Postprint Clinical Nutrition 39(4): 1242-1249

Virgin olive oil (unfiltered) extract contains peptides and possesses ACE inhibitory and antihypertensive activity*

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Abbreviations: ACE, angiotensin converting enzyme; BW, body weight; DBP, diastolic blood pressure; SDP, systolic blood pressure; SHR, spontaneously hypertensive rats.

ABSTRACT

Background & aims: The peptide and protein composition of olive oil is mostly unknown and the few studies available have not focused on the study of its low molecular weight peptides. We hypothesised that olive oil could naturally contain low molecular weight peptides with antihypertensive effect.

Methods: We produced virgin olive oil (unfiltered, var. Picual) and obtained a water-soluble peptide extract. We fractionated the peptide extract by FPLC and studied its angiotensin converting enzyme (ACE) inhibitory activity. We studied the antihypertensive effect of olive oil peptides on the systolic blood pressure (SBP) and diastolic blood pressure (DBP) using an animal model of hypertension (spontaneously hypertensive rats, SHR). The animals were randomly distributed into 3 study groups (n=8 per group) and received an oral dose of olive oil peptides (0.425 mg/Kg of BW), or a dose of Captopril (50 mg/Kg of BW) or water. SBP and DBP were registered in the rats before administration and a at 2, 4, 6, 8, 24 and 48 hours post-administration of the corresponding dose.

Results: The peptide extract and FPLC purified fractions possessed angiotensin converting enzyme (ACE) inhibitory activity. Acute oral administration of olive oil water-soluble extract produced an average blood pressure reduction of 10 mmHg at 4 h ($P<0.01$) and reached a maximum antihypertensive effect of 20 mmHg at 6 h, compared with baseline.

Conclusion: unfiltered virgin olive oil contains peptides and a water-soluble extract obtained from this oil possesses ACE inhibitory activity and in vivo antihypertensive effect.

Keywords: Olive oil, peptides, ACE, hypertension, spontaneously hypertensive rats.

INTRODUCTION

Virgin olive oil is a natural functional food which can produce cardiovascular benefits (1). According to the European Food Safety Authority (EFSA), the intake of olive oil (referred to as oleic acid or monounsaturated fat), particularly virgin olive oil, has demonstrated to produce cardiovascular benefits due to its fatty acid composition and to the antioxidant action of its naturally occurring polyphenols (mainly hydroxytyrosol and its derivatives). Indeed, EFSA has approved a number of health claims that can be applied to virgin olive oil, including the intake of monounsaturated fat and reduction of blood cholesterol (2), and the intake of olive oil polyphenols which produces the antioxidant protection of blood lipids (such as low density lipoproteins) from oxidative stress (3), among other claims.

Hypertension is a primary risk factor of cardiovascular disease which affects approximately 40% of adults aged 25 and above (4). In recent years, the effect of olive oil on the control of blood pressure has been investigated and a few human trials have shown benefits (5-7). The antihypertensive effect of virgin olive oil was suggested to be produced by the fatty acid composition (8; reviewed in 9) or by its active minor compounds such as polyphenols and triterpenoid acids. Some studies have shown that olive oil polyphenols were responsible for the anti-hypertensive effect of olive oils in hypertensive rats (10) subjects with high cholesterol levels, (11), pre-hypertensive (12, 13) hypertensive subjects (14) and coronary heart disease patients (15). A recent meta-analysis of randomised controlled trials suggested that olive oils with at least 150 ppm of polyphenol may produce beneficial effects on systolic blood pressure (16). Maslinic and oleanolic acids are the main triterpenic acids found in olive oils and pomace oils (17). Some studies with animal models have shown significant blood pressure reductions produced by the chronic administration of triterpenic acids (18, 19, reviewed in 20). The effects of olive oils enriched with triterpenic acids have been recently evaluated in metabolic syndrome patients but no effect on blood pressure was detected (21).

One of the metabolic pathways regulating blood pressure is the renin-angiotensin system, in which the angiotensin converting enzyme (ACE) plays a central role. Inhibition of ACE is a widely used strategy for the treatment of hypertension (22).

Bioactive peptides with ACE inhibitory activity have been isolated from different sources of animal and vegetable origin. The vast majority of ACE inhibitory peptides described so far, are obtained by the action of specific proteases on different sources of dietary proteins, including dairy and fermented milks, eggs, soybeans, chickpeas, peanuts, tuna, sardines, shrimp, chicken, squid, among others (reviewed in 23). The most studied and representative examples of ACE inhibitor peptides are found in hydrolysates of milk proteins, carried out with different enzymes and by fermentation of milk with different types of bacteria. The antihypertensive effects of some of these dairy peptides have been studied in animal models and in human subjects (24). However, the existence of bioactive peptides with defined functions can also occur naturally (such as in breast milk) without the use of proteases or other methods for their production (25).

