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RESEARCH ARTICLE

Pro-/antioxidant and antibacterial activity of olive leaf extracts according to bioavailability of phenolic compounds

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ABSTRACT

The olive leaf extract (OLE) is an aqueous extract from dried olive leaves which contains high amount of phytochemicals with biological activities. The aim of this work was to study the changes in phenolic compounds content, antibacterial and antioxidant activity of the OLE after an *in vitro* gastrointestinal process in presence of intestinal microorganisms. In addition, the matrix effect of major individual phenolic compounds was evaluated. The OLE individual phenolic compounds quantification was determined by HPLC. The OLE contained different phenolic compounds, such as hydroxytyrosol, oleuropein and tyrosol and after digestion, these amount bioavailable detected decreased. Additionally, the antibacterial and antioxidant activity of the extract was also significantly reduced after gastrointestinal digestion. Individual phenolic compounds of OLE showed a high antimicrobial activity against pathogenic bacteria and these showed a synergic and matrix effect in extract. Besides, these compounds showed a concentration-depend pro-/anti-oxidant activity. Therefore, although the extract is rich in phenolic compounds, the levels of these were reduced along digestion process advances, and also their antioxidant and antibacterial activity. In addition, the pro-/anti-oxidant activity shown by the extract and its major phenolic compounds must be delved into, as this may or may not be convenient depending on the future application of the extract.

Keywords: Plant extract; Antioxidant activity; Pro-oxidant activity; Antimicrobial activity; Bioavailability

INTRODUCTION

Bioactive molecules as antioxidants with very important health properties have been found in olive leaves (Martín-Vertedor et al., 2016) which are a potential natural source of polyphenolic compounds (Talhaoui et al., 2015). These could be a low-cost source to obtain bioactive compounds, and thus to provide an environmental benefit due to the exploitation of residues of olive oil industries (Briante et al., 2002). Phenolic compounds cannot be produced by the organism; therefore, these need to be incorporated through diet together with essential nutrients. In addition, the intake of products rich in polyphenolic compounds would provide a benefit for society leads to the prevention of chronic diseases. For example, specifically flavonoids are relevant in the prevention of cancers in stages of initiation, progression and promotion (Moon et al., 2006) and also attributed a positive effect on the cardiovascular system (Castro and Cambeiro, 2003). However, some authors (Wanasundara and Shahidi, 1998; Dintcheva et al., 2017) have described pro-oxidant effects of these polyphenols that could be harmful for health and, therefore, it is necessary to broaden the knowledge in this area.

From dried olives leaves can be prepared an infusion beverage, which could be utilized as aqueous extract to be added to other food products due to their antioxidant and antimicrobial activity (Delgado-Adámez et al., 2016; Amaro-Blanco et al., 2018). Talhaoui et al. (2015) indicated that the major individual phenolic compounds identified in this natural extract are caffeic acid, hydroxytyrosol, quercetin, luteolin, ligstroside, oleuropein, demethyloleuropein, vanillic acid, verbascoside and tyrosol. The main responsible for the antioxidant properties (Skerget et al., 2005) from olive leaf extract (OLE) is oleuropein which can be found in branches and olive leaves coming from residues production in virgin olive oil and table olive manufacture processes. OLE has other biological activities

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such as antivirals and anti-inflammatory activities (Bouaziz et al., 2008). Furthermore, phenolic compounds from olive leaves have antimicrobial activity against important human pathogens such as *Helicobacter pylori*, *Campylobacter jejuni*, *Staphyulococcus aureus* (Sudjana et al., 2009).

The bioavailability and absorption of phenolic compounds when these are consumed is highly controversial because these cannot be absorbed intact and are hydrolyzed by the action of bacteria present in the lower gastrointestinal tract (Karakaya, 2004). Despite this, data in this field is scarce, and thus, merge the need for exhaustive studies on the processes because its concentration implies possible beneficial or toxic effects. Currently, different models of *in vitro* digestions have been developed in order to verify their bioavailability and effectiveness during the gastrointestinal process (Bouayed et al., 2012). These digestion systems are useful tools for the understanding of the bioavailability of certain compounds in food after the digestion process.

Many studies have evaluated individually the properties of bioactive compounds, but in nature these compounds are not isolated and interact with each other, enhancing in some cases their activity, this is known as synergic effect. On the other hand, the compounds can act in one way or another depending on the medium in which they are located and the composition thereof. These modifications of its properties depending on the set of molecules that surround them, it is known as matrix effect.

Therefore, the aims of the present study were to investigate the antibacterial and antioxidant capabilities and the bioavailability of olive leaf extract after an *in vitro* gastrointestinal process in presence of certain microorganisms, as well as, to evaluate the possible synergic and matrix effect of phenolic compounds from OLE. As far as we know, no previous study includes the effect of the microorganism presence on the *in vitro* digestion in order to evaluate the bioavailability of phenolic compounds.

MATERIAL AND METHODS

Monitoring of raw material

Olive leaves were picked up from a local company within the limits of the olive-growing area "Tierra de Barros" (Badajoz, Spain). The olive leaves samplings of "Carrasqueña" cultivars. The olive leaves were vacuum-packaged (Gustav Müller VS 100, Germany) in plastic bags (500gr) and these were frozen (-80ºC) until their use.

Extraction of bioactive compounds of the olive leaves

Olive leaves were washed with distilled water and they were partially dried in a conventional-oven (model 210, Selecta® P, Spain) at 120ºC for 12min to obtain a dried material. Samples were mixed two or three times while they were in the oven to facilitate the drying process. Dried samples were ground in a domestic knife mill and were sieved to select particles between 0.5 to 3.0mm. Finally, bioactive compounds were extracted with water (60–65ºC) for 3h and the extract was filtered and centrifuged at 21036×*g* to remove solid particles from it (Martín-Vertedor et al., 2016). The OLE was frozen at -80°C until its analysis.

