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Inhibition of α-glucosidase and α-amylase by Spanish extra virgin olive oils: The involvement of bioactive compounds other than oleuropein and hydroxytyrosol

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ABSTRACT

Despite the wide use of extra virgin olive oil (EVOO) to combat several diseases, the antidiabetic and anti-cholinesterase activity of Spanish EVOO have not been assessed. In order to evaluate which compounds are responsible for these activities of five Spanish EVOOs, in addition to flavonoids, we investigated for the first time the effect of the contents of carotenoids, fatty acids (FAs), and phytoprostanes (PhytoPs) on four enzymes: α-glucosidase, α-amylase, acetylcholinesterase, and butyrylcholinesterase.

The extracts of these five Spanish EVOOs were found to contain three flavones, three carotenoids, six FAs, and seven classes of PhytoPs. The samples exhibited no *in vitro* anti-cholinesterase activity but presented strong antidiabetic activity, in the order: 'Arbequina' ≈ 'Picual' ≈ 'Cuquillo' > 'Hojiblanca' > 'Cornicabra'.

The samples showed a higher *in vitro* hypoglycemic effect than individual or mixed standards, possibly due to interaction between multiple identified compounds and/or a very complex multivariate interaction between other factors.

1. Introduction

The countries along the Mediterranean coast are the main producers of olives. Spain is one of the most important countries with respect to olive cultivation, accounting for 54.6% of global olive oil production (IOOC, 2015; FAOSTAT, 2014; Petridis, Therios, & Samouris, 2012).'

Previous studies underlined that, besides chemical composition, the quality of virgin olive oils (VOOs) is a function of cultivar, season, and agronomic conditions (Beltrán et al., 2010; Del Carlo, Ritelli, Procida, Murmura, & Cichelli, 2006).

Extra virgin olive oil (EVOO) is obtained by means that avoid any kind of alteration in the oil, allowing it to exhibit the best organoleptic features that perfectly reproduce the fruit of origin (Collado-González et al., 2016).

EVOOs can be considered as "functional food" due to their health benefits - which include anticarcinogenic, antimicrobial, hypocholesterolemic, antihypertensive, and antiinflammatory effects (Abuznait, Qosa, Busnena, El Sayed, & Kaddoumi, 2013; Loizzo, Lecce, Boselli, Menichini, & Frega, 2011). Furthermore, VOOs and olive leaves have been used traditionally as a folk remedy, to combat fevers and diseases like diabetes mellitus and to reduce the risk of developing Alzheimer's disease, as well as to reduce symptom burden after diagnosis (Loizzo et al., 2011). All of these benefits have been attributed mainly to the presence of phenolic compounds, but may also be ascribed to the occurrence of other compounds, such as carotenoids and fatty acids (FAs) (Boskou, 2006). In addition, EVOO contains other minor con-

Abbreviations: ALA, α-linolenic acid; cv., cultivar; DOY, day of the year; EVOO, extra virgin olive oil; FAs, fatty acids; GC–MS, gas chromatography coupled to mass spectrometry; HPLC-DAD, high performance liquid chromatography with diode array detection; MRM, multiple reaction monitoring; OS, oxidative stress; PhytoPs, phytoprostanes; PUFAs, polyunsaturated fatty acids; SPE, solid phase extraction; UHPLC-QqQ-MS/MS, ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry; VOO, virgin olive oil.

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stituents, such as phytoprostanes (PhytoPs) - whose content increases under the influence of both biotic and abiotic factors (Mueller, 2004). In plants, PhytoPs can function not only as defense signals, but also as endogenous mediators capable of obviating cell damage (Barbosa et al., 2015). There is evidence that PhytoPs can modulate the functioning of the immune and vascular systems in humans (Durand et al., 2011; Gutermuth et al., 2007; Karg et al., 2007). However, no work about the protective effects of PhytoPs on diabetes mellitus or neurodegenerative diseases has been published to date.

Therefore, the aim of this work was to demonstrate the ability of EVOO to exert *in vitro* health benefits, in relation to its composition. To attain this goal, the inhibition of the two major carbohydrate digesting enzymes (α-amylase and α-glucosidase) and of the two major cholinesterases (acetylcholinesterase and butyrylcholinesterase) by extracts of EVOOs rich in PhytoPs was tested. Besides, the inhibitory activities of some pure bioactive compounds (flavones, carotenoids, FAs, and PhytoPs) found in the target extracts, and of different mixtures of standards of the most abundant of these bioactive compounds, were studied.

2. Materials and methods

2.1. Plant material and experimental conditions

The study was carried out in 2015, on a farm of the company "Olimendros" located in Jumilla (Murcia, Spain). The plant material consisted of olive trees of cultivars (cvs.) 'Arbequina', 'Cornicabra', 'Picual', 'Hojiblanca', and 'Cuquillo'.

The climate of the area is strictly Mediterranean. During the experimental period, the average daily maximum and minimum air temperatures were 34 and 21 °C, respectively, and the mean annual precipitation was 122.9 mm. All the olive trees were subjected to the same cultivation practices (pruning, fertilization, irrigation, and harvesting).