Previous work carried out in our laboratory showed that olive fruit homogenates were very sensitive to protein degradation even in the presence of protease inhibitors (26). This suggested the presence of proteases in olive fruits able to produce peptides. We hypothesised that some low molecular weight peptides, either originated as a consequence of protein metabolism or as a consequence of olive oil extraction, could be transferred to olive oil and possess biological activity. In this study we report the ACE inhibitory activity of an olive oil water-soluble extract containing peptides and studied their antihypertensive effects in spontaneously hypertensive rats (SHR).

MATERIALS AND METHODS

Plant material and extraction of olive oil - Olive fruits (*Olea europea* L. variety Picual) were obtained from healthy olive trees in orchards in the province of Granada (Spain). Olive samples were hand-picked when the olives were at the turning phase of maturation (ripening index 3 and 4) according to the method described in (27), defining the ripening index as function of fruit colour in both skin and pulp. The olive fruits were carefully selected and only healthy fruits were used. For the experiments, we obtained olive oil from our own recollected olives. First, the olives were thoroughly washed with water and dried. Olive oil was extracted

using a standard method consisting of milling of the olives, soft mixing of the resulting olive paste and centrifugation of the mixture using a two-phase extraction plant. Filtration of olive oil was always avoided. Once extracted, the olive oil was immediately transferred to light protective containers and it was kept at room temperature. The extraction of the olive oil was carried out within 48 hours of the collection of the olives. The temperature of the extraction was always below 28ºC.

Preparation of olive oil extract containing peptides. ~~*Extraction of olive oil peptides*~~ Olive oil peptide extract was obtained from our olive oil preparations in several batches with a mixture of cold acetone:hexane (1:1) using an olive oil/solvent ratio of 1:2.5 (w/v), for 1 h in the cold room at 4-6°C. Then, the mixture was centrifuged at 10,000 g for 15 min at 4°C. The supernatant was carefully discarded and the precipitate (containing the peptides) was separated. The extraction process was repeated with each olive oil batch. The precipitates containing the peptides were pooled and desiccated until the next step, always maintaining a temperature below 40°C. Then, the extract was suspended in water and the mixture underwent a process of sonication (Branson 200, Branson Ultrasonics, USA) for 30 minutes and it was centrifuged at 10,000 g for 15 min at 4°C. The supernatants, containing a water-soluble peptide fraction extracted from olive oil, were collected and stored at -80ºC for further analysis and for *in vitro* and *in vivo* experiments.

Fractionation of peptides by size-exclusion chromatography - The olive oil peptides obtained with the method described above were separated by FPLC using gel filtration chromatography with a protein purification system "ÄKTApurifier" (GE Healthcare, UK) equipped with a Superdex Peptide 10/300 GL size-exclusion column with a separation range of between 7000 and 100 Da (GE Healthcare). The elution of the samples was conducted using an isocratic method with a mobile phase of 20% acetonitrile with 0.1% trifluoroacetic acid and a flow of 0.8 mL/min, for 40 min. The elution was monitored at 280 nm. Molecular mass standards of known molecular mass were used for the calibration of the size exclusion chromatographic column. The standards used were cytochrome C (12,384 Da), aprotinin (6,512 Da), Vitamin B_{12} (1,355 Da) and tryptophan (204 Da) (Sigma-Aldrich, St. Louis, MO, USA).

The injection volume of the samples and standards into the ÄKTApurifier was 200 µL. Seventeen 2-ml fractions were collected after each injection. Fractions were pooled into 6 groups (F1-F6) based on the chromatographic profile recorded at 280 nm (see Fig 2). The fractions F1-F6 were dried using a Buchi rotary evaporator R-205 (BÜCHI Labortechnik AG, Switzerland).

Peptide concentration determination - The total peptide concentration in olive oil extracts was determined by fluorescence using a protein quantification kit (FluoroProfile, Sigma-Aldrich). BSA was used as standard solution. Fluorometric quantification was carried out to 530 nm and 630 nm as excitation and emission wavelengths, respectively.

Analysis of amino acids by Gas Chromatography and Mass Spectrometry (GC-MS). The The parcial amino acid content of the water-soluble peptide fraction extracted from olive oil was analysed. Briefly, olive oil extract (containing 2-4 mg of total protein) plus 0.5 µg of DL-norleucine which was added as internal standard, were dissolved in 4 ml of 6.0 M hydrochloric acid and hydrolyzed for 20 h at 110 °C. The hydrolyzed samples obtained were taken to dryness, then added with 1 ml of dichloromethane and dried again in a rotary evaporator. The sample was dissolved with 75 µl of acetonitrile and derivatized with 75 µl N-tert-butyldimethylsilyl- N-methyltrifluoroacetamide (MTBSTFA, Sigma-Aldrich) at 100°C for 2 hours. Samples were centrifuged and the supernatants were injected (1µl per sample). A commercially available mix of seventeen amino acids (Sigma-Aldrich, not containing tryptophan, asparragin and glutamin) was used for the quantification. The concentration of amino acids was determined by GC-MS as described in (28).

