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Bioavailability of Bioactive Molecules from Olive Leaf Extracts and its Functional Value

Daniel Martín-Vertedor,[1]* María Garrido,[2] José Antonio Pariente,[2] Javier Espino[2] and Jonathan Delgado-Adámez[1]

[1]Technological Institute of Food and Agriculture (INTAEX), Extremadura Government, Avda. Adolfo Suárez s/n, 06007, Badajoz, Spain
[2]Department of Physiology, Neuroimmunophysiology, and Chrononutrition Research Group, Faculty of Science, University of Extremadura, Avda. Elvas s/n, 06006, Badajoz, Spain

Olive leaves are an important low-cost source of bioactive compounds. The present study aimed to examine the effect of *in vitro* digestibility of an olive leaf aqueous extract so as to prove the availability of its phenolic compounds as well as its antioxidant, antimicrobial, and anticancer activity after a simulated digestion process. The total phenolic content was significantly higher in the pure lyophilized extract. Phenolic compounds, however, decreased by 60% and 90% in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), respectively. Regarding antioxidant activity, it was reduced by 10% and 50% after gastric and intestinal digestion, respectively; despite this fact, high antioxidant capacity was found in both SGF and SIF. Moreover, the olive leaf extract showed an unusual combined antimicrobial action at low concentration, which suggested their great potential as nutraceuticals, particularly as a source of phenolic compounds. Finally, olive leaf extracts produced a general dose-dependent cytotoxic effect against U937 cells. To sum up, these findings suggest that the olive leaf aqueous extract maintains its beneficial properties after a simulated digestion process, and therefore its regular consumption could be helpful in the management and the prevention of oxidative stress-related chronic disease, bacterial infection, or even cancer.

Keywords: olive leaf; phenolic compounds; antibacterial activity; antifungal activity; anticancer activity.

BACKGROUND

The European Union dominates world production of olive oil (>70%), and it is also the largest consumer. Spain is the European country with the highest production of olive oil (54%), the Spanish olive oils having been recognized as high-quality oils. Extremadura, a south-western Spanish region, is the third olive oil producer in Spain after Andalucía and Castilla-La Mancha, with about 30 million of olive trees and 53 000 tonnes of annual oil production (Fuentes *et al*., 2015).

Olive leaves are important for the oil industry because they are low-cost source to obtain bioactive compounds and provide an environmental benefit because of the exploitation of residues of oil industries (Briante *et al*., 2002). Moreover, olive leaves could provide a benefit for society because the intake of products rich in polyphenolic compounds leads to the prevention of chronic diseases, thereby reducing the risk of such diseases (El Sedef and Karakaya, 2009).

In recent years, numerous initiatives have opted for the study of plant extracts for their application in the field of nutrition in order to improve food safety by replacing synthetic additives (Delgado-Adámez *et al*., 2014). Substances derived from foods and plants have recently attracted much attention because of their low toxicity, limited costs, broad availability, and potential health benefits. Extraction is one of the most widely used operations in food industry. It is mainly utilized for obtaining certain desired bioactive components initially retained in a food matrix (Pinelo *et al*., 2005). Molecules obtained by extraction present important medical and functional properties (Lee and Lee, 2010; Delgado-Adámez *et al*., 2012a). A wide range of by-products derived from food industry have been studied for this purpose, the attention being focused on those that contain high levels of phenolic compounds. The by-products obtained from fruit and vegetable processing (peel, seeds, and stones) are a special source of these compounds (Schieber *et al*., 2001; Delgado-Adámez *et al*., 2012b). Similarly, olive cake (also called wet pomace) and olive leaf have been considered to be an interesting source of phenolic compounds. In this sense, recent studies have investigated the enrichment of olive oils with a phenolic extract obtained from olive by-products, and it was observed that phenolic compounds in the enriched oils were significantly increased (Japón-Luján and Luque de Castro, 2008).

The absorption and bioavailability of phenolics in humans are controversial. It was widely believed that polyphenols could not be absorbed intact after oral ingestion but were hydrolyzed to their aglycones by bacteria enzymes in the lower gastrointestinal tract. It was further suggested that the aglycones might then be partially absorbed or may undergo further biotransformation by bacteria. Data on these aspects of phenolics are scarce and merely highlight the need for extensive

* Correspondence to: Daniel Martín-Vertedor, Technological Institute of Food and Agriculture (INTAEX), Extremadura Government, Avda. Adolfo Suárez s/n, 06007 Badajoz, Spain.
E-mail: daniel.martin@gobex.es

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investigations of the handling of phenolics by the gastrointestinal tract and their subsequent absorption and metabolism (Karakaya, 2004).

