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# MULTIPLE PROTECTIVE EFFECT OF PEPTIDES RELEASED FROM *OLEA EUROPAEA* AND *PRUNUS PERSICA* SEEDS AGAINST OXIDATIVE DAMAGE AND CANCER CELL PROLIFERATION

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ABSTRACT

The long exposition to reactive species results in oxidative stress which has been related with the development of cancer and other serious diseases. *Olea europaea* and *Prunus persica* seeds present a high protein content and preliminary results demonstrated their high potency to obtain bioactive peptides. The protective effect against oxidative damage exerted by peptides released from *Olea europaea* and *Prunus persica* seeds has been evaluated in this work. Seed hydrolysates showed protection against oxidation through four different mechanisms: inhibition of the formation of hydroxyl radicals, scavenging of free radicals, reduction of oxidizing compounds, and inhibition of lipid peroxidation. Moreover, seed hydrolysates also reduced the oxidative stress induced by an oxidizing agent on human cancer cells. Despite protection evaluated by individual mechanisms seemed to be significantly affected by the seed genotype, overall protection of seed hydrolysates was not so different. Seeds hydrolysates were not cytotoxic on normal cells but they demonstrated antiproliferative effect on human cancer cells (HeLa, PC-3, and HT-29). Peptides in all seed hydrolysates were sequenced by RP-HPLC-ESI-Q-TOF. Eighteen common peptides were observed among olive seed hydrolysates while a wider variability was observed among *Prunus* seed hydrolysates.

**Keywords:** peptides; seed; olive; prunus; oxidative damage; cell proliferation; mass spectrometry.

## 1. Introduction

Oxidative stress is derived from the exposition to high amounts of oxygen reactive species (ROS) overcoming endogenous antioxidant defense mechanisms (Roy, Galano, Durand, Le Guennec, & Lee, 2017). In addition to ROS generated in cells during mitochondrial respiration (superoxide anion and hydroxyl radicals, hydrogen peroxide, and organic peroxides), our organism is also exposed, although at a less extension, to other oxidizing molecules such as lipid peroxides and peroxides of proteins and nucleic acids. Moreover, other reactive species (RS) derived from nitrogen and sulfur also result in oxidation processes (Padilla, Mata-Pérez, Melguizo, & Barroso, 2017). Control of RS in living organisms depends on their mechanisms of generation and elimination. Different endogenous and exogenous antioxidants help control oxidation processes and guarantee a suitable balance in the generation-elimination of RS (Di Bernardini et al., 2011; Lushchak, 2014; Alpay et al., 2017). Nevertheless, under certain conditions (smoking, environmental pollutants, radiation, drugs, pesticides, industrial solvents, ozone, diseases, stress, etc.), an additional shot of antioxidants is required to prevent the damage of important biological molecules (DNA, proteins, and lipids) (Tatay, Espín, García-Fernández, & Ruiz, 2017) and the development of serious diseases (cancer, cardiovascular diseases, neurological disorders, diabetes, etc.) (Carocho & Ferreira, 2013; Da Silva, Cazarin, Batista, & Maróstica, 2014). In addition to well-known dietary antioxidants such as vitamins C and E, carotenoids, some minerals and polyphenols (Jian et al., 2017; Mirończuk-Chodakowska, Witkowska, & Zujko, 2018), peptides have also demonstrated antioxidant properties (García, Puchalska, Esteve, & Marina, 2013).

Antioxidant peptides have been observed in foods from animal and vegetable origins. Moreover, an increasing trend is the recovery of antioxidant peptides from food byproducts (García, Orellana, & Marina, 2016). Indeed, the extraction of valuable

substances (such as antioxidant peptides) from food byproducts contributes to the sustainability and protection of environmental and also reduces the cost of bioactive peptides (Toldrá, Mora, & Reig, 2016; Suwal, Ketnawa, Liceaga, & Huang, 2018). Our research team has focused part of its work in the exploitation of fruit and olive seeds on the basis of their high protein contents and high potential to obtain bioactive peptides. In a preliminary work, it has been demonstrated that olive (*Olea europeae*) and peach (*Prunus persica*) seeds could be cheap sources of peptides with antioxidant activity (Esteve, Marina, & García, 2015; Vásquez-Villanueva, Marina, & García, 2016). This conclusion was based on results obtained using just one fruit genotype and a limited number of studies.

This work proposes a deeper insight in peptides released from olive and *Prunus* seeds to demonstrate their protection against oxidative damage by studying different olive and peach seeds varieties and performing further studies that involve the investigation of different protection mechanisms, the evaluation of the protective effect using human cancer cells under oxidative stress, and the study of the antiproliferative effect on different cancer cell lines. Moreover, this work proposes the identification of peptides by reversed-phase high-performance liquid chromatography coupled to electrospray-ionization quadrupole-time-of-flight mass spectrometry (RP-HPLC-ESI-Q-TOF) and the study of the presence of peptides in relation with the protective effect of genotypes in which they were identified.

## 2. Materials and methods

### *2.1. Chemicals and samples*

All reagents were of analytical grade and water was obtained with a Milli-Q system from Millipore (Bedford, MA, USA). Hexane, acetone, trichloroacetic acid (TCA), ethanol (EtOH), methanol (MeOH), hydrochloric acid (HCl), acetonitrile (ACN), and acetic acid (AA) were obtained from Scharlau (Barcelona, Spain). Tris(hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), and phosphate buffer (PB) were from Merck (Darmstadt, Germany). DL-dithiothreitol (DTT), sodium tetraborate, β-mercaptoethanol, o-phthaldialdehyde (OPA), L-glutathione (GSH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium (ABTS), potassium persulphate, 1,10-phenantroline, ferrous sulfate, hydrogen peroxide ($H_2O_2$), potassium ferricyanide, iron (III) chloride ($FeCl_3$), linoleic acid, ammonium thiocyanate, iron (II) chloride ($FeCl_2$), trifluoroacetic acid (TFA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), trypsin, propidium iodide, antibiotics (penicillin, streptomycin, and amphotericin), fetal bovine serum, Dulbecco's Modified Eagle's Medium (DMEM), and Roswell Park Memorial Institute (RPMI) media were purchased in Sigma-Aldrich (Saint Louis, MO, USA). Tert-butylhydroperoxide (TBHP), N-acetylcysteine (NAC), and dichloro-dihydro-fluoresceindiacetate ($H_2$DCFDA) were acquired at Invitrogen (Barcelona, Spain). Alcalase 2.4 L FG was kindly donated by Novozymes Spain S.A. (Madrid, Spain). Stones from different *Prunus persica* varieties were donated by Instituto Murciano de Investigación para el Desarrollo Agrario (IMIDA, Murcia, Spain) (*Amarilla* nectarine and *Blanca* nectarine) and the Experimental Station Aula Dei (Zaragoza, Spain) (*Borracho de Jarque*, *Calanda San Miguel*, *Campiel*, *Lovell*, *Zaragozano Amarillo*, and *Zaragozano Rojo* peaches). Commercial nectarine and paraguayo were purchased in a local market

(Alcalá de Henares, Madrid, Spain). Stones from olive varieties (*Manzanilla*, *Gordal*, *Verdiel*, *Cornicabra*, and *Lechín*) were granted by the World Olive Germplasm Bank of IFAPA (Córdoba, Spain).

*2.2. Cell lines*

All cell lines (HK-2, HeLa, HT-29, and PC-3) were from the American Type Culture Collection ATCC (Rockwell, MD, USA). DMEM was the culture medium used with HK-2, HeLa, and HT-29 cell lines and RPMI was the culture medium in the case of the PC3 cell line. Media were supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin (250 ng/mL), and 10% fetal bovine serum and they were kept at 37 ºC in a humidified atmosphere with 5% $CO_2$ until use. Cells were kept at 37 ºC and 5% $CO_2$ in their culture medium.