Determination of angiotensin-converting enzyme inhibitory activity - The ACE inhibitory activity of olive oil extracts and of FPLC fractions were determined according to the method described in (29), with some modifications. This assay is based on the ability of ACE to hydrolyse the substrate o-aminobenzoylglycyl-p-nitrophenylalanyl-proline (Abz-Gly-Phe-(NO2)-Pro, Bachem Feinchemikalien, Switzerland), producing the fluorescent product o-aminobenzoylglycine (Abz-Gly). The following reagents were used: buffer A: 150 mM Tris-HCl buffer (pH 8.3), with 0.1 µM ZnCl2; buffer B: 150 mM de Tris-HCl buffer (pH 8.4), with

1125 mM NaCl; ACE solution: rabbit-lung ACE (E.C.3.4.15.1., Sigma-Aldrich), previously dissolved in 50% glycerol, was diluted in buffer A to make an enzyme concentration of 0.042 U/mL. This solution was prepared fresh every day to conduct the experiment. Substrate solution: Abz-Gly-Phe(NO_2)-Pro was dissolved in buffer B to a final concentration of 0.45 mM. This solution was also prepared every day before its use and was protected from light and kept at 4ºC. The assay was carried out using a fluorescence technique. Black polystyrene plates of 96 wells (Thermo Scientific, USA) were used. The wells contained the following reaction solutions: control = 40 µL of Milli Q water and 40 µL of ACE solution; blank = 40 µL of Milli Q water and 40 µL of buffer A; sample = 40 µL of sample and 40 µL of ACE solution; sample blank = 40 µL of sample and 40 µL of buffer A. The enzymatic reaction was initiated by adding 160 µL (final volume in each well 240 µL) of substrate solution and, immediately, the plate was mixed and incubated at 37°C in a VICTORX5 fluorometer (PerkinElmer, USA). The fluorescence generated is measured after 30 minutes using 355 and 420 nm as excitation and emission wavelengths, respectively. The ACE inhibitory activity of each sample was determined in triplicate.

The ACE inhibitory activity was calculated using the following formula:

$$\text{ACE inhibitory activity (\%)} = \frac{(\text{FC} - \text{FB}) - (\text{FS} - \text{FBs})}{\text{FC} - \text{FB}} \text{ x } 100$$

FC (Control): Fluorescence emitted after the action of ACE on the substrate, without inhibitor (i.e. sample).

FS (Sample): Fluorescence emitted after the action of ACE on the substrate, with inhibitor sample.

FB (Blank): Fluorescence emitted by the substrate.

FBs (Blank sample): Fluorescence emitted by the substrate and the sample.

188 The ACE inhibitory activity is expressed as IC_{50} which is the concentration of inhibitor required
189 to inhibit the activity of ACE by 50%.

190 *Antihypertensive activity of olive oil extract containing peptides in SHR* – The
191 antihypertensive effect of the olive oil extract was studied in the systolic blood pressure (SBP)
192 and diastolic blood pressure (DBP) of SHR. The SBP and DBP were measured by the tail-cuff
193 method (30). This model is not invasive and the only contact with the animals is the careful
194 administration of a small volume of the extract of the study, followed by the determinations of
195 the SBP and DBP. To reduce stress-induced variations in blood pressure, all measurements were
196 taken by the same person, and in the same peaceful environment. Moreover, to guarantee the
197 reliability of the measurements, a training period of two weeks prior to the real trial was
198 established, to allow the rats to be habituated to this procedure. In this period we only measured
199 the SBP and DBP of the SHR with the tail-cuff method. We investigated the antihypertensive
200 activity of a water-soluble peptide fraction extracted from olive oil as follows. Twenty-four
201 male SHR of 19-21 weeks of age were used with an average BW of 316.3 ± 12.0. SHR were
202 purchased from Charles River Laboratories (St-Germain-sur-l'Arbresle, Francia). The SHR
203 were kept at 23ºC with 12-h light/dark cycles. The animals consumed tap water and a standard
204 laboratory diet (A04 Panlab, Barcelona, Spain) *ad libitum*, during the experiments. The olive oil
205 extract containing peptides was dissolved in water and was carefully administered by oral
206 gavage directly into the stomach, between 9 and 10 am. Water was used as negative control and
207 Captopril (Sigma, USA), a well-known ACE inhibitor drug, was given as positive control. The
208 animals were randomly distributed into 3 study groups (n=8 per group) and received an oral
209 dose of olive oil extract containing peptides (0.425 mg/Kg of BW), or a dose of Captopril (50
210 mg/Kg of BW) or water. The different doses (approximately 1 mL per animal) were
211 administered by a very experienced technician and the whole procedure of gastric intubation
212 and oral administration lasted only a few seconds to minimise animal stress. The average values
213 of SBP and DBP of the SHR at baseline were 206.47±2.97 and 171.52±5.03 mmHg for the
214 Captopril group, 200.70±2.23 and 168.39±3.15 mmHg for the water group and 204.41±1.26 and
215 171.33±3.76 mmHg for the olive oil extract group, respectively. SBP and DBP were registered

in the rats at 2, 4, 6, 8, 24 and 48 hours post-administration of the corresponding dose. At least six similar consecutive measurements of SBP and DBP were taken as valid, and their averages were calculated. The equipment used was LE 5001 (Letica, Hospitalet, Spain). The animal protocol followed in the study was approved by the Bioethical Committee of Universitat Rovira i Virgili (Spain). All experiments were performed in accordance with the ARRIVE guidelines (31), the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines and the EU directive 2010/63/EU for animal experiments.