Currently, research in the field has focused the attention on understanding the mechanisms of release of certain food compounds, especially those considered beneficial for human health. However, it is crucial to develop accurate models of digestion that simulate the set of reactions that occur in the interior of the gastrointestinal tract in order to verify the potential bioavailability and effectiveness of these products. *In vivo* methods are more accurate and appropriate, but they are slow and often quite expensive. This is why researchers tend to develop *in vitro* digestion techniques that pose a quicker and cheaper alternative, despite the fact that *in vitro* methods do not manage to achieve similar levels of accuracy compared to the *in vivo* ones because of the inherent complexity of the process. Thus, a series of *in vitro* digestion models have been developed to assess structural changes, bioavailability, and digestibility of foods, indicating that *in vitro* digestion systems are common and useful tools for analyses of foods and drugs (Hur *et al*., 2011).

To further support the functional value of olive leaf extract and increase the value of the by-products generated in the olive industry, the general objective of this research was to assess the antioxidant, antimicrobial, and anticancer effects of olive leaf aqueous extract before and after *in vitro* gastrointestinal process.

MATERIALS AND METHODS

Plant materials. The study was carried out in the Technological Institute of Food and Agriculture of Extremadura (INTAEX). The olive leaves were picked up from the experimental olive (*Olea europaea* L.) orchard maintained by the Researcher Center 'Finca La Orden-Valdesequera' (Badajoz, Spain) within the limits of the olive-growing area 'Tierra de Barros'. Samplings of the olive leaves of 'Arbequina' cultivar were carried out in the morning, taking samples randomly, in perfect sanitary conditions. Specifically, 500 g of olive leaves was taken from different parts of the central area of the olive tree in three times. The samples were collected at spotted stages of maturation, using the subjective evaluation of color of the skin and flesh. After harvesting, all samples were immediately transported to the INTAEX laboratory, using ventilated storage trays to avoid compositional changes. The olive leaves were vacuum-packed (Gustav Müller VS 100, Germany) in plastic bags and stored at −80 °C until further use.

Extraction of bioactive compounds. Olive leaves used in this study were removed from leaders by hand. Olive leaves were partially dried in a conventional oven (model 210, Selecta® P, Spain) for 12 min at 120 °C to obtain the most suitable material to perform the subsequent extraction. Samples were mixed two or three times while they were in the oven to facilitate drying. Dried samples were ground in a domestic knife mill and were sieved to select particles between 0.5 and 3.0 mm. Finally, bioactive compounds were extracted from leaves with ultrapure water (1:10 *w/v*) for 3 h at 60–65 °C, and the extract was filtered and centrifuged at 21,036 ×*g* to remove solid particles (Delgado-Adámez *et al*., 2014).

Stabilization of aqueous extract. The olive leaf extracts were stored at −80 °C until they were freeze-dried in a lyophilizer (Virtis Company, Mod. Génesis 25 LL, Hücoa-Herlos). The dry extracts obtained were kept away from the light (at room temperature) in amber-colored glass bottles until further analysis.

Simulated gastrointestinal digestion. To study the behavior of the olive leaf extract during digestion, control (ultrapure water) and olive leaf extract were subjected to an *in vitro* assay following the method described by Calvo *et al*. (2012). The *in vitro* digestion was carried out in three phases. First, samples were exposed to an oral fluid which contained 1 mL of human saliva g^{-1} of extract. After that, they were mixed at slow speed using an Omni Mixer Homogenizer (Ivan Sorvall, Inc., Norwalk, Conn. USA) during 20 s. A warm bath was used to maintain the temperature around 37 °C. Second, the mix was exposed to simulated gastric fluid (SGF) containing pepsin and sodium chloride at low pH value. Finally, an intestinal digestion was simulated by exposing gastric digestion elements to a simulated intestinal fluid (SIF) containing pancreatin, lipase, as well as cholic and deoxycholic acids in PBS buffer.

The SGF was prepared according to the United States Pharmacopeia method (The United States Pharmacopeial Convention, Inc, 2000) and contained 0.2-g pepsin and 0.125-g sodium chloride in deionized water to give a final volume of 62.5 mL at pH 1.5.