Experimental design

The OLE was submitted to an *in vitro* gastrointestinal digestion and analysed at the beginning and after the different steps of the process. The phenolic compounds composition, the antioxidant and antimicrobial activity of the OLE was evaluated in order to know the changes of the bioactive compounds present in the extract during oral, gastric, short and large intestine digestion phases.

In order to evaluate the matrix effect on the antioxidant and antimicrobial activity of individual phenolic compounds present in OLE, different dilutions of the extract (at 1:10v/v and 1:100v/v) were mixed with standards of oleuropein, hydroxytirosol and tyrosol. The different combinations of compounds analysed are summarized in Table 1.

Simulated digestion of the olive leaf extract

The OLE was submitted to an *in vitro* assay in order to study the behavior of during digestion, following the method described by Martín-Vertedor et al. (2016) slightly adjusted.

After each *in vitro* digestion, the mixtures were centrifuged at 21036×g for 15min at 4ºC to remove solid particles and these were filtered through 0.22µm nylon filters.

Table 1: Experimental design followed to evaluate the matrix effect between phenolic compounds of OLE. Individual standards of phenolic compounds and a mixture (multi-standard) was evaluated as well diluted OLE mixed with standards

	Oleuropein (mg/kg)	Tyrosol (mg/kg)	Hydroxytyrosol (mg/kg)	Abbreviations
Extract 1:10v/v	-	-	-	E. 1:10
	2000	-	-	E. 1:10+Oleo
	-	2000	-	E. 1:10+Ty
	-	-	2000	E. 1:10+Hydro
Extract 1:100v/v	-	-	-	E. 1:100
	2000	-	-	E. 1:100+Oleo
	-	2000	-	E. 1:100+Ty
	-	-	2000	E. 1:100+ Hydro
Individual standards	2000	-	-	Oleo
	-	2000	-	Ty
	-	-	2000	Hydro
Multi-standard	2000	2000	2000	Mixture

HPLC analysis of phenolic compounds of the olive leaves extracts in fresh and after *in vitro* digestion

Reagents, solvents and phenolic standards

Standards of polyphenolic compounds were supplied by Extrasynthése (Genay, France), MERK-Schuchardt (Hohenbrunn, Germany), Sigma-Aldrich Chemie (Steinheim, Germany) and FlukaChemie (Steinheim, Germany). Standard solutions were prepared in methanol and stored in darkness at -20ºC. HPLC mobile phases were prepared with HPLC grade methanol and acetonitrile (Fisher chemical; Loughborough, UK) and P.A. grade formic acid (PANREAC; Barcelona, Spain).

Reversed-phase-HPLC analysis of the olive leaves extracts in fresh and after in vitro digestion.

The HPLC analysis was carried out following the method and conditions described by Cabrera-Bañegil et al. (2018). Main phenolic compounds analysis was carried out with Agilent 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany), which was equipped with a diode array detector (DAD) and fluorescence detector (FLD). Also, for this determination was used a Gemini-NX C18 column (150x4.6mm i.d., 3µm thickness, Phenomenex).

Antioxidant activity assays of the olive leaf extract

The antioxidant capacity of the extract samples was determined by the ABTS•+ method (Turoli et al., 2004). For this assay, 100µL of ABTS (2.2'-azinobis (3-ethylbenzoithiazolone 6-sulphonate)) and 10µL of the extract (fresh or after digestion, with/without individual phenolic compounds, depending on the experiment) was added in 96-well microtiter plates. The absorbance value was determined at 730nm. The results were expressed as mmolTrolox L^{-1} using a calibration curve of Trolox.

Antibacterial activity of the olive leaf extract

Antibacterial activity of OLE after gastrointestinal digestion

Bacteriostatic capacity was chosen to be determined by broth dilution method because the extracts after digestion did not exhibit bactericidal activity (data not shown). The procedure previously established by Delgado-Adámez et al. (2012a) was followed. The bacterial strains employed in this study were *Escherichia coli* (CECT 45) and *Listeria innocua* (CECT 910), Gram-negative and Gram-positive, respectively. The lyophilized extracts were solved in water and diluted (0.1%v/v) to use. Bacteriostatic activity in large intestine phase is not possible to analyze because this step include microorganism which interfere in the measure. To calculate antibacterial activity, it used the following formula:

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

$\Delta\text{Abs}_{\text{Reference}}$ corresponds to the increase in control sample absorbance.

$\Delta\text{Abs}_{\text{Assay}}$ corresponds to the increase in test sample absorbance.

Antibacterial activity of individual or mixed standard compounds with OLE.

The antibacterial activity was determined by the paper disc diffusion method described previously by Kim et al. (2000). Samples (Table 1) were dissolved in 1% of DMSO and sterilized by filtration in a 0.20µm cellulose acetate filter (Chromafil CA-20/25S). A 100µl of bacterial suspension (10^8 CFU/ml) was grown in a nutrient agar medium (Mueller-Hinton agar, Oxoid). The paper discs (6mm diameter) were placed on the agar and impregnated with 10µl of each sample. The plates were incubated at 37ºC for 24h. The antibacterial activity was measured by determination of the inhibition zone in each plate and disc.

Statistical analysis

Three replicates per treatment were analyzed. The results were statistically analyzed by SPSS 18.0 statistical software (SPSS Inc., Chicago, IL) and were analyzed by ANOVA and Tukey´s multiple range test and t-Student test. Pearson correlation was carried out to understand any relation between some analyzed parameters. Statistical significance was accepted at a confidence level $p<0.05$.