Other determinations – The content of maslinic acid and oleanolic acid were quantified in olive oil water-soluble extracts by UPLC-MS/MS as described in (32). Maslinic and oleanolic acids pure standards were obtained from Sigma-Aldrich. Methanolic stock solutions of 500 mg/L for each standard were obtained. All the samples and stock solutions were stored at – 20ºC and filtered through a 0.22 µm nylon syringe filter before the injection. Three 50 µl samples of olive oil extracts or standards were injected into the equipment and analysed. Total sterols were analysed as in Regulation (EU) No 1348/2013 (33). Alpha-tocopherol was analysed as in ISO 9936:2006 (34). Fatty acids were analysed as in (35). Total Phenolic compounds were measured as described in (36). Chlorophyll was determined as in (37).

Statistical analysis - Changes in blood pressure were expressed as absolute values of SBP and DBP before and after administration of the peptides. Data are expressed as means ± standard error of the mean (SEM). Data were analysed using one-way ANOVA followed by Bonferroni *post hoc* test. Differences of $P< 0.05$ between the groups were considered significant. SPSS statistical software version 23.0 was used for the statistical analysis (SPSS, Chicago, USA).

RESULTS

Preparation of a water-soluble peptide extract from olive oil. We obtained unfiltered olive oil from mature olives from which we produced an acetone/hexane extract. The calculated yields were 172.5±83.6 mg of dried extract and 7.24±4.1 mg of proteins per Kg of olive oil (the protein content of the dried extract was approximately 4%). From this extract, we obtained a

water-soluble peptide fraction. The calculated extraction yield was 0.09±0.02 mg of water-soluble peptides per Kg of olive oil, which represents only 1.2% of the peptides firstly extracted with organic solvents. Other compounds, mainly polyphenols were also solubilised from the unfiltered virgin olive oil and were present in the water-soluble extract of the study (Table 1).

Analysis of olive oil peptide extract. The olive oil water-soluble fraction was studied by amino acid analysis. Figure 1 shows the amino acid profile obtained by GC-MS and amino acid composition (partial) of the olive oil water-extract. This fraction was administered to SRH in the animal study (see below). The fractionation by size-exclusion chromatography (FPLC) of the same olive oil water-soluble peptide fraction revealed several peaks at 280 nm (Figure 2). Three major groups of fractions were selected, with molecular masses ranging from 5300-1600 Da (F3), 1600-700 Da (F4) and 700-200 Da (F5). These fractions contained the vast majority of the peptide content. The ACE inhibitory activity of the extracted peptides and of the FPLC-purified fractions F1-F6 was investigated *in vitro* (Table 2 and Figure 3). The water-soluble extracted peptides showed the highest activity compared with the FPLC-purified fractions.

Antihypertensive activity of olive oil water-soluble extract. We studied the antihypertensive activity of the olive oil water-soluble extract containing peptides in SHR. A single dose of olive oil extract (0.425 mg/Kg of body weight, BW) was administered to SHR and compared with Captopril (ACE inhibitor drug) and water (negative control). The nutrient content of the olive oil extract administered to the SHR is shown in Table 1. The average initial values of systolic blood pressure (SBP) and diastolic blood pressure (DBP) of the SHR before the tests were 203.8 ± 1.8 mmHg and 161.2 ± 9.4 mmHg, respectively, showing that the animals were indeed suffering from hypertension. The olive oil extract dose produced an average blood pressure reduction of 10 mmHg at 4 h ($P<0.01$) and reached a maximum antihypertensive effect of 20 mmHg at 6 h, compared with baseline (Fig 3). A non-significant trend was observed at 2 h. SBP reduction was also observed at 8 h before returning to initial values at 24 h. The SBP reduction curve obtained for Captopril was similar to that obtained with the olive oil extract but the reduction values were almost doubled and the effects lasted for 48 h. The olive oil extract produced no effect on DBP compared with controls (not shown).

DISCUSSION

In this study we report the ACE inhibitory activity of an olive oil water-soluble extract containing peptides and its antihypertensive effect on a well-established animal model of hypertension. A critical point for the isolation of the peptides was to avoid filtration steps (typically used to eliminate cloudiness) during the extraction of olive oil. In previous initial experiments carried out in our laboratory we observed that filtration, even only through a few layers of filter paper, also eliminated olive oil peptides. We investigated whether this also occurred in commercial olive oils. With the advice of a large olive oil producing industry (Deoleo, Spain), we reproduced in our laboratory the same filtration process used in their production plant and detected negligible amounts of peptides in the filtered oils (not shown), indicating that the peptides were indeed retained by the filters. For this reason, we did not use commercial oils in our investigations as virtually all commercial olive oils are subjected to a thorough filtration process.