The mix of olive leaf extract (1 g) and saliva was added to 3.6 mL of SGF and the final mixture (pH 2.2) was stirred for 20 min at 37 °C in the digester (K-349; Buchi, Flawil, Switzerland). After that, pH was adjusted to 6.5 with NaOH to inactivate pepsin. The SIF was prepared in 0.1 M PBS buffer (100 mL, pH 3.4) containing 20-mg pancreatin, 5-mg lipase, 10 mM cholic acid, and 10 mM deoxycholic acid (Lee *et al*., 2003). Then, 3.6 mL of SIF was added to the resulting product of the gastric digestion, adjusting the pH to a final value of 6.5. The mixture was stirred for 20 min at 37 °C to complete the intestinal digestion.

After each digestion step (gastric and intestinal), the digestion mixtures were centrifuged at 21,036 ×*g* for 10 min at 4 °C to remove solid particles. Finally, they were quickly stored in amber-colored glass bottles at −80 °C until further analysis.

Chemicals and instruments. HPLC analysis was performed using an Agilent Technologies series 1100 (Agilent Technologies S.L., Madrid, Spain) system equipped with an automatic injector and a diode array UV detector. HPLC mobile phase was provided by Calbiochem (USA and Canada). Folin-Ciocalteau reagent, caffeic acid, and sodium carbonate were procured from Panreac (Barcelona, Spain). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), DMSO, and 6-hidroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic

acid (Trolox) were acquired from Sigma-Aldrich (Madrid, Spain), whereas 2,2′azobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Fluka Chemicals (Madrid, Spain). All enzymes for *in vitro* digestion experiments were purchased from Sigma-Aldrich. All chemicals for the digestive fluids were obtained from Merck (Madrid, Spain). An UV–Vis spectrophotometer model HP8453 (Agilent, Madrid, Spain) was used for antioxidant potential and total polyphenol content analyses. An automatic microplate reader (Infinite M200; Tecan Austria GmbH, Groedig, Austria) was utilized for the cytotoxicity assay.

Individual phenolic compound determination. The individual phenolic compounds was analyzed in both the olive leaf extract and the extract digested in the simulated model (gastric and intestinal) following the method proposed by González-Gómez *et al*. (2009) with some minor modifications. Chromatographic separation was accomplished with a Phenomenex C18 HPLC column (150 mm × 4.6 mm, 5 μm) heated to 35 °C. The mobile phase used for the separation was composed of aqueous TFA 0.1% (A) and acetonitrile (B) in gradient mode set as follows: initial conditions, 10% of B; from 0 to 3 min, 10% of B; from 3 to 15 min, 15% of B; from 15 to 20 min the composition was kept constantly at 15% of B; from 20 to 25 min, 18% of B; and from 25 to 40 min, 30% of B. A period of 5 min was necessary for column equilibration. The flow was fixed at 0.500 mL/min for all the experiments. The injected volume was 5 μL.

Chromatographic data processing was performed using Agilent ChemStation software, and the quantification was done by external standard method. Phenolic compound quantification was achieved by the absorbance recorded in the chromatograms relative to external standards, with detection at 280 nm for oleuropein and flavanol, at 320 nm for phenolic acids and vesbascoside, at 350 nm for flavonoids, and at 360 nm for flavonols. Luteolin derivatives were quantified as milligrams of luteolin 7-O-glucoside, flavanol was quantified in milligrams of epicatechin, and flavonols were measured in milligrams of quercetin-3-O-rutinoside. The other compounds were quantified as themselves.

Antioxidant activity assay. The capacity of radical scavenging of both the olive leaf dry extract and the extract digested in the simulated model (gastric and intestinal) was assessed by the ABTS•+ method (Cano *et al*., 2000). Briefly, 1 mL of the radical cation ABTS was placed in a spectrophotometric cuvette, and 20 μL of the phenolic extract was added. The initial absorbance value at 730 nm was then compared with the absorbance obtained after 20 min of reaction. The results were expressed as Trolox antioxidant equivalents (TAE) that were expressed as mmol Trolox kg^{-1} extract. The calibration curve was done using different Trolox concentrations.

Antimicrobial activity assay

Bacterial strains. Bacterial cultures used in this study were obtained from the Spanish type culture collection (CECT) of Valencia University. The bacterial strain used in the assay of the antimicrobial activity of the extracts was *Escherichia coli 45*.

