ANTITUMOR AND HYPOTENSIVE ACTIVITY OF PEPTIDES FROM OLIVE SEEDS

Romy Vásquez-Villanueva[1], Laura Muñoz-Moreno[2], M. José Carmena[2], M. Luisa Marina[1], M. Concepción García[1]*

[1] Departamento de Química Analítica, Química Física e Ingeniería Química, Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares (Madrid), Spain.

[2] Departamento de Biología de Sistemas, Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares (Madrid), Spain

*Corresponding author (e-mail: concepcion.garcia@uah.es, telephone +34-918854915; fax +34-918854971)

ABSTRACT

Peptides with molecular weights below 3 kDa from the hydrolysis of olive seed proteins with Thermolysin (OS-3kDa), have demonstrated a high antihypertensive capacity. This fraction has been further fractionated by semipreparative RP-HPLC to obtain 8 fractions. ACE inhibitor capacity of fractions was evaluated observing the highest capacity in fraction F5. Peptides in fraction F5 were identified by RP-HPLC- and HILIC-ESI-Q-ToF and cytotoxic effect was assessed in different cell lines. Peptide LLPSY, present in this faction, was synthesized and characterized. Despite antihypertensive capacity was not as high as in fraction F5, a significant anti-proliferative capacity on two different cancer cell lines was observed. Additional studies to assess antitumor activity confirmed that this peptide showed capability to increase the adhesion capacity of tumor cells, to decrease the migration capacity of cancer cells, and to arrest cell cycle on S phase.

Keywords: peptide, olive seeds, antitumor capacity, antihypertensive capacity.

1. INTRODUCTION

Olea europaea L. is an evergreen tree found throughout the world but mainly farmed in Mediterranean countries (Terral et al., 2004; Cavalheiro et al., 2015). Olive processing generates millions of tons of waste that constitute an environmental problem due to their high amount of organic and phytotoxic compounds (Bhatnagar et al., 2014). Different strategies have been developed for the revalorization of these olive by-products (Roselló-Soto et al., 2015). Olive stones have been used for fuel production, especially in thermal processes or even to obtain biosorbents for the removal of metal pollutants (Sánchez, & San Miguel, 2016; Moubarika, & Grimib, 2015). Additionally, other studies have demonstrated that olive stones contain high-added valuable compounds such as lipids, polyphenols, free sugars, carotenoids, vitamin E, and proteins (Roselló-Soto et al., 2015, Nunes, Pimentel, Costa, Alves, & Oliveira, 2016). Moreover, olive seeds have a high protein content and the hydrolysis of these proteins has enabled to obtain peptides with antioxidant, antihypertensive, and hypocholesterolemic properties (Esteve, Marina, & García, 2015).

Peptide bioactivity mainly depends on its amino acid composition, sequence, structure, and other factors such as hydrophobicity, charge or even the binding properties of peptides (Korhonen & Pihlanto, 2003, Beaulieu, Thibodeau, Bonnet, Bryl & Carboneau, 2013). Antihypertensive peptides mainly act by the inactivation of angiotensin converting enzyme (ACE), strongly influenced by the C-terminal amino acid sequence of peptides (Erdmann, Cheung, & Schröder, 2008). ACE is a metalloprotease, zinc dependent enzyme that catalyzes the hydrolysis of the decapeptide angiotensin I, by cleavage of its C-terminal peptide, to the octapeptide angiotensin II. This peptide is

responsible for the increase in blood pressure, and also, for the inactivation of antihypertensive vasodilator bradykinin (Erdmann et al., 2008, Bernstein et al., 2013).

A person is considered hypertensive when the high blood pressure is maintained for long time and, consequently, hypertension is a risk factor of cardiovascular diseases. Despite there are synthetic drugs for the treatment of hypertension, there is a growing interest for finding alternative treatments from natural sources preventing high blood pressure without exerting side effects (Erdmann et al., 2008, Puchalska, Marina, & García, 2015). Several research works have shown the potential of ACE inhibitor peptides from marine organisms (Pangestuti, & Kim, 2017; Wilson, Hayes, & Carney, 2011), animal sources (Mane, & Jamdar, 2017), plant sources, and by-products (Rizzello et al., 2016). Concretely, ACE-inhibitory peptides have been identified in the fraction containing peptides with molecular weights (MW) below 3 kDa from the olive seed proteins hydrolysate (Esteve et al., 2015).

On the other hand, antitumor peptides from natural sources have been extensively studied. It has been reported that anti-proliferative food peptides could prevent different stages of cancer, including initiation, promotion, and progression (Gonzalez de Mejia, & Dia, 2010). Among their mechanisms of action is the inhibition of cell migration, anti-angiogenesis, and anti-proliferation, which lead to apoptosis and cytotoxic effects on cancerous cell lines (Bhutia, & Maiti, 2008). Some anti-proliferative peptides from food proteins are lectins from plants, lunasin from soybean, different peptides from hazelnuts, amaranth, milk, and egg, and widely studied peptides from marine organisms (Hernández-Ledesma, & Hsieh, 2017).

The aim of this work has been to further study the fraction containing peptides with MW below 3 kDa from the olive seed hydrolysates (OS-3kDa) that exerted high

antihypertensive capacity. For that purpose, peptides in OS-3kDa will be further fractionated and fractions will be characterized by the evaluation of its ACE-inhibitory capacity, the identification of peptides (by HILIC- and RP-HPLC-ESI-Q-ToF and *de novo* sequencing), and the study of cytotoxicity. Most interesting peptides will be synthesized and their antihypertensive and antitumor capacities will be evaluated.

2. MATERIAL AND METHODS

2.1. Materials

All reagents were of analytical grade. Water was daily obtained with a Milli-Q system from Millipore (Bedford, MA, USA). Tris(hydroxymethyl) aminomethane (Tris), hydrochloric acid, sodium phosphate, and sodium dodecyl sulphate (SDS) were from Merck (Darmstadt, German). Acetic acid (AA), N-(2-hydroxyethyl)-1-piperazinyl-N'-(2-ethanesulfonic) acid (HEPES), tripeptide hippuryl-histidyl-leucine (HHL), trifluorocetic acid (TFA), angiotensin converting enzyme (ACE) from rabbit lung, Thermolysin, pancreatic and pepsin enzymes, dimethylsulphoxide (DMSO) concentrated type-I collagen solution, propidium iodure (PI), ribonuclease A from bovine pancreas (RNase A), Bovine serum albumin (BSA), phosphate-buffered saline (PBS), trypsin, 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyltetrazolium bromide (MTT), and DMEM (Dubelcco's Modified Eagle's medium) and RPMI-1640 (Roswell Park Memorial Institute) media were purchased in Sigma-Aldrich (Saint Louis, MO, USA). Acetonitrile HPLC- and MS-grade (ACN), acetone, and n-hexane were from Scharlau Chemie (Barcelona, Spain). Peptide LLPSY was synthesized by GenScript Corp. (Piscataway, NJ, USA) with a purity > 95 %. Raw olives of Picual variety were collected in Toledo (Spain).

2.2. Cell lines

Cell lines (human cervical cancer cells (HeLa), human renal proximal tubule epithelial cells (HK-2), androgen-independent prostate cancer cells (PC-3), and triple-negative breast cancer cells (MDA-MB-468)) were from the American Type Culture Collection ATCC (Rockwell, HD, USA).