Our objective was to test the effects of unfiltered virgin olive oil water-extract containing peptides in the SHR model of hypertension, which is incompatible with the administration of organic solvents. So, our analyses and tests were carried out with the peptide fraction solubilised with water from the extract previously obtained with acetone:hexane. This is only a fraction (about 1%) of the olive oil extracted proteins, so it is likely that there are many more bioactive peptides in olive oil, yet to be studied. Several studies have reported the protein concentration of olive oils using different extraction methods but the results are somewhat controversial. Some reported values ranged 0.05-2.4 mg/Kg (38) or 0.1-0.5 mg/Kg (39) but other studies showed much higher values of 11-43 mg/Kg of oil (40).

The few studies carried out so far on olive oil proteins have not focused on the study of low molecular weight polypeptides. Regarding antihypertensive activity, the molecular sizes <3 kDa are very relevant because antihypertensive peptides (obtained by hydrolysis) typically have molecular weights between 350 Da and 3000 Da (41). Olive oil water-soluble peptide extract and FPLC purified fractions with molecular masses ranging from 5300-1600 Da (F3), 1600-700 Da (F4) and 700-200 Da (F5) showed protein content and good ACE inhibitory activity. This

suggested a possible antihypertensive effect *in vivo* so we aimed to study the effects in an animal model of hypertension.

The development of high blood pressure in SHR has clear analogies with the development of hypertension in humans (42-44). Previous SHR studies investigating the antihypertensive effects of peptide isolates originated from foods, usually administered amounts in the range of 5-500 mg/Kg of BW (23, 45-48). Compared with these studies, our dose of 0.425 mg/Kg of BW (about 0.1 mg per animal) was low. We used this small dose because the amount of peptides detected in olive oil was also low, about 7.24 mg/Kg olive oil. Although 0.1 mg of total peptides should in theory be present in about 14 g of our unfiltered olive oil, it is important to emphasise that the peptide extract used in the SHR study was a small fraction (water soluble, about 1% of the total) of the peptides present in olive oil. It is difficult to extrapolate the results from the animal study to humans. We do not know the dose at which water soluble peptides might produce antihypertensive effects in humans. If the same dose or higher would be needed, then it would exceed the nutritional boundaries because the amount of olive oil necessary to produce the effect would be much higher than the daily recommendations of fat intake. In this case, the results from this research would be more applicable to the pharma-nutrition field. However, a much lower dose of water soluble peptides might be active in humans. Also, a peptide extract containing the complete composition naturally present in olive oil (water soluble and insoluble), would be likely to have antihypertensive effect at a lower dose because it would be more hydrophobic. Even natural unfiltered virgin olive oil might be able to have antihypertensive effects at nutritional levels. Our future research in humans would clarify these important points. One of the limitations of this study was indeed that we could only investigate *in vivo* the water-soluble peptide fraction because the SHR model of hypertension is incompatible with the administration of compounds with organic solvents.

Other potentially antihypertensive olive oil minor compounds present in the water-soluble extract of our study were triterpenoid acids and polyphenols. Regarding triterpenoid acids, we could only quantify very low amounts of maslinic acid (0.052 µg/ml of extract) whilst oleanolic acid was not detectable. These can be explained by the very low solubility of

328 triterpenic acids in water (49). According to these results, the SHR of our study received
329 approximately 0.052 µg of maslinic acid per animal. This dose would have been too low to
330 produce any measurable effect in blood pressure, as previously described (18, 19). Besides, the
331 ACE inhibitory activity of maslinic acid has never been reported so we think it is very unlikely
332 that the low amounts of maslinic acid present in our extract are responsible for any of the ACE
333 inhibitory effect. However, the olive oil water-extract of our study contained measurable
334 amounts of polyphenols. As described above, olive oil polyphenols have been reported to
335 reduce blood pressure in animal models and humans (10-16). Among olive oil polyphenols,
336 oleuropein and hydroxytyrosol have been identified as the most important active molecules.
337 However, no previous reports showing the ACE inhibitory activity of olive oil polyphenols are
338 available. In fact, regarding oleuropein, one research paper reported the lack of ACE inhibitory
339 activity of oleuropein (50). The mechanisms proposed for polyphenols to produce the
340 antihypertensive effects are by increasing bioavailability of NO or acting on the expression of
341 endothelin-1 (revised in 51). However we cannot rule out the presence of other polyphenols on
342 the olive oil water-extract possessing ACE inhibitory and making a contribution to the
343 antihypertensive activity.

344 The SBP decrease obtained with our olive oil peptide extract is in line with previous
345 studies administering fermented dairy extracts (47, 52, 53), milk protein hydrolysates (54, 55),
346 or extracts of dairy foods (56), usually producing SBP reductions in the range of 10-25 mmHg,
347 4-8 hours after their oral administration (24). Some of those peptide compositions originated
348 from dairy foods constitute the basis of functional foods with demonstrated antihypertensive
349 activity in humans, including Calpis® (57) and Evolus® (58).