*2.3. Protein extraction*

*Olea europaea* and *Prunus persica* were manually pitted and seeds were extracted with a manual press. Seeds were ground with a domestic mill (for 90 s) and stored at -20 ºC until use. Ground seeds (1 g) were defatted four times with 25 mL of hexane for 30 min with shaking and, then, they were dried to 40 ºC for 24 h. Thereafter, proteins were extracted using a procedure previously developed (Vásquez-Villanueva et al., 2016). Briefly, it consists of mixing 30 mg of defatted seeds with 5 mL of a 100 mM Tris-HCl buffer (pH 7.5) containing 0.5% (w/w) SDS and 0.5% (w/w) DTT using a high intensity focused ultrasounds (model VCX130, Sonics Vibra-Cell, Hartford, CT, USA) probe (5 min, 30% amplitude), followed by centrifugation (10 min, 4000 x g) to recover the supernatant where proteins were precipitated with cold acetone (15 mL, 4 ºC, 1 h). After

centrifugation (10 min, 4000 x g) and drying at room temperature, pellets were collected and stored as protein isolates.

*2.4. Preparation of seed hydrolysates*

Protein isolates from *Olea europaea* and *Prunus persica* seeds were hydrolyzed with Alcalase (0.15 UA/g protein). Protein isolate were dissolved in a borate buffer (5 mM, pH 8.5) at a concentration of 5 mg/mL using the HIFU probe (5 min, 30% amplitude). Afterwards, the enzyme was added and the solution was incubated in a Thermomixer Compact (Eppendorf AG, Hamburg, Germany) for 4 h at 50 ºC and 700 rpm. Thereafter, temperature was kept at 100 ºC for 10 min to stop the reaction. Finally, resulting solution was centrifuged (10 min, at 6000 rpm) and the supernatant, containing peptides, was collected and stored at -20 ºC.

*2.5. Determination of peptide content*

Peptide content was determined by the OPA assay (Wang et al., 2008). This assay was based on the reaction between α-amino groups of peptides and the OPA reagent. OPA mixture (5 mL) was prepared by mixing 1.39 mL of Milli-Q water, 1 mL of 5% (w/v) SDS, 2.5 mL of 100 mM sodium tetraborate, 10 µL β-mercaptoethanol, and 100 µL of 40 mg/mL OPA in MeOH. After standing for 8 min at room temperature, the absorbance of 2.5 µL of sample with 100 µL of OPA mixture was measured at 340 nm in a spectrophotometer UV/Vis Lambda 35 (Perkin-Elmer, Walthman, MA, USA). Peptide concentration was calculated by interpolation in a calibration curve prepared with tripeptide L-glutathion (GSH) (0 – 5 mg/mL).

*2.6. Measurement of protective character of peptides*

*2.6.1. Capacity to inhibit the formation of hydroxyl radicals*

It was performed by the method of Ajibola, Fashakin, Fagbemi, & Aluko (2011) with some modifications. Hydroxyl radicals were obtained from the oxidation of Fe (II) to Fe (III) by $H_2O_2$ through the Fenton reaction. Antioxidant peptides will inhibit this oxidation reaction and, thus, the formation of hydroxyl radicals. For that purpose, 50 µL of 3 mM 1,10-phenantroline in 0.1 M PB (pH 7.4) was mixed with 50 µL of 3 mM ferrous sulphate, 50 µL of seed hydrolysate, and 50 µL of 0.01% $H_2O_2$. The mixture was incubated for 1 h at 37 ºC and 700 rpm. Then, the absorbance corresponding to the complex Fe(II)-phenantroline was measured at 536 nm. The capacity to inhibit the formation of hydroxyl radicals was calculated with the following equation:

$$\text{Hydroxyl radical formation inhibition (\%)} = \left(\frac{Abs_{sample} - Abs_{blank}}{Abs_{control} - Abs_{blank}}\right) \times 100$$

where $Abs_{sample}$ is the absorbance of the sample (seed hydrolysate), $Abs_{blank}$ is the absorbance of the digestion buffer, and $Abs_{control}$ is the absorbance of a control solution prepared by adding water instead of $H_2O_2$. GSH (1 mg/mL) was used as positive control. Each sample was measured by triplicate.

*2.6.2. Capacity to scavange free radicals*

The evaluation of the capacity of seed hydrolysates to scavenge free radicals was carried out according to the method Wiriyaphan, Chitsomboon, & Yongsawadigul (2012), with some modifications, and using ABTS radicals. The ABTS radical was obtained by oxidation of ABTS with potassium persulfate resulting in a deep green solution. The $ABTS^{\bullet+}$ stock solution was prepared by mixing 7.4 mM ABTS with 2.6 mM potassium persulfate in 10 mM PB (pH 7.4), and keeping in the dark for, at least, 16 h at

room temperature. The $ABTS^{\bullet+}$ working solution was prepared daily by diluting the $ABTS^{\bullet+}$ stock solution in 10 mM PB (pH 7.4) to achieve an absorbance of 0.70 ± 0.01 at 734 nm. Then, 100 µL $ABTS^{\bullet+}$ of the working solution was added to 1 µL of seed hydrolysate and, after mixing for 6 min, the absorbance was measured at 734 nm. ABTS radical scavenging capacity was calculated by the following equation:

$$\text{ABTS radical scavenging capacity (\%)} = \left(\frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}}\right) \times 100$$

where $Abs_{blank}$ is the absorbance of 1 µL of 10 mM PB (pH 7.4) with 100 µL $ABTS^{\bullet+}$ working solution and $Abs_{sample}$ is the absorbance of 1 µL of sample (seed hydrolysate) with 100 µL $ABTS^{\bullet+}$ working solution. Each sample was measured by triplicate.

*2.6.3. Capacity to reduce oxidizing compounds*

In order to evaluate the reducing capacity of seed hydrolysates, the method developed by Ajibola et al. (2011), with some modifications, was employed. For that purpose, 12.5 µL of seed hydrolysate was mixed with 12.5 µL of 0.2 M PB (pH 6.6) and 25 µL of 1% (w/v) of potassium ferricyanide. The mixture was incubated in the Thermomixer for 20 min at 50 ºC with shaking (700 rpm). The reaction was stopped by adding 25 µL of 10% (w/v) of TCA. Finally, 50 µL of the above solution was mixed with 40 µL of water and 10 µL of 2.5% (w/v) $FeCl_3$ and, after standing for 3 min, the absorbance was measured at 700 nm. The reducing capacity was calculated according to the following equation:

$$\text{Reducing power} = \frac{Abs_{sample}}{peptide\ content}$$

where $Abs_{sample}$ is the measured absorbance and the peptide content is that obtained by the OPA assay. The percentage of inhibition was calculated setting as maximum

reduction power the signal obtained with 1 mg/mL GSH. Each sample was measured by triplicate.

*2.6.4. Capacity to inhibit the peroxidation of lipids*

This assay was carried out with the method described by Chen, Muramoto, Yamauchi, & Nokihara (1996) with some modifications. The hydrolysate (20 µL) was mixed with 20 µL of 1.3% (v/v) linoleic acid in EtOH and 10 µL of water and incubated for 144 h at 40 ºC in the dark. The degree of oxidation of the linoleic acid was evaluated by mixing 2.5 µL of that solution with 175 µL of 75% (v/v) EtOH, 2.5 µL of 30% (w/v) ammonium thiocyanate, and 2.5 µL of 20 mM $FeCl_2$ in 3.5% (v/v) HCl. The absorbance was measured at 500 nm after keeping the mixture for 3 min at room temperature. The capacity to inhibit lipid peroxidation was calculated using the following equation:

$$\text{Lipid peroxidation inhibition capacity (\%)} = \left(1 - \frac{Abs_{sample,144\ h} - Abs_{sample,0\ h}}{Abs_{blank,144\ h} - Abs_{blank,0\ h}}\right) \times 100$$

where $Abs_{sample,144h}$ and $Abs_{blank,144\ h}$ are the absorbances corresponding to the hydrolydate and the digestion buffer, respectively, after 144 h incubation; and $Abs_{sample,0\ h}$ and $Abs_{blank,0\ h}$ are the initial absorbances corresponding to the hydrolysate and digestion buffer, respectively. Each sample was measured by triplicate.