2.3. Preparation of olive seed hydrolysates

Protein extraction was carried out following a method reported by Esteve et al. (2015). Briefly, olive seeds were manually pitted, ground, and defatted with n-hexane. Next, dried and defatted seeds were extracted with a 100 mM Tris-HCl buffer (pH 7.5) containing 0.5 % (w/v) SDS and DTT using a high intensity focused ultrasound probe (from Sonic Vibra Cell, CVX 130, Hartford, CT, USA) for 5 min at 30% amplitude. The mixture was centrifuged for 10 min at 4000 g and proteins were precipitated with cold acetone for 24 h at - 4 °C. Afterwards, acetone was removed and dried protein isolated was enzymatically digested in a phosphate buffer (pH 8.0) with Thermolysin at 0.5 g enzyme/ g protein ratio. The blend was incubated in a Thermomixer Compact (Eppendorf, Hamburg, Germany) keeping the temperature at 50 ºC. After 2 h digestion, the temperature was increased up to 100 °C for 10 min to stop the digestion. Hydrolysates were centrifuged at 4500g for 5 min and the supernatant was collected and fractionated by ultrafiltration using MW cut-off filters of 3 kDa from Merck Millipore (Tullagreen, Ireland).

2.4. Fractionation by semipreparative reversed-phase high performance liquid chromatography (RP-HPLC)

An HPLC system equipped with a quaternary pump (1100 Series, Agilent Technologies, Waldbron, Germany), an injection system, and a variable wavelength detector (both 1265 Series, Agilent Technologies) was employed. A Jupiter 4 µm Proteo 90 Å chromatographic column (250 mm x 10 mm) from Phenomenex (Torrance, CA, USA) was employed. Chromatographic conditions were: mobile phases, 0.1% (v/v) TFA in water (phase A) and in ACN (phase B); elution gradient, 15-20% B in 5 min, 20-25% B in 15 min, and 25-40% B in 15 min. Furthermore, the flow rate was 1 mL/min, the injection volume was 600 µL, and column temperature was set at 25 °C. UV detection

wavelengths were set at 254, 280 nm and, moreover fluorescence detection at excitation wavelength 280 nm and emission 360 nm. Eight fractions, collected every 5 min from minute 10, were obtained. Samples were injected 4 times and evaporated using a centrifugal evaporator (Concentrator Plus, Eppendorf, Hamburg, Germany). The remaining solid was dissolved in 2400 µL of digestion buffer. Fractions were kept at -20 °C until their use.

2.5. ACE-inhibitory capacity measurements

The ACE-inhibitory capacity was assayed following the method described by Vásquez-Villanueva et al. (Vásquez-Villanueva, Marina, & García, 2015). Shortly, 2.5 µL of sample was added to a solution containing 17.5 µL of 50 mM HEPES, 5 µL of 1.3 mg/mL HHL (freshly prepared in 50 mM HEPES (pH 8.3) and 300 mM NaCl), and 10 µL of an ACE solution (0.05 U/mL). The blend was incubated for 4 h at 37 °C and ACE was inactivated by the addition of 50 µL of cold ACN. During reaction, peptide HHL was converted into hippuric acid (HA) and they were separated by RP-HPLC using a Zorbax SB-C18 column (0.5 x 150 mm, 5 µm) from Agilent. Chromatographic conditions were: mobile phases, 0.05% (v/v) TFA in water (phase A) and in ACN (phase B); elution gradient, 5-95% B in 12 min; flow rate, 20 µL/min; injection volume, 10 µL and temperature, 25 °C. UV detection wavelength was set at 228 nm. Sample ACE-inhibition was depicted as the percentage of inhibition (Mosmann, 1983). The concentration required for the 50% inhibition of ACE activity (IC_{50}) was evaluated in those samples exceeding 50% of ACE-inhibition. For that purpose, the percentage of ACE-inhibition corresponding to, at least, six sample dilutions was plotted against the sample concentration and IC_{50} was obtained by interpolation at 50 % of ACE activity. Captopril was used as positive control.

2.6 Identification of ACE-inhibitory peptides by HILIC- and RP-HPLC-ESI-Q-ToF

Identification of peptides was carried out using an Agilent HPLC system equipped with a quaternary pump (1100 Series, Agilent Technologies, Waldbron, Germany), an injection system, and a variable wavelength detector (both 1265 Series, Agilent Technologies). This system was coupled to a Quadrupole-Time-of-Flight (Q-ToF) mass spectrometer (Series 6350), also from Agilent. Peptide separation was carried out by RP-HPLC and by hydrophilic interaction liquid chromatography (HILIC). An Ascentis Express Peptide ES-C18 column (100 mm x 2.1 mm I.D., 2.7 µm particle size) and an Ascentis Express HILIC column (100 mm x 2.1 mm I.D., 2.7 µm particle size), both, with their corresponding guard columns (5 mm x 2.1 mm, 2.7 µm particle size) from Supelco (Bellefonte, PA, USA), were employed. RP-HPLC chromatographic conditions were: mobile phases, 0.3 % (v/v) AA in water (phase A) and in ACN (phase B); elution gradient: 3% B for 3 min and 3-25 % in 30 min. Chromatographic conditions for the HILIC separation were: mobile phases, 65 mM ammonium acetate in ACN (phase A) and 65 mM ammonium acetate in water (phase B); elution gradient, 5-20% B in 20 min and 20-30% B in 15 min. Other common chromatographic conditions were: flow rate, 0.3 mL/min; column temperature, 25 °C; injection volume, 10 µL.

Mass spectrometry (MS) detection was carried out in the positive ion mode using a scanning range from 100 to 1500 m/z. Tandem MS was performed using Auto (MS/MS) mode and collision induced dissociation was set at 5 V per each 100 Da. *De novo* sequencing of peptides was carried out with PEAKS Studio 7 software from Bioinformatic Solutions Inc. (Waterloo, ON, Canada). Identified peptides showed an average local confidence (ALC, expected percentage of correct amino acids in the peptide sequence) equal or superior to 90% and a good precursor fragmentation pattern. Identifications were carried out using two independent samples injected by duplicate.

2.7. *Cytotoxicity assessment by MTT assay*

Cytotoxic effect of peptides in fraction F5 and of peptide LLPSY was estimated by cell metabolic activity measurements. For that purpose, HeLa, HK-2, PC-3, and MDA-MB-468 cells were employed and the colorimetric MTT assay reported by Mosmann et al. (1983) was followed. Shortly, cells were seeded in a 96-well plate to obtain a cell density of 1.0×10^4 viable cells/well. Culture medium was constituted by DEMEM or RPMI-1640 media containing penicillin-streptomycin and 10 % fetal bovine serum (FBS). They were kept at 37 °C in a humidified atmosphere with 5 % of CO_2 until use. Peptides were added at increasing concentrations to every cell culture. After 24 h incubation of cells with peptides, a 5 mg/mL MTT stock solution in PBS was added to each well in a 1:10 (cell volume: MTT stock solution volume) ratio and incubated for 4 h at 37 °C. Afterwards, MTT solution was carefully removed and formazan crystals, formed by viable mitochondria, were solubilized with 500 µL DMSO. The absorbance of formazan crystals was measured at a wavelength of 570 nm (with a reference wavelength at 630 nm) using automated microplate reader (Model ELX 800, Bio-tek instruments, INc., Winooski, VT, USA). Cytotoxicity was expressed as the percentage of viability of cells treated with peptides relative to that the corresponding controls. Cell viability was calculated as follow:

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

where, Abs_{Sample} and $Abs_{Control}$ is the absorbance of remaining blue formazan when cells are treated with sample (F5 fraction or peptide LLPSY) and digestion buffer, respectively.

2.8. Cell Adhesion assay

A concentrated type-I collagen solution was diluted in 10 mM glacial acetic acid and coated onto 96-well plates for 1 h at 37 °C. Plates were washed twice with PBS (pH 7.4) and cells were harvested with 0.25% trypsin/0.2% EDTA and collected by centrifugation. They were suspended in medium/0.1% (w/v) BSA (pH 7.4) and treated with 62.91 μg/mL of LLPSY peptide concentration (for PC-3 cells) and 97.56 μg/mL (for MDA-MB-468 cells) for 80 min. Control sample involved no addition of peptide. Then, cells were plated at 2.5×10^4 cells per 100 μL. The assay was finished by aspiration of wells. Cell adhesion was quantified by adding 1 mg/mL of MTT followed by 1 h incubation. DMSO (50 μL) was added to each well to dissolve formazan crystals and the absorbance at 570 nm, with a reference wavelength at 630 nm (Muñoz-Moreno et al., 2013).