350 We believe that the production of unfiltered olive oil can be a way to enrich olive oil in
351 bioactive peptides which may perhaps be beneficial in helping to control blood pressure. The
352 existence of bioactive peptides in unfiltered olive oil may allow the development of new uses
353 for this food, beyond their nutritional value, including the production of functional olive oils,
354 dietary supplements, nutraceuticals and medicinal products. This can lead to the production of

new varieties of olive oils with competitive advantages. However, filtered olive oils are more stable than unfiltered olive oils and therefore possess a longer shelf life. So maybe the results of this investigation are more applicable to the supplement/pharma-nutrition world rather than the food and nutrition field. The composition of olive oil is very much influenced by the variety of olive, location, weather conditions, olive recollection, the olive stage or ripening and the method used to produce olive oil. Another limitation of the present study is that we studied only one variety of olive oil (Picual). Although this is the most frequent variety of olive tree in Spain, other olive varieties may show different results. Apart from olive oil, a likely source of olive peptides could be olive oil waste. Olive oil extraction originates two types of by-products, solid olive pomace and liquid mill wastewater, both producing environmental problems. In view of our results, the study of the peptide composition of both olive oil by-products deserves attention. In this sense, a recent study has used olive residues as a source of protein to enzymatically produce peptide hydrolysates as a strategy for the revalorization of olive residues (59).

In conclusion, unfiltered virgin olive oil contains peptides and a water-soluble extract obtained from this oil possesses ACE inhibitory activity and *in vivo* antihypertensive effect. We are in the process of investigating the specific composition and peptide sequences present in olive oil using peptidomics.

Acknowledgements: GC-MS analyses were carried out at the Instrumental Technical Services of the Estación Experimental del Zaidín (CSIC), Granada, Spain. We thank Justa M. Amaro and Carmelo Ruiz for their valuable technical assistance, Juan de Dios Navarro Pelayo for the use of his olive tree farms and Ruth Wilson for revising the English language.

Funding: This project has been supported by European Regional Development Funds under the agreement signed between MINECO and CSIC for the realization of the RECUPERA 2020 project. Competing financial interests: the authors declare that they have no conflict of interest with the contents of this article

Author contributions: JMA-H conducted most of the experiments with olive oil, all of the *in vitro* assays, analysed the data and revised the manuscript. FIB, MM and BM tested the effects of the olive oil extract on SHR and revised the manuscript. EL-H conceived the idea for the project, obtained the funding, analysed the results and wrote the manuscript.

Data Statement - All data generated or analysed during this study are included in this published article.

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627 **FIGURE LEGENDS**

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629 **FIGURE 1.** Amino acid profile obtained by GC-MS after acid hydrolysis and derivatization
630 with N-tert-butyldimethylsilyl- N-methyltrifluoroacetamide diethyl ethoxymethylenemalonate
631 (MTBSTFA) of peptides obtained from olive oil. A, mix of seventeen amino acid standards
632 (Sigma-Aldrich); B, water-soluble peptide fraction obtained from olive oil. C, amino acid
633 composition (parcial) of the olive oil water-extract.

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635 **FIGURE 2.** Molecular weight distribution obtained by FPLC of the water-soluble
636 proteins/peptides extracted from olive oil.

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638 **FIGURE 3.** A, angiotensin-converting enzyme inhibitory activity (ACEi) of water-soluble
639 peptides extracted from olive oil. B, calibration curve obtained from the data of panel A and
640 used to determine IC_{50}.

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642 **FIGURE 4.** Systolic blood pressure (SBP) variations (mmHg) detected in spontaneously
643 hypertensive rats (SHR) at baseline and 2, 4, 6, 8, 24, y 48 h after the administration of a dose of
644 water-soluble peptides extracted from olive oil (0.425 mg/Kg BW, ●), Captopril (50 mg/Kg of
645 BW, □), or water control (○). **, significantly different compared with control ($P<0.01$) and
646 ***, significantly different compared with control and olive oil peptide extract ($P<0.01$).

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649 **TABLE 1**. Nutrient composition of olive oil water-extract administered to spontaneously
650 hypertensive rats. ND, not detected.

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Nutrient	per 1 ml of extract
Proteins (mg)	0,102
Carbohydrates	N.D.
Fats (fatty acids)	N.D.
Total polyphenols (mg)	1.01
Triterpenic acids (µg)	0.052
Sterols	N.D.
Alpha-tocopherol	N.D.
Chlorophyl	N.D.

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669 **TABLE 2.** ACE inhibitory activity (shown as IC_{50}) of olive oil water-soluble extract containing

670 peptides and of the FPLC-purified fractions F1-F6. Data are expressed as mean values ± SD.