RESULTS AND DISCUSSION

Olive leaf extract and changes after *in vitro* digestions

Phenolic compounds content of olive leaf extract and changes after in vitro digestions

The results obtained in the evaluation of the individual phenolic compounds content in OLE are shown in Table 2. Major phenolic compounds in OLE were hydroxytyrosol, oleuropein, luteolin 7-o-glucoside, apigenin 7-o-glucoside and verbascoside which represented more than 90% of the total compounds of the extract. Flavanol, flavonols and phenolic acids were the minor compounds, corresponding to the 0.8% of total phenolics. The elaboration process of the extract was optimized for the preservation of phenolics compounds (Delgado-Adámez et al., 2014). These compounds were water-soluble since we used aqueous phase to obtain the extract. Methanol or hexane were rejected owing to its toxicity (Japón-Luján and Luque de Castro, 2008). In fact, the amount of the phenolics present in the aqueous extract revealed a high amount that was considerably superior to the values found in hydromethanolic extracts (Meirinhos et al., 2005). The amount of phenolic compounds are an important factor when we assess the extracts quality (Delgado-Adámez et al., 2014).

Table 2. Polyphenols concentration of olive leaves extracts (mg/kg) in fresh and after different treatments. Results are expressed as means±SD of the three sample replicates

Phenolic compounds (mg/kg)	Olive leaves extract	Oral phase	Gastric phase	Small intestine phase	Large Intestine phase	Control large intestine phase
Phenolic acids						
Gallic acid	1.55±0.11	nd	nd	nd	nd	nd
Vanillin	0.54±0.16	nd	nd	nd	nd	nd
p-cumaric acid	0.97±0.21	nd	nd	nd	nd	nd
Ferulic acid	nd	nd	nd	nd	nd	nd
Chlorogenic acid	31.52±0.36	nd	nd	nd	nd	nd
Caffeic acid	222.50±11.20^{a}	156.40±8.20^{b}	101.50±2.10^{c}	95.40±3.40^{c}	64.60±4.20^{d}	89.60±2.40^{c}
Phenolic Alcohol						
Hydroxytyrosol	10856.00±154.60^{a}	8325.10±211.60^{b}	5255.50±141.70^{c}	4699.70±68.40^{d}	1201.40±42.15^{f}	3655.40±28.4^{e}
Tyrosol	1741.20±101.20^{a}	1625.40±25.30^{a}	741.20±21.60^{b}	655.10±10.40^{b}	265.90±9.40^{d}	469.80±10.40^{c}
Secoiridoids derivatives						
Oleuropein	22421.00±564.70^{a}	17545.70±155.40^{b}	7587.90±107.80^{c}	5645.80±55.50^{d}	1546.90±54.10^{e}	5060.70±14.80^{d}
Flavonoids						
Luteolin 7-o-glucoside	3841.50±21.90^{a}	3201.50±55.90^{b}	2502.70±25.90^{c}	2064.40±11.70^{d}	755.40±16.80f	1621.80±11.70^{e}
Apigenin 7-o-glucoside	2255.70±26.50^{a}	2100.80±12.40^{b}	687.90±9.70^{c}	564.70±9.80^{d}	257.40±19.50f	398.70±11.60^{e}
Rutin	465.90±11.40^{a}	402.10±9.70^{b}	109.90±8.40^{c}	65.90±9.70^{d}	29.80±2.60^{e}	43.90±2.60^{d}
Hidrocinamic derivatives						
Verbascoside	1005.80±10.70^{a}	955.80±11.50^{a}	356.70±10.40^{b}	269.70±11.50^{c}	101.50±11.70^{d}	225.40±9.80^{c}
Lignans						
Pinoresinol	nd	nd	nd	nd	nd	nd
Acetoxypinoresinol	nd	nd	nd	nd	nd	nd
Flavanol						
Epicatechin	25.90±0.99^{a}	21.40±2.80^{a}	9.80±2.40^{b}	8.80±2.10^{b}	3.55±2.40^{c}	4.55±2.10^{c}
Flavonols						
Quercetin-3-O-rutinoside	15.80±0.55^{a}	16.50±2.10^{a}	6.40±2.10^{b}	5.50±1.60^{b}	2.50±1,70^{d}	3.61±1.80^{c}
Quercetin-3-O-galactoside	32.60±2.10^{a}	29.80±4.50^{b}	15.80±2.10^{c}	12.60±2.10^{d}	5.40±2.10^{e}	13.90±3.60^{d}
Kaempferol	5.58±0.42	nd	nd	nd	nd	nd

$^{a-f}$: Different small letters in same row indicate significant statistical differences (Tukey's Test, $p<0.05$) during gastrointestinal digestion, nd: non-detected

OLE was submitted to an *in vitro* gastrointestinal digestion model and changes in the individual phenolic compounds were also evaluated (Table 2). The extent of digestion was evaluated by quantifying the phenols in the aqueous micellar phases after the oral, gastric, small and large intestinal phase. Amounts of individual phenols showed significant differences ($p<0.05$) after the different digestion phases. The enzymatic activity in the oral phase caused a significant ($p<0.05$) decrease on the phenolic compounds content reducing them by 30%. Main phenolic compounds like hydroxytyrosol, oleuropein and luteolin 7-o-glucoside suffered a decrease of 23, 22 and 17% respectively and other minor compounds were not detected after oral phase simulation. Therefore, the bioavaliability of phenolic compounds decreased after the oral phase.