*2.6.5. Capacity to prevent the oxidative damage induced on cancer cells*

HeLa cells (human cervical cancer cells) were seeded in 6-well plates with 2 mL of DMEM medium (density of 300,000 cells/well). One well was used as control and no oxidizing reagent was added. Rest of wells were treated with TBHP (12 µL) for half an hour to generate oxidative stress. One well with TBHP was left as control while rest of

wells were treated with 50 μL of hydrolysate or the synthetic antioxidant, NAC. All wells were incubated for 24 h at 37 ºC (5% $CO_2$). Afterwards, the culture medium was discarded and cells were washed with PB and treated with trypsin to detach cells from well walls (Uzdensky, Kolpakova, Juzeniene, Juzenas, & Moan, 2005; Huang et al., 2013; Li et al., 2014; Kaur & Esau, 2015). Thereafter, each well was moved to a tube and centrifuged for 5 min at 1300 rpm. The pellet was dissolved in 600 μL of a solution prepared with 10 μL of $H_2$DCFDA (used as oxidative stress indicator in cells) and 8 mL PB. $H_2$DCFDA reacts with intracellular ROS produced by cells during oxidative stress and is converted to highly fluorescent 2',7'-dichlorofluorescein. Thus, after incubating this solution for 30 min at 37 ºC in darkness, it was centrifuged (5 min, 1300 rpm) and pellets were washed with PB. Propidium iodide (10 μL) was added to the precipitate to stain dead cells before flow cytometry analysis with a FaCSCalibur cytometer (Beckton Dickinson, San Agustín de Guadalix, Spain). Fluorescence due to ROS formation under oxidative stress was measured at a $\lambda_{excitation}$ of 488 nm and $\lambda_{emission}$ of 585 and 530 nm corresponding to propidium iodide and probe, respectively. Experiments were carried out by triplicate.

*2.6.6. Antiproliferative effect of peptides*

Three different cancer cell lines were employed: PC-3 cells (human prostate cancer cells), HeLa cells (human cervical cancer cells), and HT-29 cells (human colorectal adenocarcinoma cells). Moreover, a normal cell line (HK-2, human renal proximal tubule cells) was also used as control. The evaluation of the effect of peptides on cell proliferation was carried out by the MTT assay. Cells, after seeding for three days, were placed in 24-well plates (at a density of 10,000 cells/well) and incubated with 50 μL of hydrolysate for 24 h (37 ºC/5% $CO_2$). Afterwards, 50 μL of 5 mg/mL MTT stock solution in PB were added to each well and incubated for 3-5 h. After removing the culture

medium (DMEM or RMPI), the blue formazan crystals, formed by viable mitochondria, were dissolved with 500 μL DMSO. Absorbance of formazan crystals was measured at a wavelength of 570 nm (with a reference wavelength at 630 nm). Percentage of cells viability was calculated as follow:

$$cell\ viability\ (\%) = \frac{Abs_{Sample}}{Abs_{Control}} \times 100$$

where, $Abs_{Sample}$ and $Abs_{Control}$ are the absorbances of remaining blue formazan when cells were treated with the hydrolysates and digestion buffer, respectively. All experiments were carried out by triplicate.

*2.7. Sequencing of peptides by RP-HPLC-ESI-Q-TOF*

Peptides in *Olea europaea* and *Prunus persica* hydrolysates were sequenced using a 6530 series high sensitivity mass spectrometry Quadrupole-Time-of-Flight (Q-TOF) coupled to a Reverse-Phase High-Performance Liquid Chromatograph (RP-HPLC), model 1100, both from Agilent Technologies (Palo Alto, CA, USA). The separation was carried out in an Ascentis Express Peptide ES-C18 column (100 mm x 2.1 mm, 2.7 μm particle size) with a guard column (5 mm x 2.1 mm, 2.7 μm particle size), both from Supelco (Bellefonte, PA, USA). Chromatographic conditions were: mobile phase A, 0.3% (v/v) AA in water; mobile phase B, 0.3% (v/v) AA in ACN; injection volume, 15 μL; flow rate, 0.3 mL/min; column temperature, 25 ºC; elution gradient for the separation of olive peptides was: 5–28% B in 30 min, 28–35% B in 5 min, 35–60% B in 2 min, and 60–95% B in 6 min; elution gradient for the separation of peach peptides was: 5–39% B in 30 min, 39–45% B in 4 min, and 45–95% B in 8 min. In both cases, a reversed gradient

from 95 to 5% B in 2 min was set at the end of the separation to recover initial eluting conditions. UV signals were recorded at 210, 254, and 280 nm.

Mass spectrometry (MS) detection was performed in the positive ion mode and the mass range was from 100 to 1500 m/z. ESI conditions were: fragmentator voltage, 200 V; nozzle voltage, 0 V; nebulizer pressure, 50 psig; capillary voltage, 3500 V; gas temperature, 350 ºC; drying gas flow, 12 L/min; and skimmer voltage, 60 V. The Jet Stream sheath gas temperature was 350 ºC and the flow was 12 L/min. MS/MS was carried out using the *Auto* mode with the following conditions: 1 precursor per cycle, active exclusion after two spectra (released after 1 min), and collision energy of 4 V per 100 Da. Two Agilent compounds, HP0921 and purine, yielding ions at 922.0098 m/z and 121.0509 m/z, respectively, were simultaneously introduced and used as internal standards throughout the analysis.

MS/MS spectra were analyzed employing PEAKS Studio Version 7 software from Bioinformatics Solutions Inc. (Waterloo, Canada) and the sequence of peptides was obtained by the *de novo* tool. A peptide sequence was accepted if an average local confidence (ALC, expected percentage of correct amino acids in the peptide sequence) equal or higher to 90% was obtained. All samples were injected by duplicated. Since *de novo* tool cannot differentiate between I and L amino acids, only isoforms with L were showed in our results, but both isoforms are equally possible.

*2.8. Statistical analysis*

Statistical analysis was performed using Statgraphics Software Plus 5.1 (Statpoint Technologies, Inc., Warranton, VA, USA). Values were expressed as mean ± standard deviation of at least three independent experiments. The analysis of variance (ANOVA) was carried out using a significant level of 0.05.

## 3. Results and discussion

### *3.1. Evaluation of the protective effect of Olea europaea seeds hydrolysates against oxidative damage*

The protective properties of olive seeds hydrolsates were evaluated based on different mechanisms of protection against oxidative damage. The inhibition of the formation of hydroxyl radicals is a very important mechanism to protect cells. Hydroxyl radical is one of the most reactive species that produces the worst effects in mammalian cells. Most hydroxyl radicals are formed *in vivo* by the Fenton reaction from $H_2O_2$ in the presence of iron ions. Hydroxyl radicals damage DNA generating 8-hydroxyguanosine which is involved in the carcinogenesis progression. As an example, in breast carcinomas, the content in 8-hydroxyguanosine is increased 8- to 17-times (Reuter, Gupta, Chaturvedi, & Aggarwal, 2010). On the other hand, ROS-scavenging is the mechanism followed by endogenous enzymes (superoxide dismutases, glutathione peroxidase, peroxiredoxins, glutaredoxin, thioredoxin and catalase) to balance ROS generation (Trachootham, Alexandre, & Huang, 2009) and it was another mechanism studied in seed hydrolysates. Additionally, the reduction of oxidizing agents also protect target molecules against oxidative damage. Moreover, the oxidation of polyunsaturated fatty acids such as linoleic acid or arachidonic acid results in oxidation products (lipid hydroperoxides) that react at cell level with proteins, nucleic acids, and glutathion altering their normal functions. Lipid oxidation has been related with various pathologies such as carcinogenesis, cardiovascular, diseases, neurodegeneration, and aging (Nam, 2011) and, thus, inhibition of this oxidation mechanism is an important protective pathway against oxidation damage. In addition to these studies, the protective effect of seeds hydrolysates against oxidative stress induced in human cancer cells was also investigated.