2.2. Oil extraction

The olives were harvested in 2015. The EVOO was extracted using an Abencor system and the oil obtained was separated by decantation. Samples for analysis were filtered and stored at -18 °C in darkness, in amber glass bottles without headspace, until analysis.

2.3. Chemicals and reagents

The PhytoPs studied (9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-D_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, *ent*-16-B_1-PhytoP, 16-B_1-PhytoP, *ent*-9-L_1-PhytoP, and 9-L_1-PhytoP) were synthesized according to our published procedures (El Fangour, Guy, Vidal, Rossi, & Durand, 2005; El Fangour et al., 2004). All extraction solvents of HPLC grade (*n*-hexane (purity ≥ 95%), methanol, and acetonitrile) and inorganic reagents (potassium sodium tartrate tetrahydrate, trisodium phosphate, sodium hydroxide, and sodium chloride) were purchased from Merck (Darmstadt, Germany). The LC-MS grade methanol was acquired from J.T. Baker (Phillipsburg, NJ, USA) and the SPE cartridges (Strata X-AW, 100 mg 3 mL^{-1}) from Phenomenex (Torrance, CA, USA). The Ellman's reagent ((5,5′-dithio bis-(2-nitrobenzoic acid) or DTNB), acetylcholinesterase (AChE) from electric eel (type VI-s, lyophilized powder), 4-nitrophenyl α-D-glucopyranoside (PNP-G), acetylcholine iodide (ATCI), butyrylcholinesterase (BuChE) from equine serum (lyophilized powder), *S*-butyrylthiocholine chloride (BTCC), galantamine, potassium phosphate monobasic, α-glucosidase (type I from baker's yeast), α-amylase (from porcine pancreas), 3,5-dinitrosalicylic acid, soluble starch, acarbose, Bis-Tris (bis (2-hydroxyethyl)amino-tris(hydroxymethyl)methane), and two carotenoids (lutein and zeaxanthin) were purchased from Sigma (St. Louis, MO, USA). Neoxanthin was purchased from CaroteNature (Lupsingen, Switzerland) and the flavonoids (apigenin, luteolin, and chrysoeriol) from Extrasynthèse (Genay, France). Authentic standards of FAs were obtained from Supelco (Bellefonte, PA, USA).

2.4. Sample extraction

Different classes of compounds present in the EVOO samples were separated by performing a dilution and a solid phase extraction (SPE), following the procedure described by Collado-González, Medina, et al. (2015). The protocol was as follows: the dilution of 1 mL of EVOO in 10 mL of *n*-hexane was re-dissolved in 2 mL of methanol and 2 mL of bis–tris buffer (0.02 M, HCl, pH 7). The resulting emulsion was subjected to SPE using a previously conditioned equilibrated Strata X-AW cartridge (100 mg 3 m L^{-1}). The cartridge was conditioned with 2 mL of several solvents: *n*-hexane, methanol and MilliQ water. In order to remove the undesirable compounds, after sample loading the cartridge was washed with 2 mL of hexane, MilliQ water, a mixture of methanol and MilliQ water (3:1, *v/v*), and acetonitrile. The target compounds were eluted with 1 mL of MeOH and dried under a nitrogen stream in order to obtain EVOO extracts as rich as possible in PhytoPs.

2.5. UHPLC-QqQ-MS/MS analyses of free phytoprostanes

The dried extract of each sample was re-dissolved in 200 μL of a mixture of solvents A/B (90:10, *v/v*), employing milliQ water/0.01% acetic acid as solvent A and methanol/0.01% acetic acid as solvent B. Reconstituted samples were sonicated for 10 min and passed through a 0.45-μm filter (Millipore, MA, USA); 20 μL of each were analyzed by UHPLC–QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany). PhytoP quantification was achieved using external calibration curves prepared with five different concentrations of each synthetic standard of these compounds.

The EVOO extracts were rather rich in PhytoPs. They also contained other bioactive compounds such as flavonoids, carotenoids, and FAs. Thus, this work has served to verify the occurrence in EVOOs of bioactive compounds other than hydroxytyrosol and oleuropein. The latter are plentiful in EVOOs and were removed by SPE using a polymer base sorbent (Strata X-AW cartridge), not a stationary phase such as C_{18} or diol (Boskou, 2006).

2.6. Phenolic compounds

The PhytoPs-rich EVOO extracts were analyzed on a C_{18} Spherisorb ODS2 column (250 x 4.6 mm, 5 μm particle size, Waters, Ireland) and two eluents were used: water (1% formic acid) as solvent A and methanol as solvent B. The injection volume of the redissolved hydromethanolic oil extract was 20 μL and elution was performed at a flow rate of 0.9 mL min^{-1}. The linear gradient started with 50% B, reaching 70% B at 35 min and 50% B at 37 min, and stopped at 40 min. Identification of the compounds was carried out in an analytical HPLC unit (Gilson) equipped with a Gilson diode array detector. All peaks from the different compounds were identified by comparing their elution order and UV–Vis spectra in the 200–400 nm range with pure standards. The quantification of phenolic compounds was achieved by matching their absorbance at 350 nm with external calibration curves produced using five different concentrations. Data acquisition and processing were performed using Unipoint System software (Gilson Medical Electronics, Villiers le Bel, France).