

Phenolic Extracts from Extra Virgin Olive Oils Inhibit Dipeptidyl Peptidase IV Activity: In Vitro, Cellular, and In Silico Molecular Modeling Investigations

Carmen Lammi ^{1,*}, Martina Bartolomei ¹, Carlotta Bollati ¹, Lorenzo Cecchi ², Maria Bellumori ², Emanuela Sabato ¹, Vistoli Giulio ¹, Nadia Mulinacci ² and Anna Arnoldi ¹

¹ Department of Pharmaceutical Sciences, University of Milan, 20133 Milan, Italy; martina.bartolomei@unimi.it (M.B.); carlotta.bollati@unimi.it (C.B.); emanuela.sabato@studenti.unimi.it (E.S.); giulio.vistoli@unimi.it (V.G.); anna.arnoldi@unimi.it (A.A.)

² Department of Neuroscience, Psychology, Drug and Child Health, Pharmaceutical and Nutraceutical Section, University of Florence, 50019 Florence, Italy; lo.cecchi@unifi.it (L.C.); maria.bellumori@unifi.it (M.B.); nadia.mulinacci@unifi.it (N.M.)

* Correspondence: carmen.lammi@unimi.it; Tel.: +39-025-031-9372

2. Material and Methods

2.1. Chemicals

Tris-HCl, ethylenediaminetetraacetic acid (EDTA) and NaCl were from Sigma-Aldrich (St. Louis, MO, USA) The DPP-IV assay kit was from (Cayman Chemicals (Michigan, USA), while Gly-Pro-amido-4-methylcoumarin hydrobromide (Gly-Pro-AMC) was from AnaSpec (Freemont, CA, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, phosphate buffered saline (PBS), penicillin/streptomycin, 24 and 96-well plates were from Euroclone (Milan, Italy), polycarbonate filters, 12 mm in diameter, 0.4 mm in pore diameter, were from Transwell Corning Inc. (Lowell, MA, USA). Tyrosol ($\geq 98\%$), hydroxytyrosol ($\geq 98\%$), and oleocanthal ($\geq 95\%$) were from Merck Life Science S.r.l. (Milano, Italy); oleuropein ($\geq 98\%$) was from Extrasynthese (Genay, France), whereas oleacin was from Phytolab (Vestenbergsgreuth, Germany).

2.3. In Vitro DPP-IV Activity Assay

The DPP-IV enzyme was provided by Cayman Chemicals (Michigan, USA), while the DPP-IV substrate (H-Gly-Pro-AMC) was from AnaSpec Inc. (Freemont, CA, USA). The experiments were carried out in triplicate in a half volume 96-well solid plate (white). Each reaction (50 μL) was prepared adding the reagents in a microcentrifuge tube in the following order: 1 X assay buffer (30 μL) [20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 1 mM EDTA], BUO and OMN EVOO extracts at final concentrations of 1, 10, 100, 500 and 1000 $\mu\text{g}/\text{mL}$ or vehicle (C) or standard oleuropein, hydroxytyrosol, tyrosol, oleacein and oleocanthal (1-1000 μM) (10 μL), and finally the DPP-IV enzyme (10 μL). Subsequently, the samples were mixed and 50 μL of each reaction was transferred in each plate well. Each reaction was started by adding 50 μL of substrate solution to each well and incubated at 37° for 30 minutes. Fluorescence signals deriving from the release of free AMC were measured using a Synergy H1 fluorescence plate reader from BioTek (excitation/emission wavelength 350/465 nm respectively).

2.4. Cell Culture

Caco-2 cells, obtained from INSERM (Paris, France), were routinely sub-cultured at 50% density and maintained at 37°C in a 90% air/10% CO₂ atmosphere in DMEM containing 25 mM of glucose, 3.7 g/L of NaHCO₃, 4 mM of stable L-glutamine, 1% non-essential amino acids, 100 U/L of penicillin and 100 µg/L of streptomycin (complete medium), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA).

2.5. *In Situ* DPP-IV Activity Assay

A total of 5 x 10⁴ Caco-2 cells/well were seeded in black 96-well plates with clear bottom. The second day after seeding, the spent medium was discarded and cells were treated with 100 µL/well of BUO and OMN extracts at the concentration of 10, 100, 500, 1000 µg/mL or vehicle (C) in growth medium for 24 h at 37°C. Afterwards, treatments were removed and Caco-2 cells were washed once with 100 µL of phosphate buffered saline (PBS) without Ca²⁺ and Mg²⁺, before the addition to each well of 100 µL of Gly-Pro-AMC substrate at the concentration of 50.0 µM in PBS without Ca²⁺ and Mg²⁺. Fluorescence signals deriving from the release of free AMC were measured using a Synergy H1 fluorescence microplate reader from BioTek (excitation/emission wavelength 350/465 nm respectively) every 1 min for up to 10 minutes.