Table 1 shows the capacity to inhibit hydroxyl radicals formation, to scavenge free radicals, to inhibit lipid peroxidation, and the reducing capacity along with the peptide concentration of seed hydrolysates obtained from five different olive varieties. The concentration of peptides in seed hydrolysates was very similar and ranged from 2.7 to 3.2 mg/mL. Despite this similar peptide contents, the protective effect of hydrolysates significantly varied depending on the studied mechanism. In general, main protective mechanism was the scavenging of free radicals followed by the inhibition of lipids oxidation. The capacity to inhibit the formation of hydroxyl radicals exerted by olive hydrolysates ranged from 14 to 52 % observing higher inhibition with seeds hydrolysates from the *Cornicabra* and *Verdiel* varieties and the lowest with the *Lechín* genotype. The capacity of hydrolysates to capture free radicals was higher than 75% for all olive seed varieties, observing the highest ability in the *Cornicabra* genotype. Reducing power ranged from 25 to 45 % (related to GSH) observing, again, for the *Cornicabra* seed hydrolysate the highest capacity and for the *Lechín*, the lowest. The inhibition of lipid peroxidation in olive seed hydrolysates ranged from 16%, corresponding to *Lechín*, to 89%, in the case of the *Manzanilla* genotype followed by *Cornicabra* with a 75% of inhibition. All these results suggested that *Cornicabra* olive genotype seemed to be the most advantageous seed to obtain peptides with protective effects while *Lechín* could be the least suitable.

These results were confirmed when HeLa cells, treated with seed hydrolysates, were submitted to oxidative stress, induced by the addition of TBHP, and intracellular ROS was measured by flow cytometry. Figure 1 shows fluorescence intensity obtained for the control (cells without hydrolysate and TBHP), for the cells under oxidizing conditions (TBHP), for the cells under oxidizing conditions treated with the synthetic antioxidant NAC, and for cells under oxidizing conditions treated with seeds hydrolysates

from *Cornicabra*, *Lechín*, and *Manzanilla* genotypes. Results demonstrated that the formation of ROS under an oxidizing environment clearly decreased when *Olea europaea* hydrolysates were added. Unlike previous results (see Table 1), there were no significant differences in the protection against oxidative stress exerted by the different olive seed hydrolysates ($p > 0.05$). This was probably due to the fact that the overall protective effect of seed hydrolysates was a result of the cooperation of the different mechanisms and it could not be attributed to any individual mechanisms (Erdmann, Cheung, & Schröder, 2008). In comparison with NAC (that enabled to decrease in a 95% the fluorescence intensity related to the control), *Cornicabra* and *Manzanilla* seed hydrolysates enabled to decrease the fluorescence intensity in a 68-96% while the olive variety *Lechín* did it in a 57%.

*3.2. Evaluation of the protective effect of Prunus persica seeds hydrolysates against oxidative damage*

Ten different *Prunus persica* varieties were studied and the same parameters assayed previously with *Olea europaea* seed hydrolysates were investigated. Table 1 groups the capacity to inhibit the formation of hydroxyl radicals, to scavenge free radicals, to inhibit the oxidation of lipids, and the reducing capacity of hydrolysates obtained from these peach varieties. Moreover, peptide content was also evaluated. Peptide contents in *Prunus persica* varieties presented a wider range than in olive seed hydrolysates. In fact, peptides contents were from 2.1-2.3 mg/mL, in paraguayo and nectarines, to 2.5-3.2 mg/mL, in peaches (*Campiel, Lovell, Borracho de Jarque, Zaragozano Rojo, Zaragozano Amarillo,* and *Calanda San Miguel*). This wider variability could be related with their genetic differences. In fact, despite all seeds were from *Prunus persica* fruits, nectarine has a recessive gene which determines the absence of fuzz on its skin, while paraguayo

is a peach mutation that results in a flat peach. Moreover, paraguayo, nectarine, and peaches differ in their flesh texture. While paraguayo and nectarine genotypes are characterized by a rapid flesh softening, peaches present a more limited softening (Haji, Yaegaki, & Yamaguchi, 2005; Wünsch, Carrera, & Hormaza, 2006; Font i Forcada, Gradziel, Gogorcena, & Moreno, 2014).

The capacity to inhibit the formation of hydroxyl radicals ranged from 9.5 to 111%, observing the highest inhibition with hydrolysates obtained from the *Lovell* and the *Calanda San Miguel* varieties. Moreover, *Borracho de Jarque* and *Campiel* showed the lowest capacity to inhibit the formation of hydroxyl radicals. The inhibition of the formation of hydroxyl radicals was, in general, much higher in *Prunus* seeds hydrolysates than in olive seed hydrolysates. Nevertheless, *Prunus persica* seeds hydrolysates showed a lower capacity to scavenge free radicals than olive seed hydrolysates. In fact, it ranged from 40%, for paraguayo, to 73%, for the commercial nectarine while all olive seed hydrolysates showed scavenging capacities higher than 75%. A low reducing capacity was observed for all peach seed hydrolysates (11-14%) while nectarines showed a higher capacity (32-42%). The percentage of lipid peroxidation inhibition was 0% in the case of *Campiel* followed by *Zaragozano Amarillo, Zaragozano Rojo*, and *Lovell*. The highest inhibition percentages were observed in the commercial nectarine, *Amarilla* nectarine, *Blanca* nectarine, and *Borracho de Jarque* peach (> 70%). From all these results, the commercial nectarine seemed to be the most profitable seed hydrolysate to obtain peptides with protective effect against oxidative damage while *Campiel* seeds were the least usable.

The protective effect of commercial nectarine, *Campiel*, and paraguayo seed hydrolysates was compared with that of a synthetic antioxidant (NAC). For that purpose, oxidative stress was induced in HeLa cells and resulting intracellular ROS was measured

(see Figure 1). Results showed that *Prunus persica* seed hydrolysates protected HeLa cells under TBHP-induced oxidative stress conditions by reducing the formation of ROS. Like in olive genotypes, significant differences observed among *Prunus* genotypes with individual mechanisms (results in Table 1) were not observed in this assay probably due to the cooperative effect of the different protection mechanisms (Erdmann et al., 2008).

3.3. *Evaluation of the protective effect of Olea europaea and Prunus persica seeds hydrolysates against cancer cell proliferation*

Cancer cell initiation and progression has been linked to oxidative stress (Reuter et al., 2010). Indeed, a maintained oxidative environment and, thus, the production of ROS for a long time can result in DNA mutations and damage and cell proliferation. In order to demonstrate that seeds hydrolysates could decrease the proliferation of cancer cells and exert protective affects, cell viability in different human cancer cell lines (HK-2, PC-3, HeLa, and HT-29) was determined using the MTT assay. MTT assay measures the metabolic activity of cells through oxidation-reduction reactions happening in mitochondria via succinate dehydrogenase system (García-Nebot, Cilla, Alegría, & Barberá, 2011). Reduction of MTT in the mitochondria results in blue insoluble formazan that is measured by spectroscopy. Figure 2 shows the viability of cells after treatment with hydrolysates obtained from different genotypes of *Olea europaea* and *Prunus persica* seeds related to a control (no peptides added, 100% viability). Cell viability in normal HK-2 cells was kept when they were treated with all *Olea europaea* and *Prunus persica* seeds hydrolysates which meant that they did not have cytotoxic effects on normal cells. However, the viability of carcinogenic cells (PC-3, HeLa, and HT-29) was suppressed to 45-87% upon treatment with hydrolysates, with the exception of HeLa cells treated with olive seed hydrolysates. Main antiproliferative effect was observed in HT-29

cells (human colorectal adenocarcinoma cells) when they were treated with *Campiel* and *Lovell* peach hydrolysates. The antiproliferative effect of *Olea europaea* and *Prunus persica* hydrolysates in cancer cells is in agreement with previous results on the protective effect of seed hydrolysates against oxidative damage.

### 3.4. *Identification of peptides by RP-HPLC-ESI-Q-TOF-MS/MS*

Peptides present in *Olea europaea* and *Prunus persica* hydrolysates were identified by RP-HPLC-ESI-Q-TOF-MS/MS. Due to a lack of databases with sequenced proteins from olive and *Prunus* seeds, peptide identification was carried out by *de novo* sequencing.

#### *3.4.1. Identification of peptides in Olea europaea hydrolysates*

The seeds hydrolysates obtained from the five olive varieties showed from 30 to 44 different peptides with 4-8 amino acids. These peptides had molecular weights (Mw) below to 1 kDa and one positive charge. Eighteen peptides were common within all olive genotypes. Additionally, there were other common peptides among some olive genotypes and other peptides that seemed to be specific to every genotype. Sequences of all common and specific peptides with ALC > 90% are grouped in Table 2. Additional peptides with ALC below to 90% were not listed in Table 2.