2.9. Wound healing assay

MDA-MB-468 and PC-3 cells were incubated in 24-well plates and a small wound area was performed in the confluent monolayer with a scraper. Cells were incubated in the absence (control) or presence of peptide LLPSY at concentrations of 62.91 μg/mL (in the case of PC-3 cells) and 97.56 μg/mL (in the case of MDA-MB-468 cells). Three representative fields of each wound were photographed by a Nikon Diaphot 300 inverted microscopy at different times (0 and 24 h). Wound areas of samples were averaged and assigned a value of 100% at 0 h (Muñoz-Moreno et al., 2013).

2.10. Cell cycle analysis

PC-3 and MDA-MB-468 cells were grown in 6-well plates. After 24 h, the culture medium was removed and replaced with RPMI-1640 medium containing 10% FBS and 1% antibiotic/antimycotic (penicillin/ streptomycin/amphotericin B) for 16 h. After that,

cells were subjected to various treatments for 8 h. Then, cells were washed with PBS and detached with 0.25% trypsin/0.2% EDTA. After centrifugation at 500g for 5 min at 4 ºC, the pellets were mixed with ice-cold ethanol (70%) and kept at 20 ºC for 30 min. After ethanol removing by centrifugation, the pellets were washed with PBS and centrifuged again. The supernatants were discarded and the pellets suspended in PBS, 0.2 mg/mL RNase A and 20 mg/mL PI before flow cytometry analysis. Results obtained were analyzed with the Cyflogic v 1.2.1 program (Turku, Finland) (Muñoz-Moreno, Bajo, Prieto & Carmena, 2017).

2.11 Data analysis

Data were subjected to one-way ANOVA and differences were determined by Bonferroni's multiple comparison test with *GraphPad Prism software*. Each experiment was repeated at least three times. Data are the means of individual experiments and presented as mean ± SEM; $p < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

A previous work, reported by our research team, revealed that olive seeds and fruit seeds are sustainable and cheap sources of peptides with antihypertensive activity and that most active ones were always concentrated in the fraction, obtained by ultrafiltration, that contained peptides with MW below 3 kDa (Esteve et al., 2015, Vásquez-Villanueva et al., 2015; González-García, Marina, & García, 2015). In the present work, this fraction (OS-3kDa) has been further studied in order to obtain a deeper knowledge on peptides showing high bioactivity. For that purpose, OS-3kDa ultrafiltration fraction was next fractionated by semipreparative RP-HPLC and peptides in most active fractions were characterized.

3.1. ACE-inhibitory capacity of fractions obtained from OS-3kDa by semipreparative RP-HPLC

Figure 1 depicts the chromatogram yielded by the OS-3kDa and the fractions collected. Moreover, Figure 1 also shows the percentage of ACE inhibition reached by the 8 collected fractions. Fractions F1, F3, F4, and F5 showed the highest percentage of ACE-inhibition (65-74%) while fractions F2 and F8 were the less active. Additionally, the IC_{50} was measured for those fractions exceeding 50% of ACE-inhibition (F1, F3, F4 and F5) and results are shown in Figure 1. Fraction F5 showed the lowest IC_{50} value (3.6 ± 0.9 µg/mL). This value was even lower than the obtained for the whole OS-3kDa (29.6 ± 0.5 µg/mL), which means that this fraction was, at least, eight times more active than OS-3kDa. This is a high antihypertensive activity in comparison with purified peptides from red algae, where the most active fractions reached an IC_{50} of 57.2 ± 5.0 µg/mL (Wu et al., 2017) or even fractions derived from caprine kefir with IC_{50} values higher than 21 µg/mL (Quirós, Hernández-Ledesma, Ramos, Amigo, & Recio, 2005). This result encouraged to think that highly antihypertensive peptides similar to those

observed in soybean (VLIVP (IC_{50} = 1.69 μM)) (Puchalska, García & Marina, 2014), corn (LQP (IC_{50} = 2.0 μM), LSP (IC_{50} = 1.7 μM), and LRP (IC_{50} = 0.29 μM), and milk (VPP (IC_{50} = 9.13 μM) and IPP (IC_{50} = 5.15 μM)) (Puchalska, Marina & García, 2012) could be obtained from the F5 olive seed fraction. All IC_{50} values were quite higher than the ACE-inhibitory drug exerted by Captopril®, which was used as positive control in our experiments. Indeed, Captopril reached 100% of ACE-inhibition and showed an IC_{50} = 5.0 ng/mL. In order to identify peptides with high bioactivity, F3, F4 and F5 fractions were next analyzed by HPLC-MS/MS.

3.2. *Identification of peptides in fraction F5 by RP-HPLC- and HILIC-Q-ToF-MS*

Amino acid sequencing of peptides in fractions F3, F4, and F5, that exerted the lowest IC_{50}, was carried out by RP-HPLC-ESI-Q-ToF. Table 1 summarizes the peptides identified by MS/MS for these 3 fractions. Antihypertensive peptides are usually short size, present a significant amount of hydrophobic amino acids (A, F, I, L, M, P, W and V), and also they contain aromatic amino acids (F, H, Y) in the first three C-terminal positions (Amado, Vázquez, González, Esteban-Fernández, Carrera, & Pineiro, 2014; Ghassem, Babji, Said, Mohmoodani, & Aeihara, 2014; Balti, Bougatef, Sila, Guillochon, Dhulster, & Nedjar-Arroume, 2015). Sixteen and fifteen peptides could be identified in F3 and F4 fractions, respectively. All of them showed less than 10 amino acids and MW below 1 kDa. The contribution of hydrophobic and aromatic amino acids was 54%, in the fraction F3, and 57%, in the fraction F4. A further analysis of peptides was carried out in fraction F5 due to its higher antihypertensive activity and the lower number of identified peptides. In addition to RP-HPLC, fraction F5 was also separated by hydrophilic interaction liquid chromatography (HILIC) that enabled a better separation of most polar peptides. Identified peptides showed typical features of

antihypertensive peptides, presenting 4-6 amino acids and MW below 1 kDa. When separation was carried out by RP-HPLC, 7 peptides were identified and the contribution of hydrophobic and aromatic amino acids residues was around 84%, much higher than in the case of F3 and F4 fractions. When using HILIC, 4 peptides were identified and, unlike those peptides observed by RP-HPLC, a lower amount of hydrophobic and aromatic amino acids (around 76%) was observed within sequences. The hydrophobic and aromatic amino acid content in F5 was higher than the content in F3 and F4, that could explain its high antihypertensive activity. Additionally, MS/MS fragmentation of peptide LLPSY, identified by both chromatographic modes is shown in Figure 2. This peptide was also observed in the most antihypertensive fraction obtained from an Arbequina variety olive seed by Esteve et al. (2015).

3.3. Cytotoxicity of peptides in fraction F5

Cytotoxicity of peptides in fraction F5 was evaluated by the MTT assay using two different cell lines, one healthy line (HK-2 cells) and one cancer line (HeLa cells). Fraction F5 did not show cytotoxic effects in any cell line, exerting cell viability values higher than 80%, even at higher concentrations than the IC_{50} (data not shown).

3.4. Characterization of the synthesized peptide LLPSY

Within peptides identified in fraction F5, peptide LLPSY was selected for its synthesis and study since it was the only one that had been previously identified as antihypertensive peptide in the olive seed (Esteve et al., 2015). The percentage of ACE-inhibition of peptide LLPSY was 95.2% and the IC_{50} was 23.6 ± 0.3 μg/mL (39.9 ± 0.5 μM). Surprisingly, this value was higher than the calculated previously for the whole fraction F5 (3.6 ± 0.9 μg/mL). Furthermore, the resistance of LLPSY to gastrointestinal enzymes was also evaluated. Results showed a decrease in the percentage of ACE-

inhibition to 11.6%. Similar behavior was observed in some synthesized peptides from caprine kefir peptides. In both cases, this behavior could be justified by the partial or total hydrolysis of the peptide, due to gastrointestinal enzymes, and to the lack of activity of resulting peptides or amino acids (Quirós et al., 2005).