Determination of antimicrobial activity. The antimicrobial activity was studied following the procedure previously established by Delgado-Adámez *et al*. (2012a). These analyses were carried out in both the olive leaf dry extract, and the extracts digested in the simulated model (gastric and intestinal). The extracts were first solved in water and then diluted to the highest concentration (0.1% *v/v*) to be tested. Afterwards, serial dilutions were made in a concentration range from 0.1% to 0.001% (*v/v*). The target microorganism was cultured in Mueller-Hinton broth (MHB) at 37 °C for 24 h. After that, the suspensions were diluted with 0.5 McFarland standard turbidity and diluted again (1/1000 ratio) in MHB. It was added 180 μL of MHB containing diluted bacteria (~10^5 Colony-Forming Units (CFU) mL^{-1}) and 20 μL of the different solutions of the extracts per well in 96-well microtiter plates. A positive control (containing inoculums but no extracts) and negative control (containing extracts but no inoculums) were included on each microplate to verify any change in absorbance of the olive leaf extract. The contents of the wells were mixed and the microplates were incubated at 37 °C for 24 h under aerophilic conditions. Absorbances at 450 nm were measured at 0 and 24 h in a plate reader. Turbidity readings were related to bacterial growth. The inhibitory effect was calculated using the following formula:

$$\%\text{Inhibition} = \frac{\Delta\text{Abs}_{\text{Reference}} - \Delta\text{Abs}_{\text{Assay}}}{\Delta\text{Abs}_{\text{Reference}}} \times 100$$

where:$\Delta\text{Abs}_{\text{Reference}}$ is the increase in absorbance of control sample.$\Delta\text{Abs}_{\text{Assay}}$ is the increase in absorbance of test sample.

Anticancer activity assay

Cell culture. Human histiocytic leukemia (U937) cell line (ECACC No. 85011440) was grown in RPMI 1640 medium (Lonza, Barcelona, Spain) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C. Given that previous works has proven the efficiency of different olive leaf extracts against leukemia cells (Abaza *et al*., 2007; Fares *et al*., 2011; Samet *et al*., 2014), we used U937 cells to study whether the olive leave extracts maintained their anticancer potential after a simulated digestion.

Cytotoxicity assay. The cytotoxic effects of the olive leaf extract on leukemic U937 cell line were studied by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. The cells were seeded at a density of 5×10^4 cells/well in 24-well plates. The olive leaf dry extract (1 g) was dissolved in 9 ml distilled H_2O and then serially diluted in a concentration range from 0.1% to 0.001% (*v/v*). The aqueous extract digested in the simulated model (both gastric and intestinal digestion) was also serially diluted in a concentration range from 0.1% to 0.001% (*v/v*). Five microliters

of each concentration of both the aqueous extract and the digested-like extracts was applied to the wells of a 24-well plate containing sub-confluent cell cultures. After 24 h of incubation, MTT solution (5 mg/mL) was then added to each well, and the formazan precipitate was dissolved in 200-μL DMSO after 1 h of incubation at 37 °C. The content of the wells was homogenized on a microplate shaker for 5 min. The optical densities (OD) were measured on a microplate reader at a test wavelength of 490 nm and a reference wavelength of 650 nm to cancel out the effect of cell debris. All tests and analyses were run in duplicate, and mean values were recorded. The cell survival curves were calculated as percentage of control values (untreated samples).

Statistical analysis. For statistical studies, SPSS 17.0 software was used (SPSS Inc. Chicago, IL, USA). All analyses were done in quintuple, unless otherwise indicated. Data were expressed as means ± SEM and were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's multiple range test to analyze the effect of the digestion process. The effect of the dilution was analyzed in the antimicrobial and anticancer assays as well. The significance level was set at $p < 0.05$.

RESULTS

Phenolic compounds and antioxidant activity in the olive leaf extract

The individual phenolic compounds were quantified by HPLC using external standard calibration curves for each individual phenolic compound. The linearity range of the analytical procedure ranged from 98.5 to 99.3% of the studied compounds. We used standard phenol concentrations and injected the correspondent standard solution three times. All calibration curves, which were obtained as a function of the integrated peak area, were linear throughout the studied range, with determination coefficients (r^2) > 0.99 for all components. The results obtained in the evaluation of the individual phenolic content are shown in Table 1. Significant quantitative differences were observed in a wide number of phenolic compounds in the olive leaf extract. Thus, it was found that the most abundant compound in the olive leaf extract was oleuropein. In fact, oleuropein represented more than 50% of the total identified compounds. Apart from oleuropein, bioactive molecules such as