Some of these sequences were previously observed, according to BIOPEP database. Indeed, LVVD, KIPL, and NIFY peptides yielded antihypertensive bioactivities while NLLN and LLDA showed antibacterial properties (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008). All peptides contained a high amount of hydrophobic amino acids such as valine (V), leucine/isoleucine (L/I), proline (P), alanine (A), and methionine

(M) and aromatic amino acids such as phenylalanine (F), tyrosine (Y), tryptophan (W), and histidine (H). Presence of high amounts of hydrophobic and aromatic amino acids is a typical feature within antioxidant peptides (Erdmann et al., 2008; Esteve et al., 2015). Hydrophobic amino acid residues enable hydrogen-transfer and lipid peroxyl radical trapping and increase the accessibility to hydrophobic targets (Sarmadi & Ismail, 2010; Vásquez-Villanueva et al., 2016). Amino acids with aromatic residues allow the electron transfer and promote radical-scavenging properties in peptides. On the other hand, the imidazole-containing amino acids (histidine) show ability to hydrogen-transfer, lipid peroxyl radical trapping, and to metal ion-chelation. Histidine-containing peptides were observed in all olive genotypes (ALMSPH, SHTLVY, VVVVPH, ALMAPH, HTLY, WSMH, LMAPH, LVTPH, HTLVY).

A comparison of the signal intensity of the 18 common peptides in the five olive varieties showed that they were very similar among varieties with the exception of the *Manzanilla* genotype that showed slightly higher intensities. Peptides VVVVPH and FDGEVK had been previously observed by our research team in another olive genotype, *Arbequina* (Esteve et al., 2015). Figure 3(a) displays, as example, the total ion chromatogram (TIC) of an *Olea europaea* seed hydrolysate obtained with the *Cornicabra* variety and the mass spectra of peptides VVVVPH (common to all olive genotypes) and QQFL (only present in *Cornicabra* with ALC > 90%).

A further comparison of the TIC corresponding to the *Cornicabra* genotype, that showed a high protective effect (according to results showed in Table 1), with that from the *Lechín* genotype, with lower capacity, was performed. In addition to the 18 common peptides observed in all olive varieties, 13 additional peptides (with ALC > 90%) were common between *Cornicabra* and *Lechín* hydrolysates. Moreover, 7 peptides observed in *Cornicabra* were also observed in *Lechín* but with ALC percentages below to 90%

(LLLPQ, NLFY, TLLF, STLF, AEAF, AQVL, and NLPAE). Only peptides SFVVLK, QQFL, and LVTPH were present in *Cornicabra* and not in *Lechín*.

*3.4.2. Identification of peptides in Prunus persica hydrolysates*

*Prunus persica* seed hydrolysates showed from 8 to 36 peptides with 4-10 amino acids. Sequences of all common and specific peptides obtained by PEAKS software using the *de novo* tool are displayed in Table 3. Additional peptides to those grouped in Table 3 were observed in all *Prunus* genotypes with ALC below to 90%.

All peptides had a Mw lower than 1.3 kDa and presented one positive charge except NDNRNQLLR and QAREPDNRLQ which had two positive charges. Some of these sequences were found in the BIOPEP database. As examples, peptides TLAL and LPSY were described in BIOPEP for their antihypertensive activity and peptides LLDI, KGVL, VLPH, ANAL, and TQLI for their antibacterial activity (Minkiewicz et al., 2008). Most usual amino acids within peptide sequences were hydrophobic. Most different chromatographic profile was observed for the paraguayo seed hydrolysate while the rest of *Prunus* seed hydrolysates showed more similarities. Only two common peptides (HLPLL, NLPLL) were present in all *Prunus persica* genotypes. These peptides had been previously observed in other *Prunus* genus seeds (cherry and plum seeds) (González-García, Marina, & García, 2014; García, Endermann, González-García, & Marina, 2015). Three additional peptides (LTLQ, NLGNPE, and GLYSPH) were common to nine of the ten varieties (with the exception of paraguayo) being two of them (NLGNPE and GLYSPH) also present in cherry and plum seed hydrolysates (González-García et al., 2014; García et al., 2015). Despite these peptides were common among varieties, there were clear differences on their signal intensities. Indeed, peptide HLPLL

showed the highest intensity in all *Prunus persica* genotypes with the exception of *Lovell* peach and in paraguayo. Peptide NLPLL showed the highest signal in the *Lovell* peach and *Amarilla* nectarine but it yielded a specially low signal in the *Borracho de Jarque* peach and in paraguayo. A huge signal corresponding to peptide NLGNPE appeared in *Zaragozano Amarillo*, *Zaragozano Rojo*, *Borracho de Jarque*, and *Calanda San Miguel* peaches. Also, the peptide GLYSPH presented a high signal in *Lovell*, *Zaragozano Amarillo*, and *Zaragozano Rojo* peaches. Moreover, the signal corresponding to peptide LTLQ in *Lovell* peach was significant in comparison with other genotypes.

All nectarine genotypes (commercial nectarine, *Amarilla* nectarine, and *Blanca* nectarine) showed 7 additional common peptides while the two *Zaragozano* peaches presented 20 additional common peptides, some of them also observed in plum and cherry seeds (LYTPH, YLSF, KGVLY, LLAQ, DGDPLL, and VVNE) (González-García et al., 2014; García et al., 2015). Figure 3(b) shows, as example, the TIC obtained for the commercial nectarine with the mass spectra corresponding to peptides HLPLL (common to all *Prunus persica* genotypes) and ALNVN (only observed in commercial nectarine).

Comparison of peptides identified in the commercial nectarine (variety showing the highest protective effect according to results in Table 1) and *Campiel* (variety showing the lowest protective effect) enabled to discover that peptides ALNVN and HLPSYVN were present in the commercial nectarine but they were not observed in *Campiel* or in any other *Prunus persica* seed hydrolysates. Additionally, other peptides (QAREPDNRLQ, HAVLTQ, NDNRNQLLR, LYTPH, TFVLPH, LLAQAL, NVFSGF, and SLLDL) were found in the commercial nectarine and in other *Prunus* seed hydrolysates but they were not observed in the *Campiel* seed hydrolysate.

## Conclusions

Peptides released from *Olea europaea* and *Prunus persica* seeds exert protection against oxidative damage. Protective effect seemed to be mediated through multiple mechanisms that included the inhibition of the formation of hydroxyl radicals, the scavenging of free radicals, the reduction of oxidizing compounds, and the inhibition of lipid peroxidation. Significant differences within olive and *Prunus persica* genotypes were observed by these studies. Moreover, seed hydrolysates were also capable to reduce the oxidative stress induced in human cervical cancer cells although no significant differences were observed among genotypes. Probably the overall protection observed in seed hydrolysates was as a consequence of the cooperative effect of the different mechanisms and it was not related to any individual mechanism. Studies on cell viability demonstrated the no cytotoxic effect of hydrolysates in normal HK-2 cells and the antiproliferative effect in malignant cells from human prostate cancer, colorectal adenocarcinoma, and cervical cancer. Eighteen common peptides were observed in all olive seed hydrolysates while only two peptides were common among all *Prunus persica* seeds. Identified peptides contained high amounts of hydrophobic amino acids and imidazole-containing amino acids. *Olea europaea* and *Prunus persina* seeds are cheap sources of peptides with demonstrated and significant protection properties against oxidation that can be useful for the preparation of new functional foods and nutraceuticals.

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**Figure captions**

**Figure 1**. ROS production in HeLa cells under normal (control) and under oxidizing (addition of TBHP) conditions. Cells under oxidizing conditions were treated with the synthetic antioxidant NAC or with peptide hydrolysates obtained from different *Olea europaea* (*Cornicabra*, *Lechín*, and *Manzanilla*) and *Prunus persica* (nectarine, *Campiel*, and paraguayo) genotypes. Values are expressed as mean ± standard deviation of at least three independent experiments and $p < 0.05$ were significant. Significant differences, obtained by ANOVA, are indicated by a letter (a-d).