After being subjected to the action of gastric acids *in vitro*, the initial amount of phenolic compounds was reduced by 35-70%. In this step, the compounds that suffered an important reduction were hydroxytyrosol, tyrosol, oleuropein, apigenin 7-o-glucoside and verbascoside which decreased 51, 57, 66, 69 and 64% respectively compared to the fresh extract and 36, 54, 56, 67 and 62% compared to the oral step. Therefore, phenolic compounds showed important reductions before the intestinal phase.

In the small intestine phase, the content analyzed was also decreased with respect to gastric phase where the most important decrease was suffered by oleuropein which lost a 25% of its concentration.

Finally, the lowest values found corresponded to OLE subjected to large intestine phase where there was a release the range of 6 to 29% of phenolic compounds to be assimilated in the intestinal tract. The majority of OLE phenolic compounds like hydroxytyrosol, tyrosol, oleuropein, luteolin 7-o-glucoside, apigenin 7-o-glucoside and verbascoside were reduced to 11, 15, 7, 19, 11 and 10% respectively in compare with the initial concentration. Therefore, from the initial extract most phenolic compounds are lost (around 90%) and only the 10% would be available. It is important to remark, the remaining amount of phenolic compounds after digestion was very significant, specially, hydroxytyrosol

and oleuropein, which are the one of the most abundant phenols in extract.

The same decrease tendency was obtained by other researchers (Bouayed et al., 2012; Martín-Vertedor et al., 2016) which found that the phenolic compounds were progressively degraded after digestion process, around 50% in different apple varieties and 90% in OLE respectively. In simple matrices, these compounds are unprotected and therefore these are released slowly and easily assimilated by organism but the complexity of some food matrices has a protective character that helps to maintain the compounds amount after digestion process (Acosta-Estrada et al., 2014). A high bioavailability was found when the compounds were mixture with an oily matrix which are more absorbable than the compounds in an alimentary matrix (de Valle-Prieto et al., 2017).

Despite the high reduction during digestion, the OLE presented a considerable amount of phenolic compound after the digestion process. This fact is relevant by the amount of hydroxytyrosol, tyrosol, oleuropein which are known to possess high antioxidant activity (Carrasco-Pancorbo et al., 2005). These evidences can be interesting to slow the oxidative processes during the food digestion.

Antioxidant activity of olive leaf extract and changes after in vitro digestions

OLE presented high antioxidant activity measured by ABTS method (Fig. 1). The value of 15.9mmol Trolox L^{-1} corresponds to the extract and it is higher antioxidant activity than those reported in other extracts such as plum extract (Delgado-Adámez et al., 2012b). In fact, our olive leaves extract had higher antioxidant activity, 15-fold more than other foods such as virgin olive oil (Delgado-Adámez et al., 2014). Subsequently, after oral phase the antioxidant capacity was maintained similar to the fresh extract. However, it significantly decreased with olive leaves matrix degradation in next phases. The lowest antioxidant content was found in the final phase in large intestine, where it maintained the 34% of its initial antioxidant activity. These results are in concordance with the phenolic compounds decrease after gastrointestinal simulation, particularly with the oleuropein, hydroxytyrosol and tyrosol, phenolic compounds with high antioxidant capacity (Carrasco-Pancorbo et al., 2005).

Other researchers (He et al., 2015), showed that changes in temperature, pH and digestion process can generate an important decrease and inhibition of the main proteins and compounds in fruit juice-milk beverage and consequently in its activities. This are in concordance with the results of our research.

Concretely, the different polyphenol concentration of OLE present a positive correlation (Pearson's correlation coefficient) to the antioxidant activity throughout gastrointestinal digestion. The caffeic acid ($r=+0.87$, $p<0.01$), hydroxytyrosol ($r=+0.97$, $p<0.001$), tyrosol ($r=+0.98$, $p<0.001$), oleuropein ($r=+0.97$, $p<0.001$), luteolin 7-o-glucoside ($r=+0.96$, $p<0.001$), apigenin 7-o-glucoside ($r=+0.95$, $p<0.001$), rutin ($r=+0.93$, $p<0.01$), verbascoside ($r=+0.97$, $p<0.001$), epicatechin ($r=+0.96$, $p<0.001$), quercetin-3-O-rutinoside ($r=+0.95$, $p<0.001$) and quercetin-3-O-galactoside ($r=+0.98$, $p<0.001$). These results show that there is an important relationship between the decrease in antioxidant capacity of OLE and the bioavailability of its phenolic compounds, but despite that reduction, antioxidant activity remained at a high level.

Antimicrobial activity of olive leaf extract and changes after in vitro digestions

Fig. 2 shows the antimicrobial activity of OLE against *Escherichia coli* (Gram-negative bacteria) and *Listeria innocua*

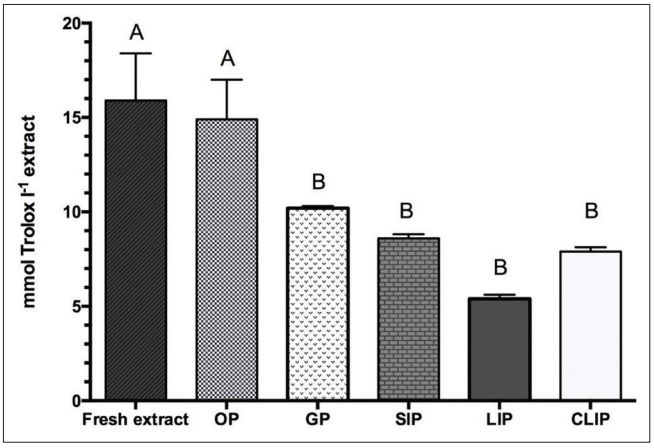

Fig 1. Antioxidant activity of OLE fresh and after gastrointestinal digestions phases. Fresh extract, after oral phase (OP), gastric phase (GP), short intestine phase (SIP), large intestine phase (LIP) and control large intestine phase (CLIP). Results are showed means±SD. A-C Means not sharing a common capital letter show significant differences ($p<0.05$) by Tukey's multiple test between treatments.