Table 1. Composition of the individual phenolic compounds and antioxidant activity obtained in the olive leaf aqueous extract and after gastric and intestinal digestion *in vitro*

Phenolic compounds (mg/kg^{-1})	Olive leaves extract		Simulated gastric digestion		Simulated intestinal digestion	
Phenolic acids						
Gallic acid	1.73 ± 0.14		nd		nd	
Vanillin	0.47 ± 0.15		nd		nd	
p-Cumaric acid	0.81 ± 0.16		nd		nd	
Ferulic acid	nq		nq		nq	
Chlorogenic acid	30.72 ± 0.82		nd		nd	
Caffeic acid	201.5 ± 21.2	b	18.7 ± 0.19	a	nd	
Phenolic alcohol						
Hydroxytyrosol	10 924.7 ± 541.6	c	3195.1 ± 457.6	b	1124.4 ± 142.8	a
Tyrosol	1681.2 ± 111.4	c	369.8 ± 71.6	b	170.2 ± 11.0	a
Secoiridoid derivatives						
Oleuropein	22 541.8 ± 1057.6	c	4050.6 ± 122.5	b	1447.9 ± 155.5	a
Flavonoids						
Luteolin 7-o-glucoside	3741.9 ± 120.1	c	698.7 ± 44.8	b	268.9 ± 17.6	a
Apigenin 7-o-glucoside	2147.1 ± 124.9	c	587.4 ± 11.1	b	257.4 ± 19.5	a
Rutin	357.4 ± 14.7	c	50.7 ± 11.6	b	25.7 ± 8.8	a
Hidrocinamic derivatives						
Verbascoside	914.4 ± 20.7	c	196.4 ± 17.1	b	79.6 ± 14.6	a
Lignans						
Pinoresinol	nd		nd		nd	
Acetoxypinoresinol	nd		nd		nd	
Flavanol						
Epicatechin	21.73 ± 0.37		nd		nd	
Flavonols						
Quercetin-3-O-rutinoside	14.89 ± 0.67		nd		nd	
Quercetin-3-O-galactoside	30.57 ± 1.55	b	11.7 ± 8.6	a	nd	
Kaempferol	5.58 ± 0.42		nd		nd	
Antioxidant activity (mmol Trolox l-1 extract)	15.6 ± 2.01	c	10.3 ± 0.15	b	8.4 ± 0.01	a

Results are expressed as mean ± SEM of three sample replicates. Different small letters in the same row indicate significant statistical differences (Tukey's test, $p < 0.05$) among treatments. nd: not detected; nq: not quantified.

hydroxytyrosol, luteolin, apigenin, and verbascoside were found at high concentration in the extract. Phenolic acids such as vanillin, p-cumaric acid, and caffeic acid were minor compounds, provided its concentration corresponded to 0.5% of total phenolics.

Nevertheless, as depicted in Table 1, the amount of individual phenols showed significant variations ($p < 0.05$) after the different experimental conditions. Thus, the effect of digestion was evaluated by quantifying the phenols in the aqueous micellar phases after the gastric and intestinal digestion steps, which was calculated back to 1 g of olive leaf extract. These findings were compared with the values obtained for the undigested extract in order to address the influence of the digestion process into the aqueous olive leaf extract. Following the digestion process, on average the initial amount of phenolic compounds was reduced by 60% after being subjected to the action of gastric acids *in vitro*. Therefore, 40% of the phenolic compounds would be available to be absorbed by the organism and would enter into the intestinal tract. The lowest values found corresponded to olive leaf extract subjected to intestinal digestion where there was a reduction of 90% of phenolic compounds with respect to the olive leaf fresh extract. That means that 10% of phenolic compounds would be available to be assimilated in the intestinal tract.

Concerning the antioxidant activity of the olive leaf extract, it was measured by the ABTS method and reached a concentration of 15.60 mmol Trolox kg^{-1} extract (Table 1). Interestingly, after gastric and intestinal digestions we found a high antioxidant capacity, i.e. 10.30 and 8.40 mmol Trolox kg^{-1} extract, respectively, which means that it was reduced by 34% and 46%, respectively, compared to the fresh olive leaf extract.