**Figure 2.** Viability of a normal cell line (HK-2 cells) and three different carcinogenic cell lines (PC-3 cells (human prostate cancer cells), HeLa cells (human cervical cancer cells), and HT-29 cells (human colorectal adenocarcinoma cells)) treated with different *Olea europaea* (a) and *Prunus persica* (b) seed hydrolysates. Values are expressed as mean ± standard deviation of at least three independent experiments and $p < 0.05$ were significant. Significant differences, obtained by ANOVA, are indicated by letters within every cell line.

**Figure 3**. TIC corresponding to an *Olea europaea* seed hydrolysate (*Cornicabra*) and mass spectra of peptides VVVVPH (common to all olive genotypes) and QQFL (only present in *Cornicabra* with ALC > 90%) (a) and TIC corresponding to a *Prunus persica* seed hydrolysate (commercial nectarine) and mass spectra of peptides HLPLL (common to all *Prunus persica* genotypes) and ALNVN (only present in nectarine with ALC > 90%) (b).

Table 1. Peptide content and capacity to prevent the oxidative damage through different mechanisms exerted by *Olea europaea* and *Prunus persica* seed protein hydrolysates.

| Seed hydrolysate | Peptide content (mg/mL) | Capacity to inhibit the formation of hydroxyl radicals (%) | Capacity to scavenge ABTS radicals (%) | Reducing capacity (%) | Capacity to inhibit the peroxidation of lipids (%) |
|---|---|---|---|---|---|
| ***Olea europaea genotypes*** | | | | | |
| **Manzanilla** | 2.7 ± 0.1 | 24.0 ± 1.5 | 78.2 ± 1.9 | 40.5 ± 0.4 | 89.2 ± 0.3 |
| **Gordal** | 3.2 ± 0.1 | 31.4 ± 2.7 | 82.4 ± 1.1 | 25.6 ± 0.5 | 54.5 ± 1.8 |
| **Verdiel** | 3.0 ± 0.2 | 48.7 ± 5.3 | 75.3 ± 1.7 | 31.2 ± 2.1 | 71.8 ± 3.3 |
| **Cornicabra** | 2.7 ± 0.2 | 52.0 ± 0.4 | 86.5 ± 0.9 | 44.6 ± 1.7 | 75.1 ± 0.3 |
| **Lechín** | 2.9 ± 0.1 | 14.0 ± 1.0 | 77.4 ± 2.9 | 24.8 ± 0.1 | 15.6 ± 2.7 |
| ***Prunus persica genotypes*** | | | | | |
| **Commercial paraguayo** | 2.1 ± 0.1 | 47.7 ± 1.2 | 40.1 ± 1.0 | 19.8 ± 0.1 | 49.8 ± 0.2 |
| **Commercial nectarine** | 2.1 ± 0.1 | 78.9 ± 0.8 | 72.6 ± 0.9 | 42.3 ± 4.6 | 80.6 ± 4.4 |
| **Amarilla nectarine** | 2.3 ± 0.1 | 70.0 ± 3.3 | 67.5 ± 5.2 | 32.5 ± 1.3 | 73.8 ± 0.1 |
| **Blanca nectarine** | 2.2 ± 0.1 | 51.9 ± 3.2 | 61.9 ± 5.9 | 31.6 ± 0.7 | 70.4 ± 0.2 |
| **Borracho de Jarque*** | 2.9 ± 0.1 | 9.5 ± 0.6 | 59.1 ± 0.4 | 12.4 ± 0.3 | 71.0 ± 2.2 |
| **Calanda San Miguel*** | 3.2 ± 0.1 | 104.3 ± 1.7 | 66.1 ± 0.8 | 10.9 ± 1.3 | 31.0 ± 1.0 |
| **Lovell*** | 2.6 ± 0.1 | 110.7 ± 0.4 | 63.1 ± 2.2 | 14.5 ± 0.1 | 14.0 ± 2.4 |
| **Campiel*** | 2.5 ± 0.1 | 12.7 ± 7.5 | 51.0 ± 1.6 | 13.4 ± 0.8 | 0.0 ± 3.4 |
| **Zaragozano Amarillo*** | 3.1 ± 0.1 | 13.1 ± 5.2 | 63.5 ± 1.9 | 11.4 ± 0.5 | 11.7 ± 3.3 |
| **Zaragozano Rojo*** | 3.0 ± 0.3 | 73.5 ± 1.4 | 62.6 ± 3.2 | 12.6 ± 0.8 | 12.9 ± 3.8 |

* These *Prunus persica* genotypes were peaches.

Table 2. Sequences of peptides observed in the *Olea europaea* genotypes[a].

| **Common *Olea europaea* genotypes** | |
|---|---|
| **Peptide sequences** | **Seed genotypes** |
| KLPLL, WSPLNN, TLPLL, ALMSPH, FVVLK, SSPLL, KLGNF, SHTLVY, VVVVPH, VVLK, ALMAPH, HTLY, VFDGE, FLPH, TLVY, WSMH, QGDLL, WNVN | All genotypes |
| FLLF, ADLY, KLPL | All genotypes except *Verdiel* |
| KVSSPL, LSPL | All genotypes except *Cornicabra* |
| SVNDL, SEGGVTE, LMAPH, LTYL | All genotypes except *Manzanilla* |
| MVPVPVN | *Manzanilla*, *Gordal*, and *Cornicabra* |
| RPLSPT, DLYNPR | *Manzanilla*, *Cornicabra*, and *Lechín* |
| LVTPH, SFVVLK, NLFY, AEAF | *Gordal*, *Verdiel*, and *Cornicabra* |
| NVDLE, TLPLLN, LVLF | *Gordal*, *Verdiel*, and *Lechín* |
| NDVFK, DVYNPR | *Gordal*, *Cornicabra*, and *Lechín* |
| LLPVLL | *Gordal* and *Verdiel* |
| NLPAE | *Gordal* and *Cornicabra* |
| LLDA | *Gordal* and *Lechín* |
| VDLE, SMPVDVL | *Verdiel*, *Cornicabra*, and *Lechín* |
| **Specific *Olea europaea* genotypes** | |
| **Peptide sequences** | **Seed genotypes** |
| FDGEVK, DFVVLK, HTLVY, TNLE | Manzanilla |
| YTSSPLL, ALPPGLT, DNVFK, LLQPV | Gordal |
| NLPAELV, NVPNLGQQ, VFGSQ | Verdiel |
| DASPLNN, LLLPQ, TLLF, STLF, AQVL, QQFL | Cornicabra |
| LLPVL, TLPLLL, VLAEAF, NLLN, LVVD | Lechín |

[a] All identified peptides showed ALC > 90%.

Table 3. Sequences of peptides observed in the *Prunus persica* genotypes[a].