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Sample	**IC_{50} (µg prot/ml)**
Olive oil water-soluble extract containing peptides	2,5 ± 0,
F1	67,5 ± 5,8
F2	174,3 ± 0,4
F3	47,6 ± 2,4
F4	138,6 ± 7,4
F5	98,0 ± 5,0
F6	140,1 ± 11,6

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FIGURE 1. Amino acid profile obtained by GC-MS after acid hydrolysis and derivatization with MTBSTFA of peptides obtained from olive oil. A, mix of seventeen amino acid standards (Sigma-Aldrich); 1, Ala; 2, Gly; 3, Val; 4, Leu; 5, Ile; 6, L-norLeu; 7, Pro; 8, Met; 9, Ser; 10, Thr; 11, Phe; 12, Asp; 13, Cys; 14, Glu; 15, Lys; 16, His; 17, Tyr; 18, Trp; 19; Cys. B, water-soluble peptide fraction obtained from olive oil. C, amino acid composition (parcial) of the olive oil water-extract. N.D, not detected.

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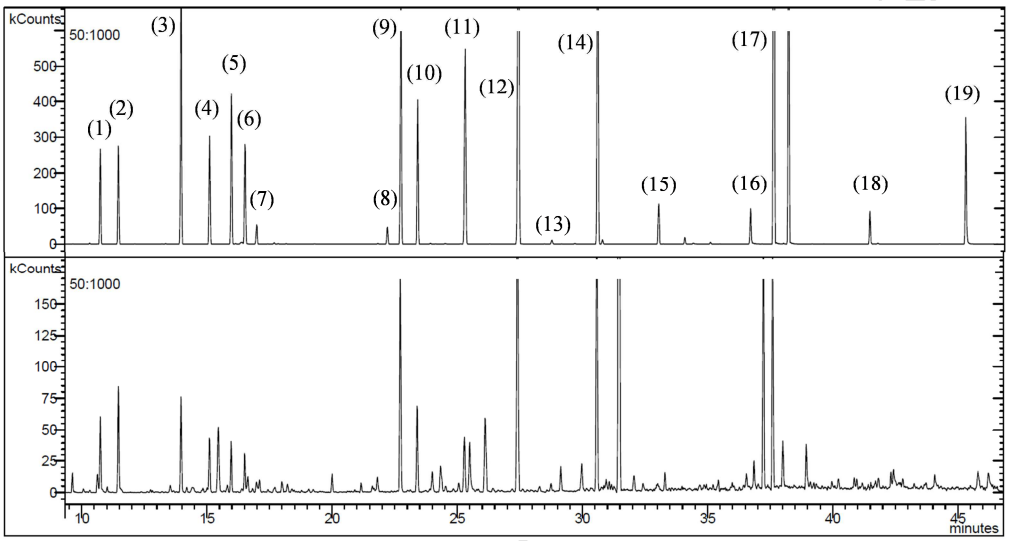

C.

Amino Acid	pmols ± SD
L-Alanine	283 ± 31
L-Arginine	62 ± 4
L-Aspartic acid	194 ± 43
L-Cystine	N.D.
L-Glutamic acid	193 ± 76
Glycine	270 ± 62
L-Histidine	523 ± 72
L-Leucine	383 ± 14
L-Lysine	321 ± 14
L-Methionine	54 ± 2
L-Phenylalanine	130 ± 8
L-Proline	82 ± 24
L-Serine	206 ± 47
L-Threonine	184 ± 16
L-Tryptophan	N.D.
L-Tyrosine	65 ± 23
L-Valine	302 ± 8
L-Isoleucine	231 ± 11

695 **FIGURE 2.** Molecular weight distribution obtained by FPLC of the water-soluble peptides

696 extracted from olive oil.