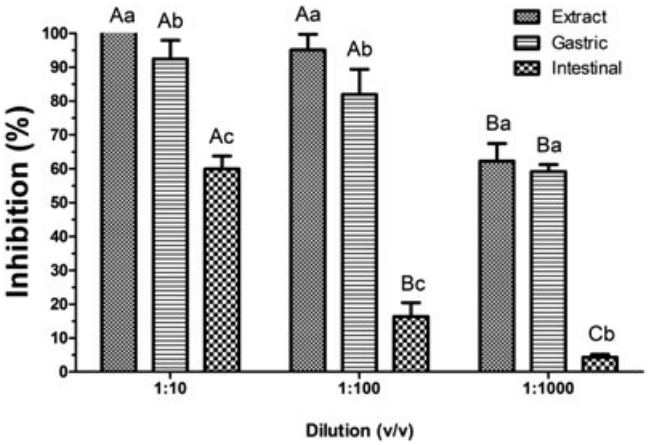

Figure 1. Percentage of inhibition in the aqueous olive leaf extract before and after gastric and intestinal digestion *in vitro* in *Escherichia coli*.. Results are expressed as mean ± SEM of three sample replicates. Different capital letters over the columns indicate significant statistical differences (Tukey's test, $p < 0.05$) along the dilutions in each treatment. Different small letters over the columns indicate significant statistical differences (Tukey's test, $p < 0.05$) among treatments in each dilution.

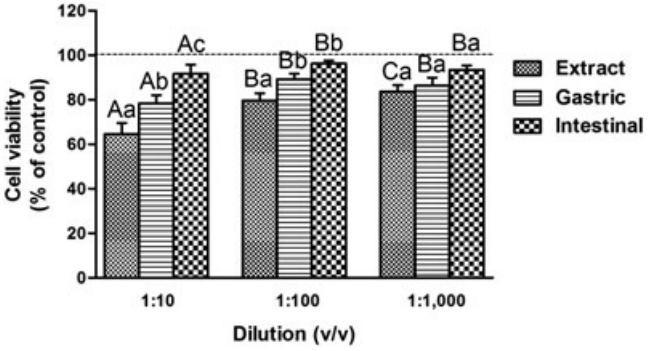

Figure 2. Dose-dependent cytotoxic effect of the aqueous olive leaf extract. After 24 h of exposure to the aqueous or the *in vitro* digested extracts (gastric and intestinal digestion), their citotoxicity towards U937 cell line was determined by the MTT assay. Values are presented as means ± SEM of six independent experiments and expressed as percentage of control values (untreated samples). Different capital letters over the columns indicate significant statistical differences (Tukey's test, $p < 0.05$) along the dilutions in each treatment. Different small letters over the columns indicate significant statistical differences (Tukey's test, $p < 0.05$) among treatments in each dilution.

Antimicrobial activity of the olive leaf extract

The olive leaf aqueous extract and its *in vitro* digestions were screened for their antimicrobial activity against *E. coli* at different dilutions (Fig. 1). The aqueous extract inhibited almost completely the growth of *E. coli* when diluted 1:10 and 1:100, while the inhibition was around 60% for the dilution 1:1000. Certainly, the chemical composition of the olive leaf extract conditioned the antimicrobial effects observed. Additionally, after *in vitro* gastric digestion model, an inhibition superior to 85% was observed in the first two dilutions (1:10, 1:100), while the inhibition was higher than 60% in 1:1000 dilution. Finally, the intestinal digestion produced an inhibition of 60%, 15%, and 5% in the three dilutions, respectively.

Cytotoxic activity displayed by the olive leaf extract

The olive leaf aqueous extract was also used to assay its cytotoxic activity against U937 cells at different dilutions. As shown in Fig. 2, a general dose-dependent decrease in the survival of U937 cells was observed after treatment with the olive leaf aqueous extract. At the highest concentration tested (1:10), the aqueous extract exhibited a substantial, statistically significant ($p < 0.05$) cytotoxic effect, thus reducing the cell viability by 40%. The intermediate concentration (1:100) was partially efficient in diminishing cell viability. In fact, the olive leaf extract provoked a significant ($p < 0.05$) diminution in U937 cell viability (~20%). At the lowest concentration tested (1:1000), the olive leaf aqueous extract displayed poor cytotoxicity. More importantly, after the simulated *in vitro* digestion, either gastric or intestinal, the olive leaf extract retained the cytotoxic potential. As a matter of fact, at the highest concentration tested (1:10), the olive leaf extract diminished U937 cell viability by 20% and 10% after the gastric and intestinal digestion, respectively. At lower concentrations (1:100 and 1:1000), the olive leaf extract showed negligible cytotoxicity.