In parallel, cytotoxicity of peptide LLPSY was evaluated using several cell lines, one healthy line (HK-2 cells) and three cancer lines (HeLa, PC-3, and MDA-MB-468). Unlike the whole F5 fraction, LLPSY caused a different cytotoxicity in the cell lines (see Figure 3). It did not show cytotoxic effects at any concentration in healthy HK-2 cells and in cancer HeLa cells. However, LLPSY peptide treatment showed a strong anti-proliferative effect dose-dependent manner in PC-3 and MDA-MB-468 cancer cells after 24 h (Figure 3C and 3D). Indeed, both cancer cell lines showed IC_{50} values lower than 100 μg/mL (IC_{50} = 86.1 ± 2.6 μg/mL for the prostate cancer PC-3 cells and 97.6 ± 1.9 μg/mL for the breast cancer MDA-MB-468 cells). In the same direction, the antiproliferative effects of lunasin peptide and common bean have been defined in colon and breast cancer cells (Dia, & Gonzalez de Mejia, 2011; Chan, Zhang, & Ng, 2013, Wu, Zhang, Wang, & Ng, 2011). Taking into account these results, and that LLPSY presents common characteristics with other antitumor peptides (Hernández-Ledesma et al., 2017, Kim et al., 2013; Chan et al., 2013; Wu et al., 2011), further studies to assess anti-carcinogenic activity of this peptide were next carried out.

3.5. Effect of peptide LLPSY on several tumoral processes

Additional studies to evaluate the capability of peptides to modify the adhesion and migration processes of cancer cells in presence of peptide LLPSY and its capacity to arrest cell cycle were performed.

Cell adhesion is an essential aspect of cell survival for metastatic tumor cells. When cell adhesion is disrupted, cancer cells are able to migrate to other healthy tissues and could lead to metastatic processes (Fernández-Martínez, Bajo, Sánchez-Chapado, Prieto, & Carmena, 2009). Cell adhesion assay was carried out by treating PC-3 and MDA-MB-468 cells with LLPSY peptide at concentrations corresponding to the IC_{50} (86.1 μg/mL for PC-3 and 97.6 μg/mL for MDA-MB-468). Figure 4A shows that the treatment with LLPSY resulted in increasing cellular adhesion in both cell lines, observing a higher effect on breast cancer cells (37% increase) than in prostatic cancer cells (21% increase).

Furthermore, in order to evaluate whether LLPSY could modify the capability of cancer cells to migrate to other tissues, the wound-healing assay was employed. For that purpose, after performing the wound on the cells monolayer, it was treated with LLPSY peptide at concentrations corresponding to the IC_{50} (86.1 μg/mL for PC-3 and 97.6 μg/mL for MDA-MB-468). Figure 4B shows that the presence of peptide significantly retarded the close of the wound as compared to untreated cancer cells (controls) after 24 h of treatment. Lowering migration capability of cells in presence of peptide was more significant on breast cancer cells (63%) than on prostate cancer cells (49%). In this regard, other peptides derived from food, like those present in soybean, have demonstrated inhibitory effect on cancer cell migration (Lima, Oliveira, Saúde, Mota, & Ferreira, 2017).

The cell cycle consists of several well controlled phases or stages. Briefly, in G_1 phase, the cell grows in size and synthesizes all it needs to duplicate DNA in S phase. In G_2 phase, cell growths rapidly and prepares for mitosis in M phase. In M phase, chromatids are separated. After daughter cells are separated in cytokinesis, they can enter again to G_1 phase or can arrive to a resting phase (G_0 phase), which could result in an apoptosis

process. In order to find out the reasons involved on the anti-proliferative effects in presence of peptide LLPSY on PC-3 and MDA-MB-468 cells, the cell cycle analysis was carried out. Figure 5 shows that the treatment of cells with peptide resulted in an increase of the number of cells in sub G_0 phase and a decrease of the number of cells in G_2/M phase. These results could indicate that peptide LLPSY arrests cell cycle on S phase. Similar results were observed for other peptides such as lunasin from soybean that showed capacity to arrest cell cycle in S phase on breast cancer cells (MDA-MB-231) (Hsieh, Hernández-Ledesma, & de Lumen, 2010) and at G_2/M phase on metastatic colon cell line KM12L4 (Dia et al., 2011) or peony polysaccharides that arrested cervix cancer cell cycle at S phase (Zhang, Shi, Thakhur, Hu, Zhang, & Wei, 2017).

Take all together, the results suggest that LLPSY peptide shows antiproliferative and antimetastatic effects on prostate and breast cancer cells. However, further studies are needed to confirm the antitumor capacity of LLPSY from olive seeds.

4. CONCLUSIONS

Fraction containing peptides with MW less than 3 kDa from an olive seed hydrolysate has demonstrated high antihypertensive capacity (IC_{50} = 29.6 ± 0.5 µg/mL). A further fractionation by semipreparative RP-HPLC enabled to concentrate highly antihypertensive peptides in a subfraction exerting an IC_{50} = 3.6 ± 0.9 µg/mL. The analysis of this fraction by LC-MS/MS required the use of reverse phase and hydrophilic liquid interaction chromatography for the comprehensive identification of peptides. Ten peptides with common characteristic with antihypertensive peptides were identified. This fraction did not show cytotoxic effects on HeLa and HK-2 cell lines. Nevertheless, synthetic peptide LLPSY, identified in previous fraction, showed a huge anti-proliferative effect on aggressive stages of prostate cancer cells (PC-3) and breast cancer cells (MDA-MB-468). Further studies to asses this effect by the evaluation of the capacity of LLPSY peptide to modify cell adhesion, migration, and cell cycle processes enabled to confirm the potential antitumor character of this peptide from the olive seed.

5. REFERENCES

Amado, I. R., Vázquez, J. A., González, P., Esteban-Fernández, D., Carrera, M., & Pineiro, C. (2014). Identification of the major ACE-inhibitory peptides produced by enzymatic hydrolysis of a protein concentrate from cuttlefish wastewater. *Marine Drugs*, *12*, 1390–1405.

Balti, R., Bougatef, A., Sila, A., Guillochon, D., Dhulster, P., & Nedjar-Arroume, N. (2015). Nine novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (*Sepia officinalis*) muscle protein hydrolysates and antihypertensive effect in spontaneously hypertensive rats. *Food Chemistry, 170*, 519-525.

Beaulieu, L., Thibodeau, J., Bonnet C., Bryl, P., & Carbonneau, M. (2013). Evidence of anti-proliferative activities in blue mussel (*Mytildus Edulis*) by-products. *Marine Drugs, 11,* 975-990.

Bernstein, K. E., Ong, F. S., Blackwell, W. B., Shah, K. H., Giani, J. F., González-Villalobos, R. A., Shen, X. Z., & Fuchs, S. (2013). A Modern Understanding of the traditional and nontraditional biological functions of angiotensin-converting enzyme. *Pharmacological Reviews, 65,* 1-46.

Bhatnagar, A., Kaczala, F., Hogland, W., Marques, M., Paraskeva, C., Papadakis, V., & Sillanpää, M. (2014) Valorization of solid waste product from olive oil industry as potential adsorbents for water pollution control: A review. *Environmental Science and Pollution Research, 21,* 268-298.

Bhutia, S., & Maiti, T. K. (2008). Targeting tumors with peptides from natural sources. *Trends in Biotechnology, 26,* 210-217.

Cavalheiro, C. V., Picoloto, R. S., Cichoski, A. J., Wagner, R., de Menezes, C. R., Zepkra L. Q., & Barin S. J. (2015). Olive leaves offer more than phenolic compounds-

Fatty Acids and mineral composition of varieties from southern Brazil. *Industrial Crop and Products, 71*, 122-127.