(Gram-positive bacteria). The extract had the same antimicrobial activity against the two microorganisms; however, it was significantly modified after digestions. The fresh extract had a high antibacterial activity, and after gastric digestion it maintained more of 90% of its activity however in final intestinal digestion the OLE lost around 40% of its antimicrobial activity, same results that showed in antioxidant capacity. These results are in contrast to what other authors have observed in other plants extracts like plum leaves extracts and grape seeds aqueous extract (Delgado-Adámez et al., 2012a, 2012b) in which the antibacterial activity was higher against gram-negative bacteria. In spite of the important reduction of phenolic compounds during digestion, the antimicrobial activity remained in an important level after gastrointestinal simulation process. A possible hypothesis can be that the decrease in phenolic compounds during digestion can generate transformations in them and the accumulation of other compounds with antibacterial activity. Or also, that the antibacterial activity provided by OLE is also due to the action of non-phenolic compounds.

Olive leaf extract and matrix effect of the pure major phenolic compounds

Antioxidant activity of olive leaf extract and pure major phenolic compounds

Fig. 3 shows the antioxidant activity of individual standards (tyrosol, hydroxytirosol and oleuropein) and/or dilutions of OLE (1:10 and 1:100) as well as the mixture of individual standards (multi-standard) of phenolic compounds. The results show that the fresh extract has dose-dependent antioxidant activity, because this activity decreased when OLE concentration was diluted. Pure standard compounds have an important antioxidant activity when these acts individually; tyrosol had the greatest capacity and oleuropein the lowest. This activity increased when the compounds were together; the mixture of major phenolic compounds in OLE (multi-standard) did not show a synergic effect because this activity was lower than the sum of its individual activities.

When individual compounds were added to diluted OLE (1:10 and 1:100), different effects were observed

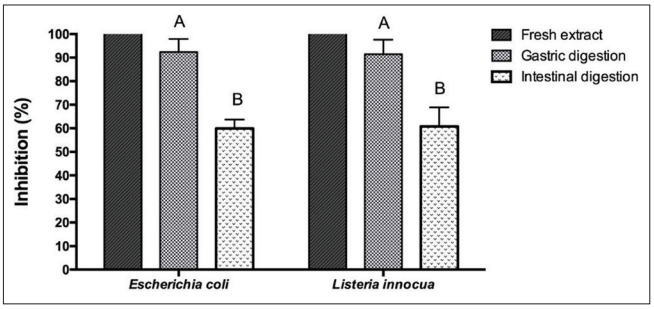

Fig 2. Bacteristatic activity of fresh extract, fresh extract after gastric digestion and fresh extract after intestinal digestion (short intestine phase) against *Escherichia coli* and *Listeria innocua*. Antibacterial activity is showed as means with standard deviations. A-C Means not sharing a common capital letter show significant differences ($p<0.05$) by Student t-test in the same microorganism group.

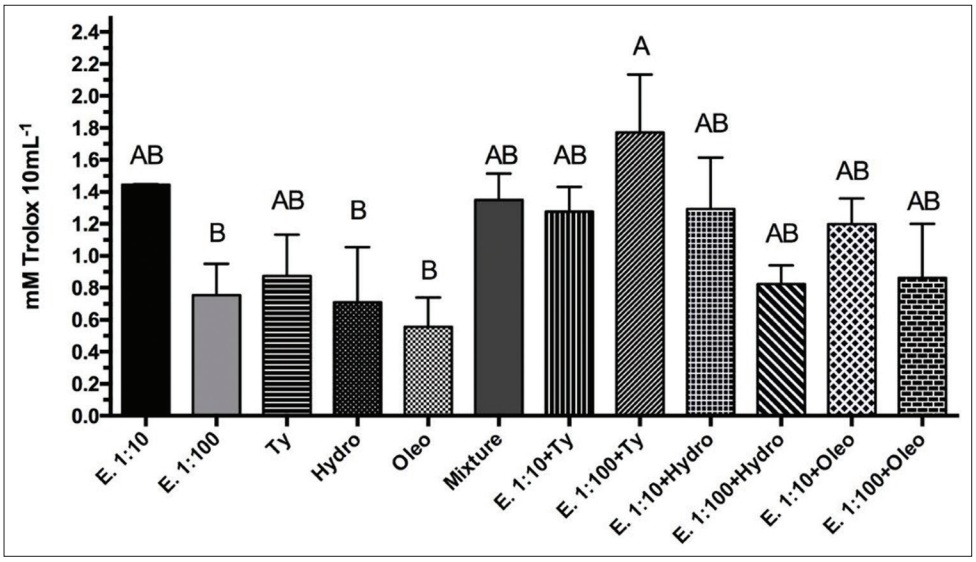

Fig 3. Antioxidant activity of hydroxytyrosol, tyrosol, oleuropein, fresh extract (in different dilutions), fresh extract (in different dilutions) with added standards phenolic compounds and the compounds mixture. Antioxidant activity is showed as means with standard deviations in mM Trolox $10ml^{-1}$. A-B Means not sharing a common capital letter show significant differences ($p<0.05$) by Tukey's multiple test between different samples.