DISCUSSION

Olive leaves are a cheap raw material that can be used as an adequate supply for products with high added value (Briante *et al*., 2002) and a potential source of natural antioxidants because of its high content in phenolic compounds. It is important to note that the use of methanol or hexane as extractants has been previously rejected owing to its toxicity (Japón-Luján and Luque de Castro, 2008). Thus, we have assayed an aqueous phenolic-rich extract that could be used as a new product for oil industry, given that it is easily obtained and shows feasibility of production. Olive leaves were selected based on previous research whereby we evaluated different aqueous extracts obtained from olive leaves and olive cake so as to determinate the optimal conditions for phenolic extraction (Delgado-Adámez *et al*., 2014). In this way, two variables that potentially influence the process, i.e. drying time and temperature, were optimized in a multivariate study, using total phenolic compounds as independent variable. We found that the extract with less drying time, i.e. 12 min, and higher temperature, i.e. 120 °C, stood out with the significantly highest total phenolic content. Nonetheless, the individual phenolic compounds have been also analyzed herein in order to characterize the olive leaf extract before and after being subjected to the *in vitro* gastrointestinal digestion model (gastric and intestinal steps). Individual bioactive molecules such as oleuropein, hydroxytyrosol, luteolin, apigenin, and verbascoside were found at high concentration in the aqueous extract, while others like vanillin, p-cumaric acid, and caffeic acid were minor compounds. All these compounds were previously reported to occur in olive leaves (Pereira *et al*., 2007a). The quantification of the phenolics present in the aqueous extract revealed a high amount of these compounds that was considerably superior to the values found in hydromethanolic extracts of the same and others olive cultivars, as previously reported (Meirinhos *et al*., 2005). Besides, lignans such as pinoresinol and acetoxypinoresinol were not detected, which is in accordance with the results found by Paiva-Martins and Gordon (2001). Concerning the *in vitro* digestion processing, most of the phenolic acids present in the olive leaf aqueous extract, except for caffeic acid, were found in both the SGF and the SIF in their free form, which is rather unstable during gastric digestion. More importantly, the remaining amount of phenolic compounds after gastric and duodenal digestion was very significant, especially the concentration of oleuropein, which is the most abundant phenol in olive leaves (Japón-Luján and Luque de Castro, 2008). This fact could have great interest because of the protective effects of oleuropein against oxidative damage (Paiva-Martins and Gordon, 2001). Indeed, the ingestion of olive leaf extracts with a high content of phenolics could be desirable for its use with preventive and/or therapeutic purposes (Somova *et al*., 2003).

Regarding the antioxidant activity of the olive leaf extract tested herein (roughly 15 g Trolox kg^{-1} extract), it is clearly higher compared to those previously found in plum leaf extracts, which ranged from 5.08 ± 0.44 to 1.85 ± 0.44 g Trolox kg^{-1} extract (Delgado-Adámez *et al*., 2012b). Besides, when compared to virgin olive oil, our olive leaf extract had 15-fold higher antioxidant activity than virgin olive oil. For instance, Samaniego *et al*. (2007) referred high antioxidant activity with values of 1.00 mmol Trolox kg^{-1} oil in 'Picual' extra-virgin olive oil, whereas Calvo *et al*. (2012) presented values of total antioxidant activity of 1.35 mmol Trolox kg^{-1} oil in 'Morisca' and 'Picual' monovarietal oils. In this sense, some researchers evaluated strategies for the development of a virgin olive oil enriched with aqueous extracts obtained from olive leaf and olive cake in order to increase the dose of phenolic compounds ingested in the diet without the drawback of a high calorie intake (Delgado-Adámez *et al*., 2014). This fact could be especially appealing for the olive industry given that it adds further support to the functional value of virgin olive oil and, at the same time, increases the value of olive by-products. Moreover, European Food Safety Authority (EFSA) has recently accepted the olive leaf aqueous extract as a safe product and, therefore, the olive leaf extract could be used as food additive. In fact, olive leaf extract contain many potentially bioactive compounds that may have antioxidant, antihypertensive, antiatherogenic, antiinflammatory, anticancer, hypoglycemic, and hypocholesterolemic properties (De Marino *et al*., 2014; Fakhraei *et al*., 2014; Olmez *et al*., 2015; Kishikawa *et al*., 2015).