Chan, Y.S., Zhang, Y., & Ng, T.B. (2013). Brown Kidney Bean Bowman-Birk Trypsin Inhibitor is Heat and pH Stable and Exhibits Anti-proliferative Activity. *Applied Biochemistry and Biotechnology, 169*, 1306-1314.

Dia, V. P., & Gonzalez de Mejia, E. (2011). Lunasin induces apoptosis and modifies the expression of genes associated with extracellular matrix and cell adhesion in human metastatic colon cancer cells. *Molecular Nutrition and Food Research, 55*, 623–634.

Erdmann, K., Cheung, B. W. Y., & Schröder, H. (2008). The possible roles of blood-derived bioactive peptides in reducing the risk of cardiovascular disease. *Journal of Nutritional Biochemistry, 19*, 643-654.

Esteve, C., Marina, M. L., & García, M. C. (2015). Novel strategy for the revalorization of olive (*olea europaea*) residues based on the extraction of bioactive peptides. *Food Chemistry, 167*, 272-280.

Fernández-Martínez, A. B., Bajo, A. M., Sánchez-Chapado, M., Prieto J. C., & Carmena, M. J. (2009). Vasoactive intestinal peptide behaves as a pro-metastatic factor in human prostate cancer cells. *The Prostate, 69*, 774-786.

Ghassem, M., Babji, A.S., Said, M., Mahmoodani, F., & Aeihara, K. (2014). Angiotensin I-converting enzyme inhibitory peptides from snakehead fish sarcoplasmic protein hydrolysate. *Journal of Food Biochemistry, 38*, 140–149.

Gonzalez de Mejia, E., & Dia, V. P. (2010). The role of nutraceutical proteins and peptides in apoptosis, angiogenesis, and metastasis of cancer cells. *Cancer and Metastasis Reviews, 29*, 511–528.

González-García, E.; Puchalska, P., Marina, M. L.; García, M. C (2015). Fractionation and identification of antioxidant and angiotensin-converting enzyme-inhibitory peptides obtained from plum (*Prunus domestica L*.) stones. *Journal of Functional Foods, 19,* 376-384.

Hernández-Ledesma, B., & Hsieh, C. (2017). Chemopreventive role of food-derived proteins and peptides: A review. *Critical Reviews in Food Science and Nutrition, 57,* 2358-2376.

Hsieh, C., Hernández-Ledesma, B., & de Lumen, B. O. (2010). Lunasin a novel seed peptide, sensitizes human breast cancer MDA-MB-231 cells to aspirin-arrested cell cycle and induces apoptosis. *Chemico-Biological Interaction, 186,* 127-134.

Kim, E. K., Kim, Y. S., Hwang, J. W., Lee, J. S., Moon, S. H., Jeon, B. T., & Park P. J. (2013). Purification and characterization of a novel anticancer peptide derived from Ruditapes philippinarum. *Process Biochemistry, 48,* 1086–90.

Korhonen, H., & Pihlanto, A. (2003). Food-derived bioactive peptides- opportunities for designing future foods. *Current Pharmacology Design, 9,* 1297-1308.

Lima, A., Oliveira, J., Saúde, F., Mota, J., & Ferreira, R. B. (2017). Proteins in soy might have a higher role in cancer prevention than previously expected: soybean protein fractions are more effective mmp-9 inhibitors than non-protein fractions, even in cooked seeds. *Nutrients, 9,* doi:10.3390/nu9030201.

Mane, S., & Jamdar, S. N. (2017). Purification and identification of ACE-inhibitory peptides from poultry viscera protein hydrolysate. *Journal of Food Biochemistry, 41,* doi:10.1111/jfbc.12275

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of lmmunological Methods, 65*, 55-63.

Moubarika, A., & Grimib, N. (2015). Valorization of olive stone and sugar cane bagasse by-products as biosorbents for the removal of cadmium from aqueous solution. *Food Research International, 73*, 169-175.

Muñoz-Moreno, L., Arenas, M. I., Schally, A. V., Fernández-Martínez, A. B., Zarka, E., González-Santanter, M., Carmena, M. J., Vacas, E., Prieto, J. C., & Bajo, A. M. (2013). Inhibitory effects of antagonist of growth hormone-releasing hormone on growth and invasiveness of PC3 human prostate cancer. *International Journal of Cancer, 132*, 755-765.

Muñoz-Moreno, L., Bajo, A. M., Prieto, J. C., & Carmena, M. J. (2017). Growth hormone-releasing hormone (GHRH) promotes metastatic phenotypes through EGFR/HER2 transactivation in prostate cancer cells. *Molecular and Cellular Endocrinology*, *446*, 59-69.

Nunes, M. A., Pimentel, F. B., Costa, A. S. G., Alves, R. C., & Oliveira, M. B. P. P. (2016). Olive by-products for functional and food application: Challenging opportunities to face environmental constraints. *Innovative Food Science and Emerging Technologies, 35*, 139-148.

Pangestuti, R., & Kim, S. (2017). Bioactive peptide from marine origin for the prevention and treatment of non-communicable diseases, *Marine Drugs, 15*, doi: 10.3390/md15030067

Puchalska, P., García, M. C., & Marina, M. L. (2014). Development of a capillary high performance liquid chromatography–ion trap-mass spectrometry method for the

determination of VLIVP antihypertensive peptide in soybean crops. *Journal of Chromatography A, 1338*, 85-91.

Puchalska, P., Marina, M. L., & García, M. C. (2012). Development of a reversed-phase high-performance liquid chromatography analytical methodology for the determination of antihypertensive peptides in maize crops. *Journal of Chromatography A, 1234*, 64-71.

Puchalska, P., Marina, M.L., & García, M.C. (2015). Isolation and characterization of peptides with antihypertensive activity in foodstuffs. *Critical Reviews in Food Science and Nutrition, 55*, 521-51.

Quirós, A., Hernández-Ledesma, B., Ramos, M., Amigo, L., & Recio, I. (2005). Angiotensin-converting enzyme inhibitory activity of peptides derived from caprine kefir. *Journal of Dairy Science, 88*, 3480-3487.

Rizzello, C. G., Tagliazucchi, D., Babini, E., Rutella, G. S., Taneyo Saa, D. L., & Gianotti, A. (2016). Bioactive peptides from vegetable food matrices: Research trends and novel biotechnologies for synthesis recovery. *Journal of Functional Foods, 27*, 549-569.

Roselló-Soto, E., Koubaa, M., Moubarik, A., Lopes, R. P., Saraiva, J. A., Boussetta, N., Grimi, N., & Barba, F. J. (2015). Emerging opportunities for the effective valorization of wastes and by-products generated during olive oil production process: Non-conventional methods for the recovery of high-added value compounds. *Trends in Food Science and Technology, 45*, 296-310.

Sánchez, F., & San Miguel, G. (2016). Improved fuel properties of whole table olive stones via pyrolytic processing. *Biomass and Bioenergy, 92*, 1-11.

Terral, J. F., Alonso, N., Buxó i Capdevila, R., Chatti, N., Fabre, L., Fiorentino, G., Marinval, P., Pérez Jordá, G., Pradat, B., Rovira, N., & Alibert, P. (2004). Historical biogeography of olive domestication (*Olea europaea L.*) as revealed by geometrical morphometry applied to biological and archaeological material. *Journal of Biogeography, 31*, 63–77.

Vásquez-Villanueva, R., Marina, M. L., & García, M. C. (2015). Revalorization of a peach (*Prunus Persica (L.) Batsch*) byproduct: Extraction and characterization of ACE-inhibitory peptides from peach stones. *Journal of Functional Foods, 18*, 137–146.

