved in the absorption of the saccharide from the lumen of the small intestine to the bloodstream.

In summary, our results support the hypothesis that OA may facilitate a better control of postprandial hyperglycaemia and oxidative stress, so contributing to the preservation of insulin signalling. While its aptitude for inhibiting α -glucosidase is powerful, its ability for acting as scavenger of radical species is only moderate. This last aspect indicates that the efficient antioxidant activity exhibited by OA in cell cultures and experimental animals is likely to result from a more intricate and multi-factorial mechanism. In recent years, there have been increasing evidence to support the assertion that OA reinforces the cellular antioxidant response by modulating the expression of key genes, including antioxidant proteins and NADPH-producing enzymes, responsible for maintaining the GSH level and its transportation into mitochondria. In this nutri genomic effect of OA, the activation of the nuclear factor erythroid 2 p45-related factor 2 (Nrf2) seems to play a protagonist role (Wang *et al.*, 2010; Castellano *et al.*, 2013).

Obesity, insulin resistance and T2DM are considered the first pandemics of the 21st century. Therefore, National Health Systems are obliged to

introduce urgent measures which delay or avoid their progression. In this sense, OA might be used in future preventive and therapeutic strategies. Despite the limited number of studies in humans, it is accepted that OA is a safe phytochemical. None of these trials have recorded significant adverse effects (Xu, 1980; Minich *et al.*, 2007; Song *et al.*, 2006; Chen *et al.*, 2010; Rada *et al.*, 2015). Nevertheless, further investigations in humans will be necessary to extend the use of OA in the design of new drugs and functional foods, which allow for personalized diets and nutrigenomic approaches for the prevention of high-prevalence chronic disorders such as diabetes.

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