Interestingly, the digested extracts maintained high antioxidant capacity, which may be positively correlated to the amounts of hydroxityrosol, tyrosol, and, especially, oleuropein found after gastric and intestinal digestion because they are known to possess high antioxidant activity (Somova *et al*., 2003; Carrasco-Pancorbo *et al*., 2005). This is of outstanding importance considering that many functional foods are currently designed to provide a high intake of bioactive molecules so as to reduce the risk of diseases associated with aging and oxidative stress (Giugliano and Esposito, 2005; Covas, 2008). Nevertheless, despite the fact that the preparation of olive leaf extract may result in an increase in the bioavailability of phenols in plasma, these promising results should be further confirmed *in vivo* by the determination of the phenolic metabolites (glucuronide, methylated, and sulfated derivatives) in plasma samples after the consumption of olive leaf extract. Indeed, the knowledge of phenolics' bioavailability is essential to understand their conjugations and bioactivities in the organism. Thus, there is evidence that when phenolics are absorbed in the free form, their absorption and conjugation follow the same pathways as that of flavonoids. In addition, phenolic metabolites can retain a strong antioxidant activity and might still exert a significant antioxidant action *in vivo* (Heleno *et al*., 2015).

As for the assay of antimicrobial activity, the entire extracts were used because they may be more beneficial than isolated constituents because a bioactive individual component can change its properties in the presence of other compounds present in the extracts (Borchers *et al*., 2004; Lee and Lee, 2010). Herein, we proved that not only the aqueous extract but also the digested extracts were able to strongly inhibit the growth of *E. coli*. Other researchers have also reported antimicrobial capacity of leaf phenolics, such as walnut leaves (Pereira *et al*., 2007b), olive leaves (Pereira *et al*., 2007a; Lee and Lee, 2010), plum leaves (Delgado-Adámez *et al*., 2012b), and hazelnut leaves (Oliveira *et al*., 2008). The high content of oleuropein and other phenolic compounds identified in the extract, which contributed to

its antioxidant activity, might contribute to its antimicrobial properties as well (Sudjana *et al*., 2009; Lee and Lee, 2010). In particular, oleuropein, has exhibited antibacterial activity against a wide variety of Gram-positive and Gram-negative human pathogenetic bacterial strains (Pereira *et al*., 2007a; Sudjana *et al*., 2009), antifungal properties (Aziz *et al*., 1998), as well as antiviral actions mostly against enveloped viruses (Ma *et al*., 2001; Micol *et al*., 2005). Similarly, chlorogenic acid has been shown to have strong antimicrobial activity (Davidson and Branen, 1981), and is usually present in olive leaf and drupe extracts (Brahmi *et al*., 2013). The active portion of chlorogenic acid, according to Grodzinska-Zachwieja and Kahl (1966), is caffeic acid, a hydroxycinnamic acid. Several hydroxycinnamic acid derivatives have been found to have antimicrobial effects against several microorganisms, including *E. coli* (Lee and Lee, 2010). During the digestion these compounds are reduced, which may be directly correlated with the lower antimicrobial activity found with respect to that obtained in aqueous extracts. Anyway, our findings demonstrated that the use of olive leaves as nutraceuticals may diminish the risk of *E. coli* infections, particularly in the gastric and intestinal tract as observed with 1:10 and 1:100 dilutions, which is probably because of the protective actions provided by its phenolic compounds.

In relation to the anticancer assay, our findings suggested that the digested olive leaf extract could preserve its anticancer potential against human histiocytic leukemia U937 cells, as observed after the *in vitro* digestion processing, thereby indicating that the extract may be able to act at systemic level. Despite the fact that previous research has already tested the anti-proliferative and/or cell killing abilities of different olive leaf extracts against leukemia cells (Abaza *et al*., 2007; Fares *et al*., 2011; Samet *et al*., 2014), none of them has proved whether the extracts maintained their anticancer properties once they entered the gastrointestinal tract. Therefore, olive leaf extracts warrant further investigation into their potential antileukemic benefits.

Overall, our results suggested that the olive leaf aqueous extract, which possessed high content in phenolic compounds, could be absorbed by the gastrointestinal tract, and its regular consumption may be helpful in the management and the prevention of oxidative stress-related chronic diseases, bacterial infection, or cancer. In fact, even after simulated digestion, the olive leaf extract depicted *in vitro* antioxidant, antimicrobial, and antitumor activities.

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Conflict of Interest

The authors have no conflict of interest to declare.

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