Wilson, J., Hayes, M., & Carney, B. (2011). Angiotensin-I-converting enzyme and prolyl endopeptidase inhibitory peptides from natural sources with a focus on marine processing by-products. *Food Chemistry, 129*, 235-244.

Wu, Q., Cai, Q., Yoshida, A., Sun, L., Liu, Y., Liu, G., Su, W., & Cao, M. (2017). Purification and characterization of two novel angiotensin I-converting enzyme inhibitory peptides derived from R-phycoerythrin of red algae (*Bangia fusco-purpurea*). *European Food Research and Technology*, *243*, 779-789.

Wu, X., Sun, J., Zhang, G., Wang, H., & Ng, T.B. (2011). An antifungal defensin from Phaseolus vulgaris cv. 'Cloud Bean'. *Phytomedicine, 18*, 104-109.

Zhang, F., Shi, J.-J., Thakurm K., Hu, F., Zhang, J.-G. & Wei, Z.-J. (2017). Anti-cancerous potential of polysaccharide fractions extracted from peony seed dreg on various human cancer cell lines via cell cycle arrest and apoptosis. *Front Pharmacology, 8*, 1-13.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Economy and Competitiveness (ref. AGL2016-79010-R), and the Comunidad of Madrid and European funding from FEDER program (S2013/ABI-3028 AVANSECAL-CM). R.V.-V. thanks the University of Alcalá for her pre-doctoral contract.

FIGURE CAPTIONS

Figure 1. Chromatogram obtained by semipreparative RP-HPLC of peptides with MW below 3 kDa from the olive seed hydrolysate, ACE-inhibitory capacity of fractions (F1-F8) expressed as % of ACE- inhibition, and IC_{50} values of most active fractions.

Figure 2. Fragmentation spectrum of peptide LLPSY in fraction F5 of the olive seed hydrolysate.

Figure 3. Cell viability of human cell lines HK-2 (A), HeLa (B), PC-3 (C), and MDA-MB-468 (D) after treating with peptide LLPSY. The results are expressed as percentage of control value. Data are mean ± SEM of at least three independent experiments; ***p < 0.001.

Figure 4. (A) Cell adhesion and (B) cell migration capacity of PC-3 prostate and MDA-MB-468 breast cancer cells in presence or absence of synthesized peptide LLPSY at 0 min (□) and at 80 min (■). (B) The results are expressed as percentage of control value. Data are mean ± SEM of at least three independent experiments; **p < 0.01; ***p < 0.001.

Figure 5. Analysis of cell cycle in prostate cancer PC-3 cells (A) and in breast cancer MDA-MB-468 cells (B) after treatment with 87.1 μg/mL and 97.56 μg/mL of peptide LLSPY, respectively. The results are shown as % of cells in each phase of the cycle compared to untreated control. Data are mean ± SEM of at least three independent experiments; *p < 0.05; **p < 0.01.

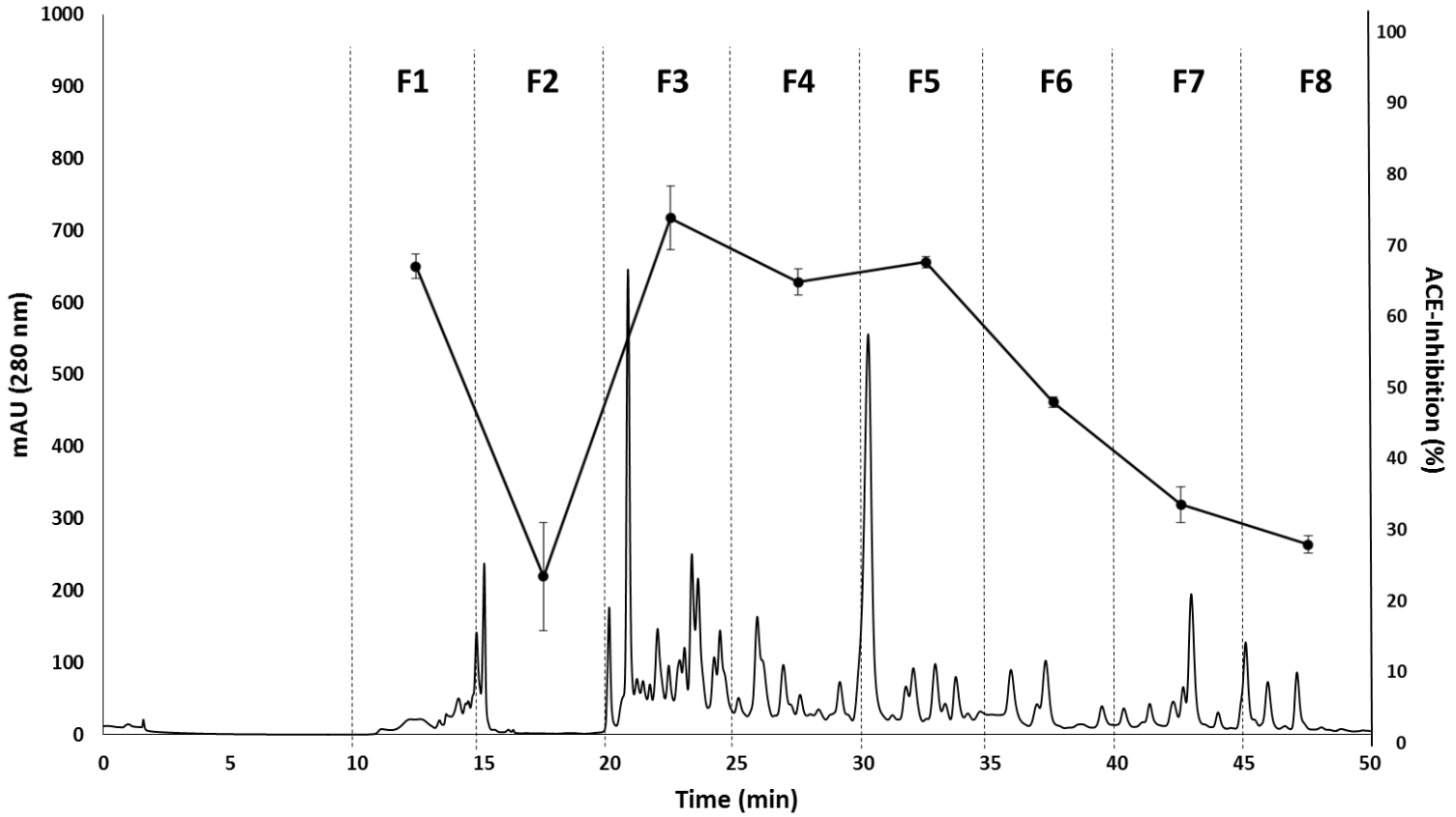
Table

Table 1. Sequence, ALC, mass, retention time, and mass accuracy obtained in the identification of peptides in fractions F3, F4, and F5 collected by semipreparative RP-HPLC from an olive seed hydrolysate by RP-HPLC- and HILIC-ESI-Q-ToF (in the case of fraction F5).