depending on the individual standard added to the extract. In the case of OLE with oleuropein or hydroxytyrosol, the antioxidant activity between different dilutions of OLE was the same when these compounds were added. However, in the case of OLE with tyrosol, the antioxidant activity in 1:10 dilution was lower than 1:100 dilution. This fact could be explained because the concentration of phenolic compounds reaches to a top level and its antioxidant activity was converted to pro-oxidant activity, so the increases of the antioxidant activity are not a sum of the values of both separately, as it could be expected. Some authors have already observed these effects. Melo et al. (2016) reported that açai seed extracts applied in bulk soybean oil had a concentration-dependent pro-oxidative activity. Moreover, other authors (Wanasundara and Shahidi, 1998), have also indicated that one of the molecules responsible of this pro-oxidant effect in plants extracts are the chlorophylls, and when these are eliminated of plant material, the extract returned to have a high antioxidant activity. In addition, according to our results, not only the chlorophylls could be responsible for the pro-oxidant activity, but also the concentration of individual phenolic compounds.

The preservation of phenolic compounds of OLE could be desirable for its use with preventive and/or therapeutic purposes due to the protective effect of this group of phenolic compounds against oxidative damage in human red blood cells (Paiva-Martins and Gordon, 2001). For this reason, many functional foods are currently designed with the goal of providing a high intake of active biomolecules to reduce the risk of diseases associated with aging and oxidative stress (Covas, 2008). For this reason, the goal of this study is to know in depth the relationship between major phenolic compounds of the extracts and their antioxidant activity.

Antibacterial activity of olive leaf extract and pure major phenolic compounds

The results of the antimicrobial activity of the individual phenolic compounds and the mixture of them with the OLE by diffusion disc method are shown in Fig. 4. The OLE antibacterial results are different that the showed by broth dilution method because this method (paper diffusion disc) only measure the bactericide activity not the bacteriostatic activity. The standard of hydroxytyrosol and the mixture of hydroxytyrosol, tyrosol and oleuropein (Mixture) presented the highest antimicrobial activity against Gram-negative *E. coli* bacteria with significant differences (Fig. 4A) respect to Hydro and Oleo standards. Diluted OLE and the extracts with standards did not show antibacterial activity. The mixture of pure standards with OLE (1:10 and 1:100) did not present synergic effect between them because the antimicrobial activity was not increased as we could expect. Other researchers (Sanhueza et al., 2017) have explained that the synergic effect cannot increase because this effect is generally not due to a single compound, but the effect is due to the combined action of several compounds which can act positively and/or negatively. However, the same study has also reported a great synergistic potential by increasing the amount of phenolic compounds in grape pomace to enhance its antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*. When the antimicrobial activity of the OLE with different dilutions was evaluated, we have not found activity against this bacterium and also, when the OLE was mixed with the individual standards, antimicrobial activity was not found against this bacterium. Therefore, the addition of standards to fresh OLE showed a negative matrix effect because it did not allow that the phenolic standards compounds develop their antimicrobial activity.

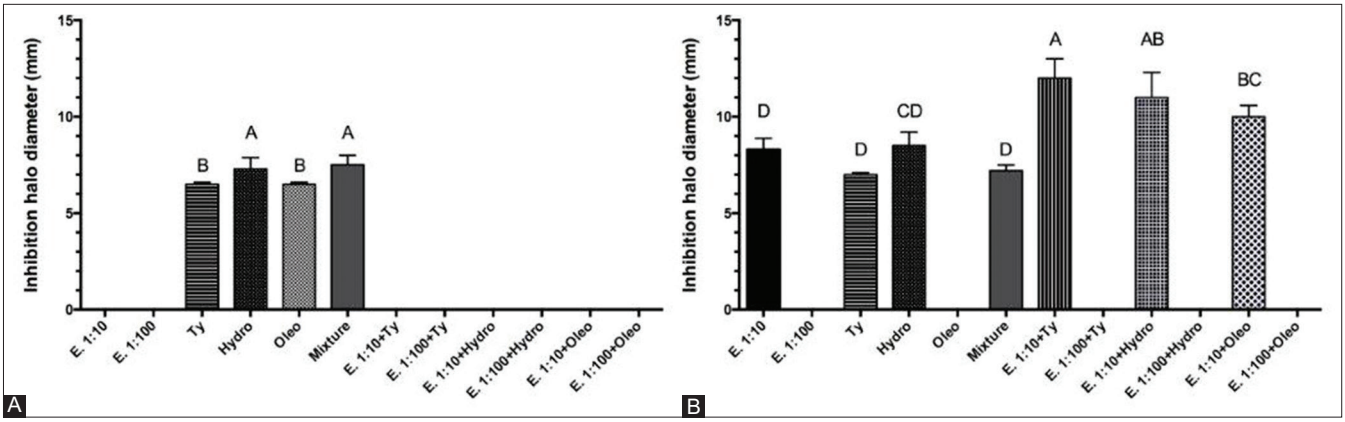

Fig 4. Bactericide activity of hydroxytyrosol, tyrosol, oleuropein, fresh extract (in different dilutions), fresh extract (in different dilutions) with added compounds and the compounds mixture against *Escherichia coli* (A) and *Listeria innocua* (B). Antibacterial activity is showed as means with standard deviations in millimeters. A-D Means in the same microorganism not sharing a common letter show significant differences ($p<0.05$) by Tukey's multiple test between different compounds assays.