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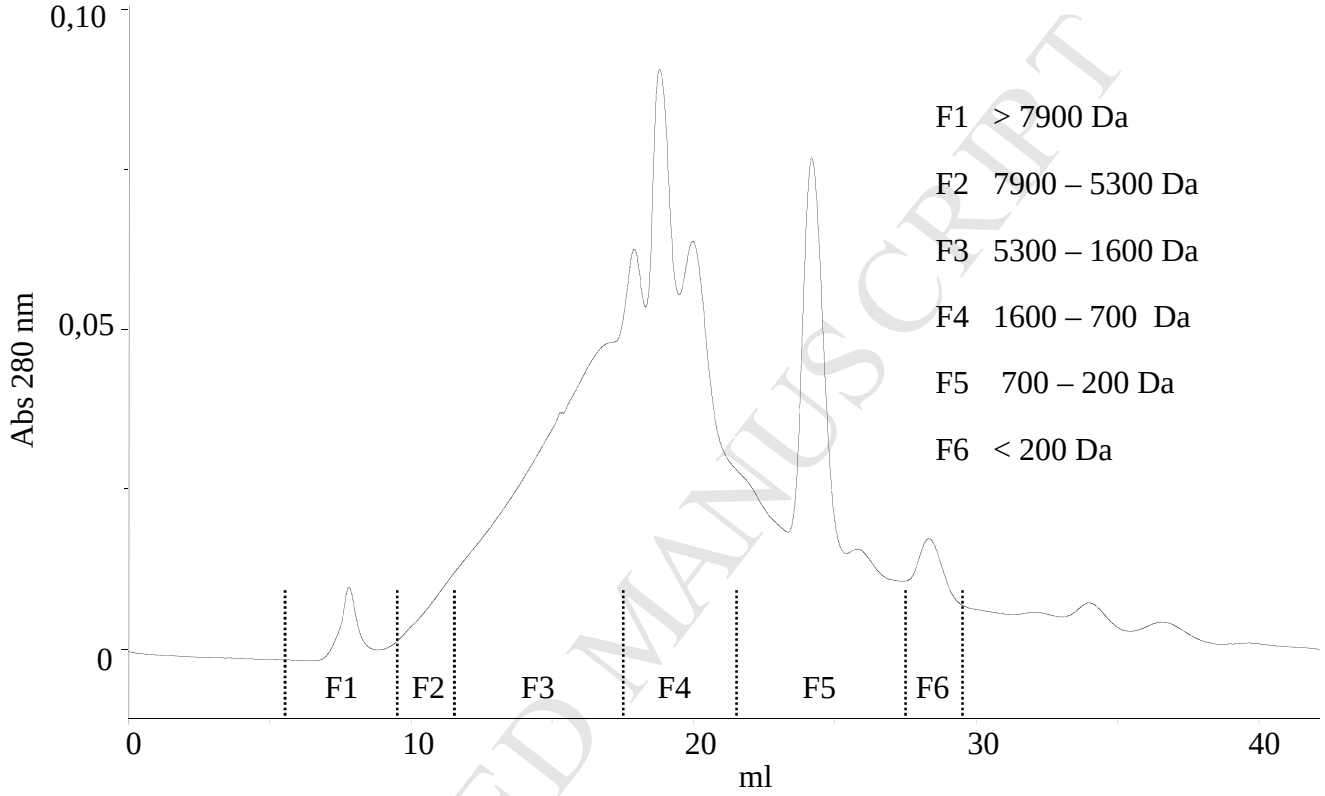

699 **FIGURE 3.** A, angiotensin-converting enzyme inhibitory activity (ACEi) of olive oil water-
700 soluble extract containing peptides. B, calibration curve obtained from the data of panel A and
701 used to determine IC50.

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703 **A.**

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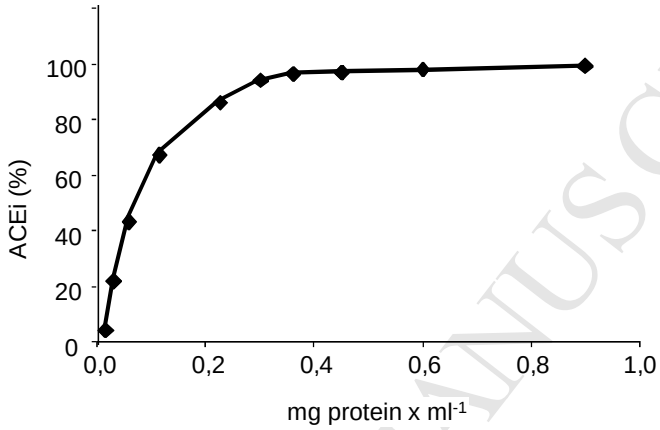

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707 **B.**

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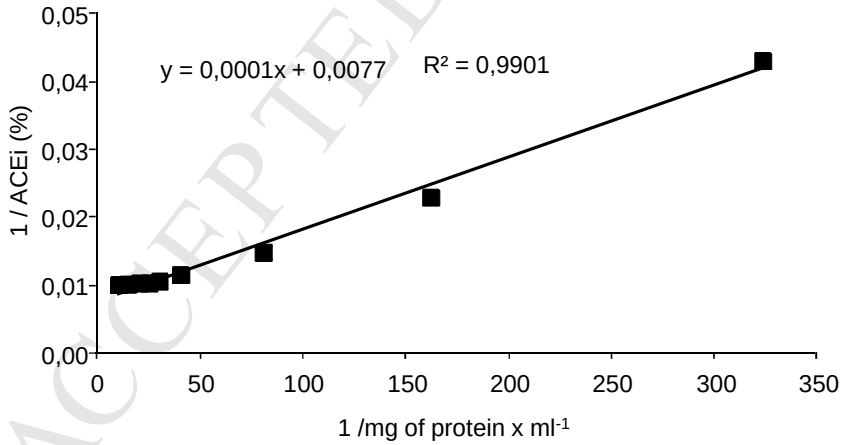

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713 **FIGURE 4.** Systolic blood pressure (SBP) variations (mmHg) detected in spontaneously
714 hypertensive rats (SHR) at baseline and 2, 4, 6, 8, 24, y 48 h after the administration of a dose of
715 water-soluble peptides extracted from olive oil (0.425 mg/Kg BW, ●), Captopril (50 mg/Kg of
716 BW, □), or water control (○). **, significantly different compared with control (P<0.01) and
717 ***, significantly different compared with control and olive oil peptide extract (P<0.01).
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