	Peptide sequence	ALC (%)	Mass (Da)	Retention time (min)	Mass accuracy (ppm)
F3					
RP-HPLC	VVLED	94	573.301	16.2	5.6
	VSVDD	94	533.2333	4.74	6.5
	LGLGD	94	473.2485	16.83	9.4
	LMAPH	94	567.2839	9.4	3.4
	FEET	94	524.2118	5.91	6.7
	LVLAK	94	542.3792	13.24	2.3
	LMSPH	94	583.2788	5.57	3.2
	VVVVPH	93	648.3959	17.27	2.5
	VSVND	92	532.2493	3.87	5.0
	LVTPH	91	565.3224	7.95	1.2
	YEPTPR	91	761.3708	7.61	4.5
	MDMS	91	482.1505	4.99	4.7
	VDLE	91	474.2325	12.36	6.7
	VVVVPHN	90	762.4388	16.34	4.3
	LVVN	90	443.2744	7.51	2.4
	LLDE	90	488.2482	8.24	1.5
F4					
RP-HPLC	LLVN	98	457.29	16.39	0.9
	LLVD	97	458.274	18.38	3.5
	DEFR	96	565.2496	5.42	8.4
	LGLGD	95	473.2485	16.97	9.4
	LVVD	95	444.2584	12.65	3.9
	VLVD	95	444.2584	11.63	3.9
	YDYNDDEFR	94	1235.4731	24.06	3.1
	VLAD	94	416.2271	4.35	4.4
	VGGTL	93	445.2536	11.83	8.2
	VFDD	92	494.2013	10.37	5.4
	FLPH	92	512.2747	14.45	1.6
	VAEL	91	430.2427	14.01	7.4
	VAVLE	91	529.3112	18.28	3.8
	ESTL	91	448.2169	4.85	4.5
	LLDT	91	460.2533	14.17	4.2
F5					
RP-HPLC	KLPL	96	469.3264	20.43	2.2
	LVLT	94	444.2948	18.43	2.1
	LLNY	93	521.2849	21.49	4.9
	LMAPHW	93	753.3632	29.5	5.7
	LMAPH	92	567.2839	10.72	2.6
	VFDD	91	494.2013	10.52	4.2
HILIC	LLDY	95	522.2689	1.59	0.8
	LPAE	94	428.2271	27.24	0.8
	PEFS	91	478.2063	24.56	0.7
RP-HPLC and HILIC	LLPSY	91[a],[b]	591.3268	25.53[a], 1.79[b]	4.0[a], 0.5[b]

[a] Parameters obtained by RP-HPLC and [b] parameters obtained by HILIC.

Figure 1
Click here to download Figure: Figure 1.docx



Fraction	IC_{50} (µg/mL)
F1	31.2 ± 5.6
F3	10.5 ± 3.5
F4	5.7 ± 0.6
F5	3.6 ± 0.9

Figure 1.

Figure 2

Click here to download Figure: Figure 2.docx

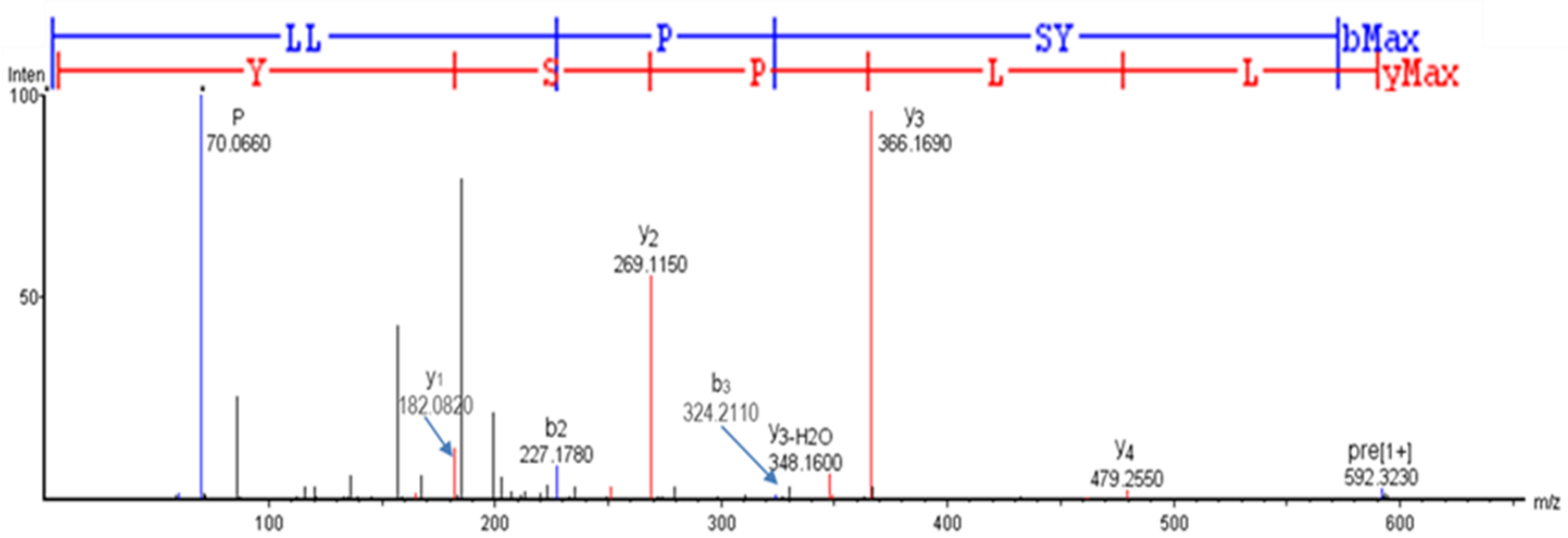
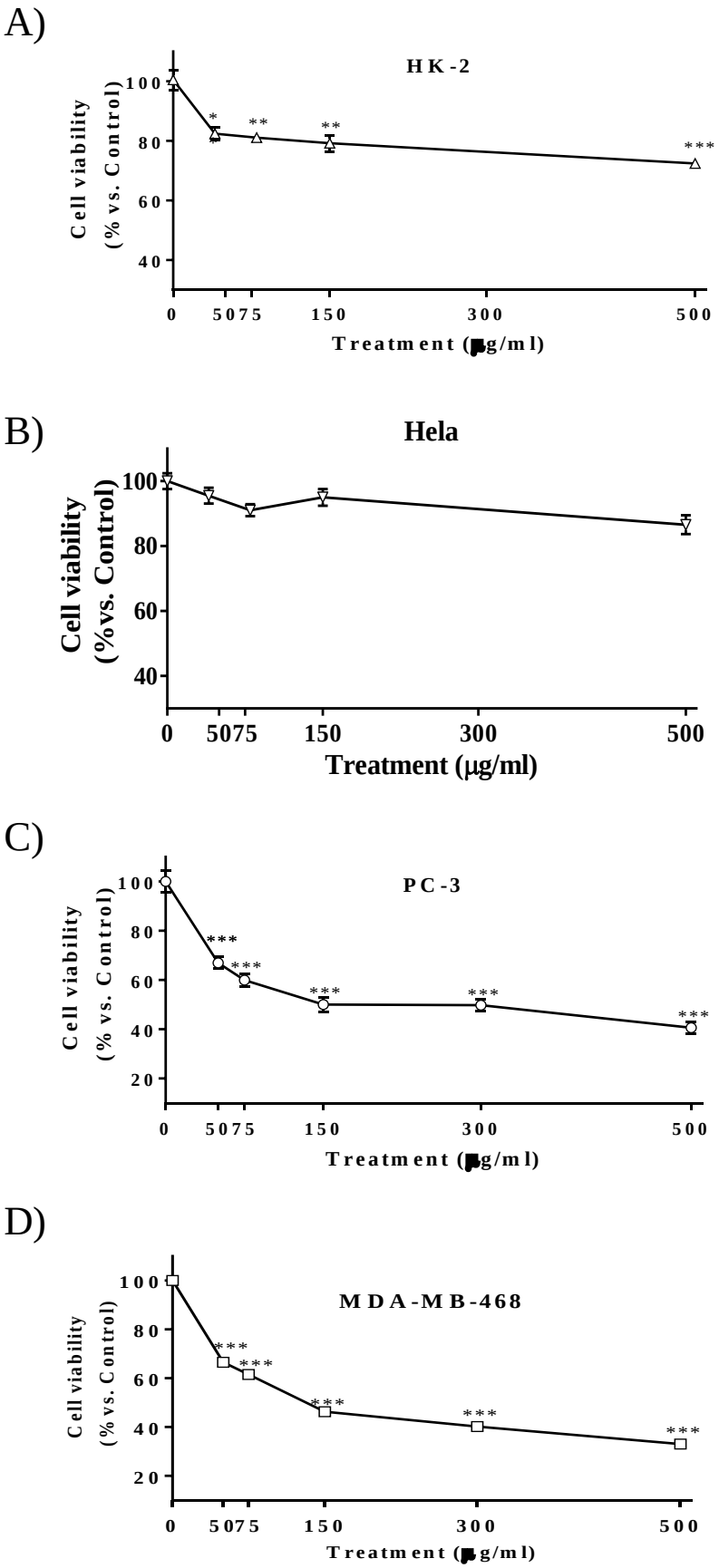