| Common *Prunus persica* genotypes | | | |
|---|---|---|---|
| **Peptide sequences** | **Seed genotypes** | **Peptide sequences** | **Seed genotypes** |
| HLPLL, NLPLL | All genotypes | YVNAPQ | Commercial nectarine, *Blanca* nectarine, *Campiel*, and *Lovell* peach |
| LTLQ, GLYSPH, NLGNPE | All genotypes except the commercial paraguayo | YFAF | Commercial nectarine, *Blanca* nectarine, and *Zaragozano Rojo* peach |
| YLSF | All genotypes except *Lovell* peach | TFVLPH, HAVLTQ, NVFSGF, TLAL | Commercial nectarine and *Blanca* nectarine |
| LLDL | All peach and nectarine genotypes except the commercial nectarine | LLAQAL | Commercial nectarine and *Borracho de Jarque* peach |
| KGVL | All peach and nectarine genotypes except *Blanca* nectarine | NDNRNQLLR | Commercial nectarine and *Zaragozano Rojo* peach |
| ALYTPH, FLLF | All peach and nectarine genotypes except *Calanda San Miguel* peach | HLPSY | *Amarilla* nectarine, and peach genotypes except *Lovell* and *Zaragozano Rojo* peaches |
| EGAGGALE | All peach and nectarine genotypes except *Borracho de Jarque* peach and *Blanca* nectarine | ALPDE | *Amarilla* and *Blanca* nectarines, *Borracho de Jarque*, *Calanda San Miguel*, and *Campiel* peaches |
| KLLQPV | All peach genotypes except *Calanda San Miguel* peach, and *Amarilla* nectarine | WNVN | *Amarilla* and *Blanca* nectarines, and *Lovell* and *Zaragozano Amarillo* peaches |
| FVSPF | All peach genotypes except *Campiel* peach, and *Amarilla* nectarine | FNPQ | *Amarilla* nectarine, and Borracho de Jarque and *Calanda San Miguel* peaches |
| DGDPLL | All peach genotypes | NHLPLL | *Amarilla* nectarine, Borracho de Jarque, *Calanda San Miguel*, and *Zaragozano Rojo* peaches |
| LVLF | All *Zaragozano* and nectarine genotypes and *Borracho de Jarque* peach | LPLLR | *Amarilla* nectarine and *Calanda San Miguel* peach |
| LYTPH | All *Zaragozano* and nectarine genotypes, and *Borracho de Jarque* and *Lovell* peaches | APGALLY | *Blanca* nectarine and *Borracho de Jarque* peach |
| KGVLY | All *Zaragozano* and nectarine genotypes, and *Borracho de Jarque* and *Campiel* peaches | HLPLLQ | *Borracho de Jarque*, *Calanda San Miguel*, and *Zaragozano Amarillo* peaches |

| Common *Prunus persica* genotypes | | | |
|---|---|---|---|
| **Peptide sequences** | **Seed genotypes** | **Peptide sequences** | **Seed genotypes** |
| NAFLN | All *Zaragozano* and nectarine genotypes except the commercial nectarine | QLLR | *Borracho de Jarque*, *Calanda San Miguel*, *Campiel*, and *Zaragozano Amarillo* peaches |
| LVAV | All *Zaragozano* genotypes, *Amarilla* and *Blanca* nectarines, and *Borracho de Jarque* peach | GAGYGPQ | *Borracho de Jarque* and *Calanda San Miguel* peaches |
| LLAQ | All *Zaragozano* genotypes, *Amarilla* nectarine, and *Borracho de Jarque* peach | NLPLLQ | *Borracho de Jarque*, *Calanda San Miguel*, and *Lovell* peaches |
| VVNE | All *Zaragozano* genotypes, *Amarilla* nectarine, *Calanda San Miguel*, and *Lovell* peaches | VNAPQL | *Borracho de Jarque*, *Calanda San Miguel*, and *Zaragozano Amarillo* peaches |
| QAREPDNRLQ | All *Zaragozano* genotypes, commercial nectarine, and *Borracho de Jarque* peach | HAVLT | *Borracho de Jarque* and *Lovell* peaches |
| VSLL | All *Zaragozano* genotypes, *Blanca* nectarine, and *Campiel* peach | NLPLLL | *Calanda San Miguel* and *Zaragozano Amarillo* peaches |
| VLPH | All *Zaragozano* genotypes, and *Lovell* peach | AVLT | *Calanda San Miguel* and *Lovell* peaches |
| DHLPLL | All *Zaragozano* genotypes | HGVLQ | *Calanda San Miguel* and *Campiel* peaches |
| SLLDL | All nectarine genotypes | DGDPLLN | *Lovell* and *Zaragozano Rojo* peaches |

| Specific *Prunus persica* genotypes | |
|---|---|
| **Peptide sequence** | **Seed genotypes** |
| VLLDL, RAPSVS, VLVE, LVRVQ, SLPVPLDPA | Commercial paraguayo |
| HLPSYVN, ALNVN | Commercial nectarine |
| LPSYS, APGAL, AFGPE, ANAL | Amarilla nectarine |
| LDLS, LVAVNL, LLLPL | Borracho de Jarque |
| NLGDPS, LPLLW, LPLLQ, LPSY, GHPVAL, LPSLPK, ADLF, WAGGALE, LPLLF, NVNPE, TQLL | Calanda San Miguel |
| LVAVSLL, LVDGF, VELT, YQLS | Campiel |
| LSVVN, ALPDEV, HLPSYV, LPYPLGPN, SGFDT | Lovell |
| ETLAL | Zaragozano Amarillo |
| NTQLLAQ | Zaragozano Rojo |

[a] All identified peptides showed ALC > 90%.