Moreover, the phenolic compounds standards that presented antimicrobial activity against *L. innocua*, a Gram-positive bacterium (Fig. 4B), were tyrosol and hydroxytyrosol. In this case, the oleuropein standard did not show any activity. In addition, the fresh extract (dilution 1:10) had antibacterial activity against this bacterium while the dilution 1:100 did not show activity. Besides, the activity of the phenolic mixture was similar to tyrosol and less than hydroxytyrosol, and thus, this did not show synergy activity because it did not increase exponentially.

However, when individual standards were added to OLE (1:10), the antimicrobial activity was significantly increased. This can be explained because exist a positive matrix effect, due to the fact that when the standards were inoculated into the fresh extract, the growth of the bacteria was clearly neutralized in compared to the pure standards with significant differences. According to other studies, the matrix effect of OLE can play a key role on the ability of phenolic compounds to neutralize microorganisms (Cebeci and Sahin-Yesilcubuk, 2014). The positive matrix effect can be explained by the positive synergic effect of the fresh extract over the pure standards. This may be due to the action of other compounds presents in the extract and which its antimicrobial activity has not been yet analyzed.

The mechanism of action of phenolic compounds has not been yet determined. However, it has been observed that these can inactivate essential enzymes, react with cell membrane or alter the function of the genetic material (Martillanes et al., 2017). The differences in the antimicrobial activity of the OLE between two microorganisms employed in this study, can be explained due to the structural differences in the bacterial cell walls of the Gram-positive and gram-negative groups. On the other hand, the great diversity of compounds presents in OLE may also play a key role in the differences found in the matrix effect, which are much present in nature and sometimes these extracts and natural antioxidant do not act as we expect (Delgado-Adámez et al., 2016).

For these reasons, olive leaf extracts can be used as a suitablefood additive with high added value (Briante et al., 2002) and a potential source of natural antioxidants and antimicrobial compounds due to its high content in phenolic compounds, overall taking into account that EFSA (European Food Safety Authority) has accepted the olive leaves aqueous extract as a safe product. Therefore, the intake of phenolic compounds from this extract would allow their use as a new product due to its great benefits. Furthermore, the ingestion of olive leaf extract with high phenolic compounds content, with antioxidant and antimicrobial activity could be desirable for its use for therapeutic purposes and to increase the shelf-life of food (slowing down the oxidation reactions and the microbial growth).

CONCLUSIONS

The olive leaf extract contained high amounts of phenolic compounds such as tyrosol, hydroxytyrosol and oleuropein. When the olive leaf extract was submitted to conditions similar to digestion process total phenolic compounds were significantly reduced, but much of the total antioxidant and antibacterial activity were maintained. Therefore, the levels of antioxidant or antimicrobial activity of the extract make it interesting to be intake could possess also a therapeutic use.

The study of the bioavailability and the synergistic effect of phenolic compounds is complex but necessary to advance in the possible applications of extracts. In this case, OLE presented phenolic compounds which produce a synergic and positive matrix effect against the proliferation of *Listeria innocua* but in other cases a pro-oxidant effect depending of the levels of individual phenolic compounds. Further studies need to be developed to evaluate in depth to these interactions because there is not much literature on this subject that shows evidence of the pro-oxidant (toxic) factors that phenolic compounds could carry on.

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Conflict of interest

The authors declare that there is no conflict of interest.

Authors' contributions

JRP conducted the experiments and wrote the original draft. DMV designed the experiments. RR reviewed and edited the article to be published. JDA conceptualized the study, supervised the study, reviewed and edited the article to be published.

REFERENCES

Acosta-Estrada, B. A., J. A. Gutiérrez-Uribe and S. O. Serna-Saldívar. 2014. Bound phenolics in foods, a review. Food Chem. 152: 46-55.

Álvarez, E. and O. Cambeiro. 2003. Actividad biológica de los flavonoides (I). Acción frente al cáncer. Offarm. 22: 130-140.

Amaro-Blanco, G., J. Delgado-Adámez, M.J. Martín and R. Ramírez. 2018. Active packaging using an olive leaf extract and high pressure processing for the preservation of sliced dry-cured

shoulders from Iberian pigs. Innov. Food Sci. Emerg. Technol. 45: 1-9.

Bouayed, J., H. Deußer, L. Hoffmann and T. Bohn. 2012. Bioaccessible and dialysable polyphenols in selected apple varieties following *in vitro* digestion vs. their native patterns. Food Chem. 131, 1466–1472. https://doi.org/10.1016/j.foodchem.2011.10.030

Bouaziz, M., I. Fki, H. Jemai, M. Ayadi and S. Sayadi. 2008. Effect of storage on refined and husk olive oils composition: Stabilization by addition of natural antioxidants from Chemlali olive leaves. Food Chem. 108: 253-262.

Briante, R., M. Patumi, S. Terenziani, E. Bismuto, F. Febbraio and R. Nucci. 2002. *Olea europaea* L. leaf extract and derivatives: Antioxidant properties. J. Agric. Food Chem. 50: 4934-4940.

Cabrera-Bañegil, M., F. Pérez-Nevado, A. Montaño, R. Pleite and D. Martín-Vertedor. 2018. The effect of olive fruit maturation in Spanish style fermentation with a controlled temperature. LWT Food Sci. Technol. 91: 40-47.

Carrasco-Pancorbo, A., L. Cerretani, A. Bendini, A. Segura-Carretero, M. Del Carlo, T. Gallina-Toschi, G. Lercker, D. Compagnone and A. Fernández-Gutiérrez. 2005. Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil. J. Agric. Food Chem. 53: 8918-8925.

Cebeci, F. and N. Şahin-Yesilcubuk. 2014. The matrix effect of blueberry, oat meal and milk on polyphenols, antioxidant activity and potential bioavailability. Int. J. Food Sci. Nutr. 65: 69-78.