Figure 2

Figure 3
Click here to download Figure: Figure 3.docx



1

2 **Figure 3.**

Figure 4
Click here to download Figure: Figure 4.docx

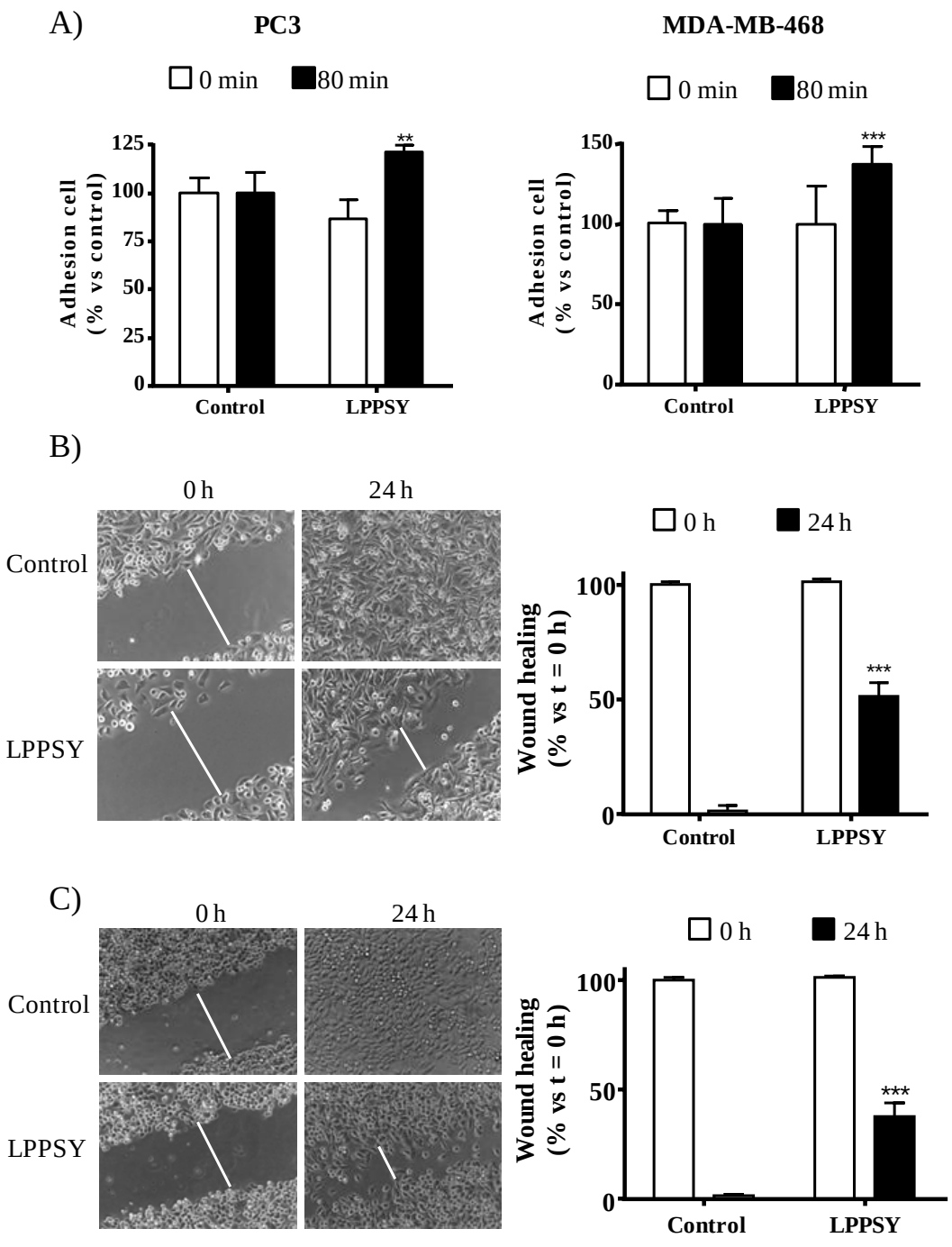
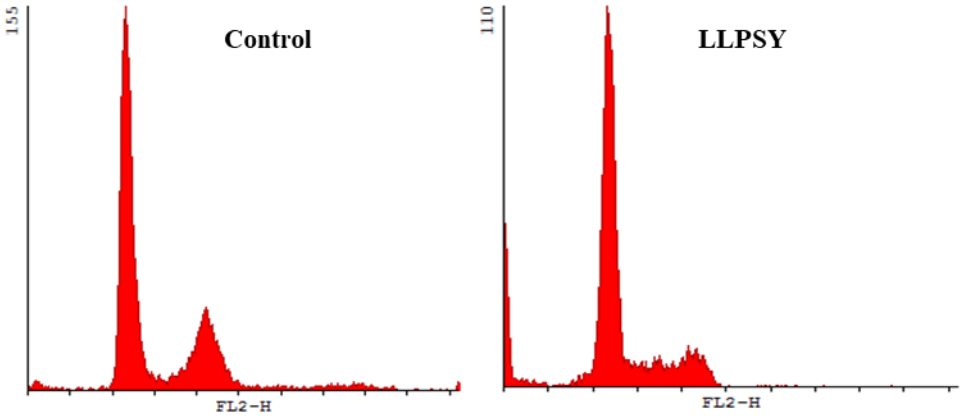


Figure 4.

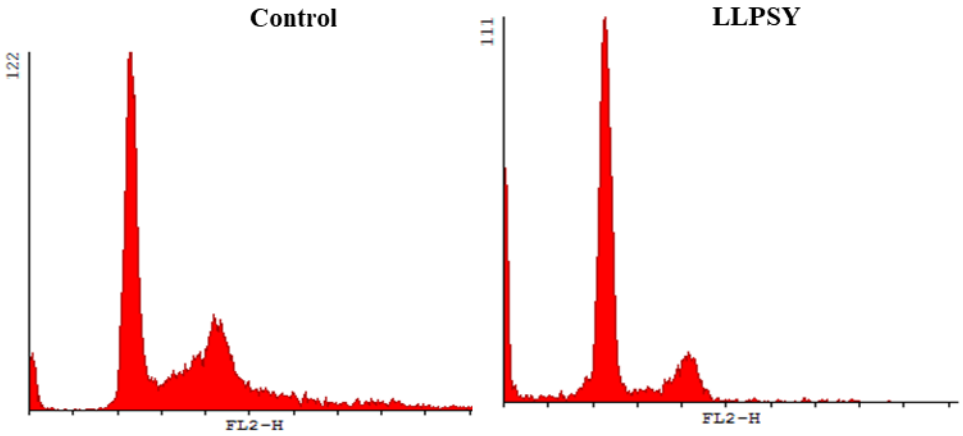
Figure 5
Click here to download Figure: Figure 5.docx

A) PC3



	SubG$_0$	G$_1$	S	G$_2$/M
CONTROL	1.13 ± 0.12	53.82 ± 0.32	6.53 ± 0.46	37.29 ± 0.52
LLPSY	6.26 ± 0.36 *	54.46 ± 1.90	9.57 ± 0.25	25.48 ± 0.27 **

B) MDA-MB-468



	SubG$_0$	G$_1$	S	G$_2$/M
CONTROL	3.28 ± 0.52	49.59 ± 0.56	12.53 ± 0.46	39.14 ± 0.62
LLPSY	7.65 ± 0.42 *	48.414 ± 1.89	9.37 ± 0.52	21.11 ± 0.72 **

Figure 5.