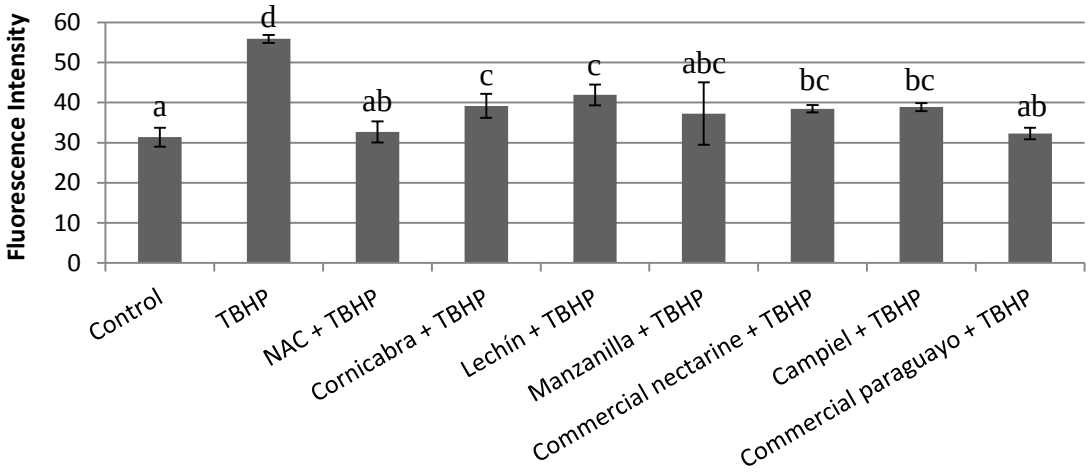



Fig.1

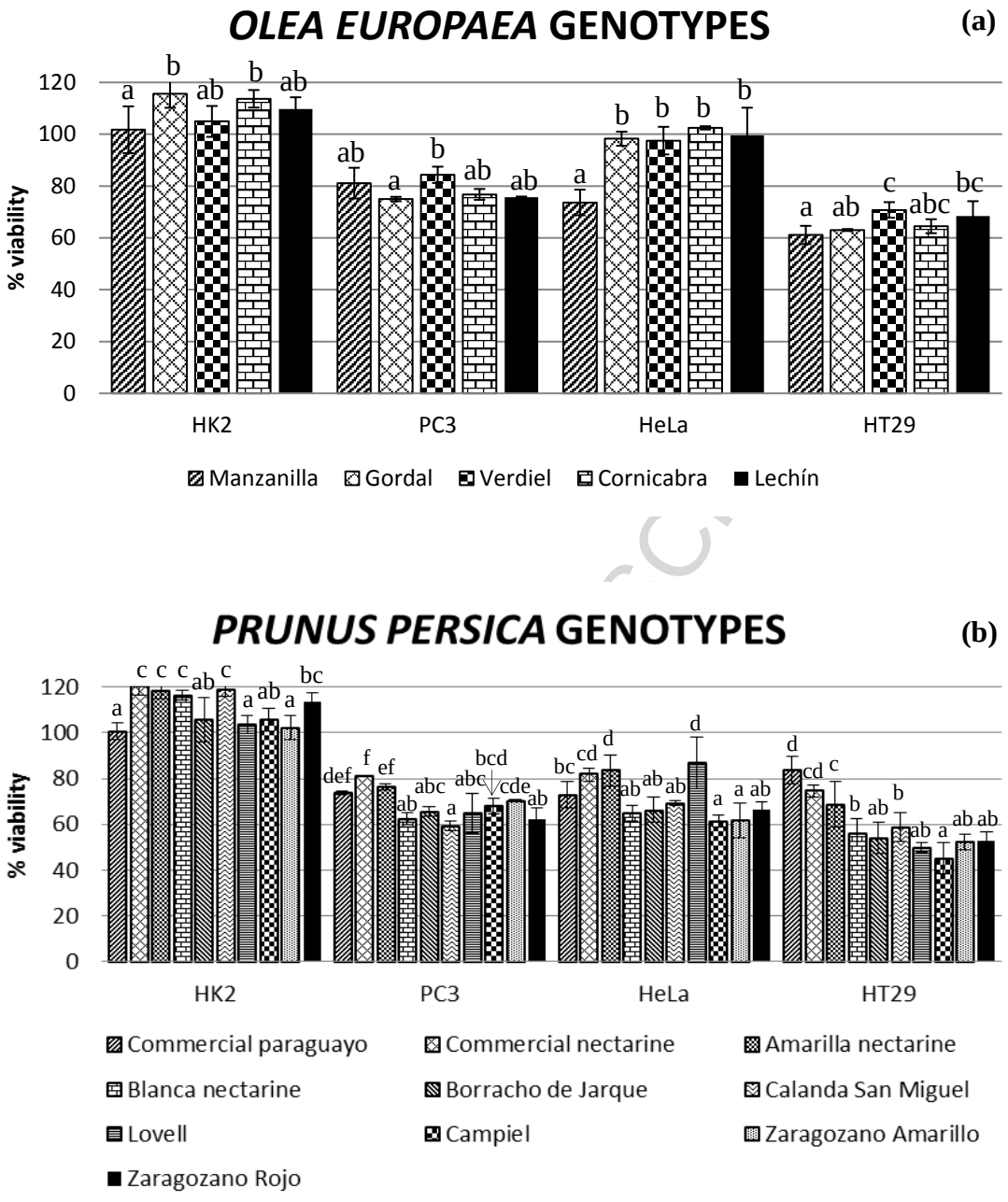



Fig.2

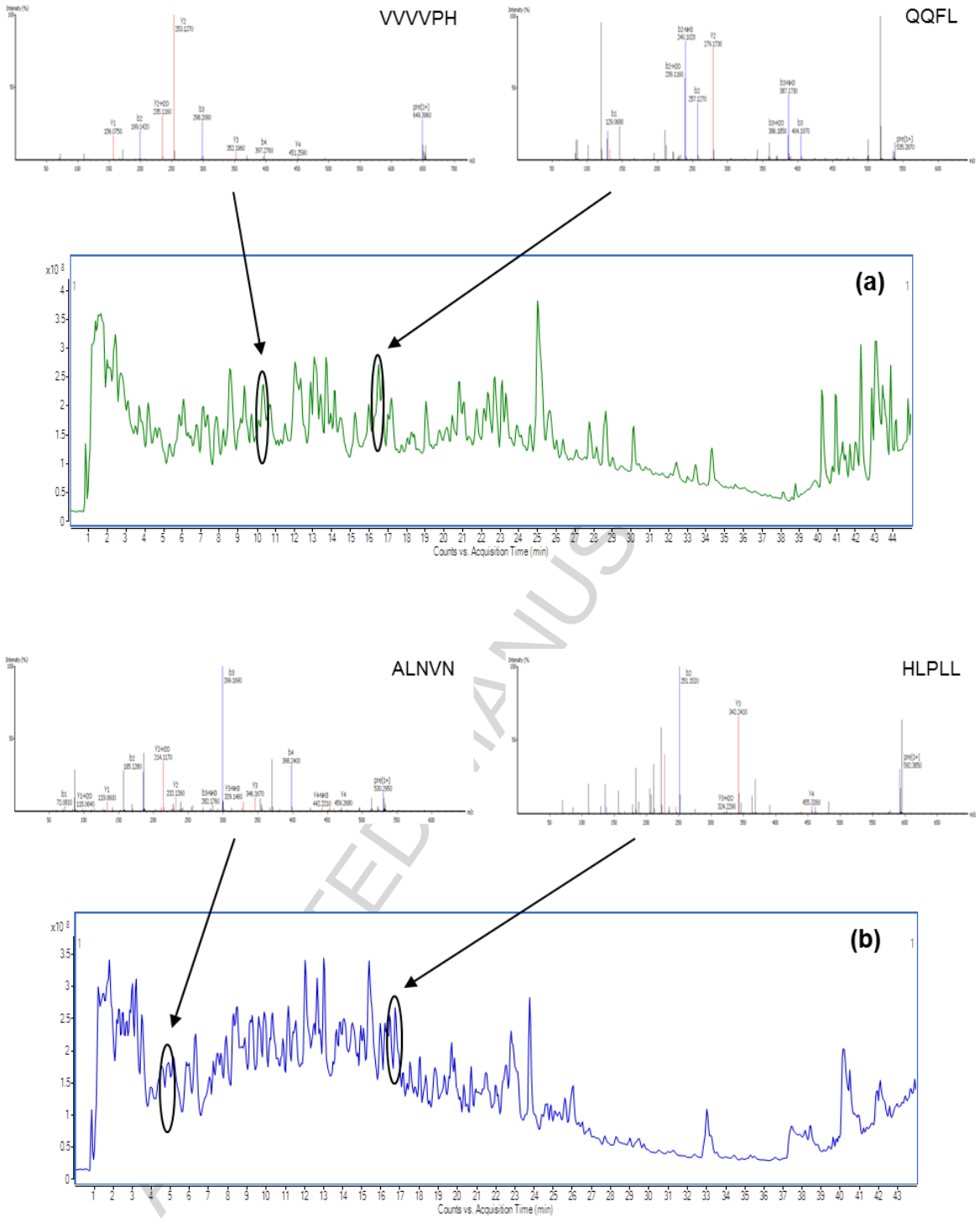
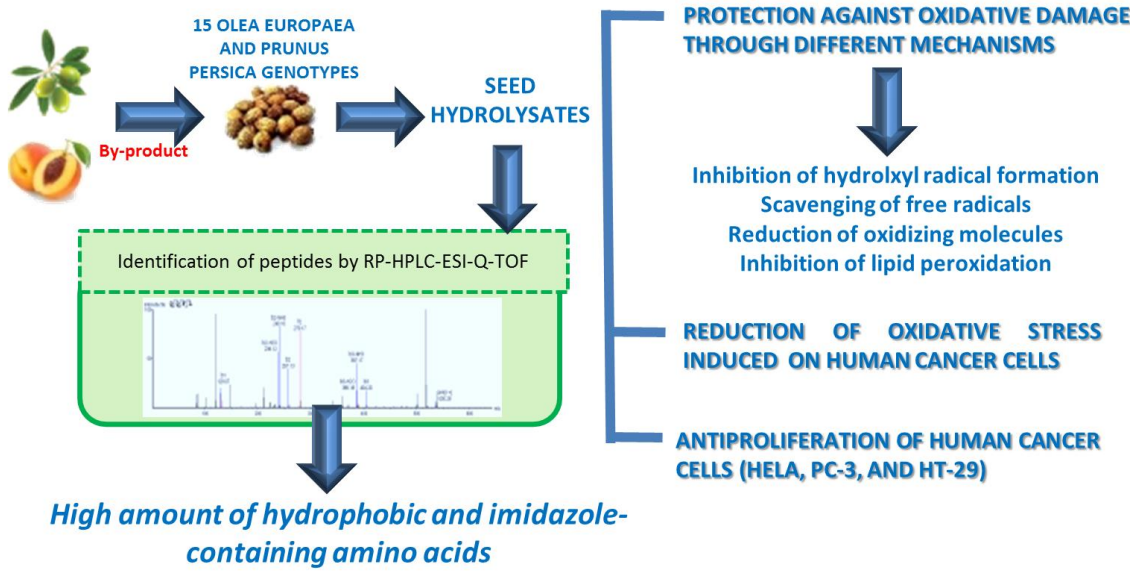



Fig. 3




Graphical abstract

***Highlights***

- Peptides in olive and *Prunus* seed hydrolysates exert protection against oxidation.

- Protective effect was mediated through multiple mechanisms.

- Seed hydrolysates reduced the oxidative stress induced in human cervical cancer cells.

- Seed hydrolysates demonstrated antiproliferation of different human cancer cells.

- Peptides contained high amounts of hydrophobic and imidazole-containing amino acids.