Covas, M. I. 2008. Bioactive effects of olive oil phenolic compounds in humans: Reduction of heart disease factors and oxidative damage. Inflammopharmacology. 16: 216-8.

Delgado-Adámez, J., E. Bote, V. Parra-Testal, M.J. Martín, R. Ramírez. 2016. Effect of the olive leaf extracts *in vitro* and in active packaging of sliced Iberian Pork Loin. Packag. Technol. Sci. 29: 649-660.

Delgado-Adámez, J., M. N. F. Baltasar, M. C. A. Yuste and D. Martín-Vertedor. 2014. Oxidative stability, phenolic compounds and antioxidant potential of a virgin olive oil enriched with natural bioactive compounds. J. Oleo Sci. 63: 55-65.

Delgado-Adamez, J., E. Gamero Samino, E. Valdes Sanchez and D. Gonzalez-Gomez. 2012a. In vitro estimation of the antibacterial activity and antioxidant capacity of aqueous extracts from grape-seeds (*Vitis vinifera* L.). Food Control. 24: 136-141.

Delgado-Adamez, J., M. F. Fernandez-Leon, B. Velardo and D. Gonzalez-Gomez. 2012b. In vitro assays of the antibacterial and antioxidant activity of aqueous leaf extracts from different *Prunus salicina* Lindl. cultivars. Food Chem. Toxicol. Toxicol. 50: 2481-2486.

Dintcheva, N. T., R. Arrigo, M. Baiamonte, P. Rizzarelli and G. Curcuruto. 2017. Concentration-dependent anti-/pro-oxidant activity of natural phenolic compounds in bio-polyesters. Polym. Degrad. Stab. 142: 21-28.

De Valle-Prieto, M., B. J. Delgado-Adámez, M. V. Gil, S. Martillanes, M. N. Franco and D. Martín-vertedor. 2017. Virgin olive oil enriched with lutein-zeaxanthin from *Spinacia oleracea*. J. Oleo Sci. 468: 463-468.

He, Z., B. Yuan, M. Zeng, G. Tao and J. Chen. 2015. Effect of simulated processing on the antioxidant capacity and *in vitro* protein digestion of fruit juice-milk beverage model systems. Food Chem. 175: 457-464.

Japón-Luján, R. and M.D. Luque De Castro. 2008. Liquid-liquid extraction for the enrichment of edible oils with phenols from olive leaf extracts. J. Agric. Food Chem. 56: 2505-2511.

Karakaya, S. 2004. Bioavailability of phenolic compounds. Crit. Rev. Food Sci. Nutr. 44: 453-64.

Kim, M. S., D. C. Lee, J. E. Hong, I. S. Chang, H. Y. Cho, Y. K. Kwon and H. Y. Kim. 2000. Antimicrobial effects of ethanol extracts from Korean and Indonesian plants, Korean J. Food Sci. Technol. 32: 949-958.

Martillanes, S., J. Rocha-Pimienta, M. Cabrera-Bañegil, D. Martín-Vertedor and J. Delgado-Adámez. 2017. Application of phenolic compounds for food preservation: Food additive and active packaging. In: M. Soto-Hernández (Ed.), Phenolic Compounds Biological Activity. InTechOpen, London.

Martín-Vertedor, D., M. Garrido, J. A. Pariente, J. Espino and J. Delgado-Adámez. 2016. Bioavailability of bioactive molecules from olive leaf extracts and its functional value. Phyther. Res. 30: 1172-1179.

Meirinhos, J., B. M. Silva, P. Valentão, R. M. Seabra, J. A. Pereira, A. Dias, P. B. Andrade and F. Ferreres. 2005. Analysis and quantification of flavonoidic compounds from Portuguese olive (*Olea europaea* L.) leaf cultivars. Nat. Prod. Res. 19: 189-195.

Melo, P. S., L. O. R. de Arrivetti, S. M. de Alencar and L. H. Skibsted. 2016. Antioxidative and prooxidative effects in food lipids and synergism with α-tocopherol of açaí seed extracts and grape rachis extracts. Food Chem. 213: 440-449.

Moon, Y. J., X. Wang and M. E. Morris. 2006. Dietary flavonoids: Effects on xenobiotic and carcinogen metabolism. Toxicol. *In Vitro*. 20: 187-210.

Paiva-Martins, F. and M. H. Gordon. 2001. Isolation and characterization of the antioxidant component 3,4-dihydroxyphenylethyl 4-formyl-3-formylmethyl-4-hexenoate from olive (*Olea europaea*) leaves. J. Agric. Food Chem. 49: 4214-4219.

Sanhueza, L., R. Melo, R. Montero, K. Maisey, L. Mendoza and M. Wilkens. 2017. Synergistic interactions between phenolic compounds identified in grape pomace extract with antibiotics of different classes against *Staphylococcus aureus* and *Escherichia coli*. PLoS One. 12: e0172273.

Škerget, M., P. Kotnik, M. Hadolin, A. R. Hraš, M. Simonič and Ž. Knez. 2005. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. Food Chem. 89: 191-198.

Sudjana, A. N., C. D'Orazio, V. Ryan, N. Rasool, J. Ng, N. Islam, T. V. Riley and K. A. Hammer. 2009. Antimicrobial activity of commercial *Olea europaea* (olive) leaf extract. Int. J. Antimicrob. Agents. 33: 461-463.

Talhaoui, N., A. Taamalli, A. M. Gómez-Caravaca, A. Fernández-Gutiérrez and A. Segura-Carretero. 2015. Phenolic compounds in olive leaves: Analytical determination, biotic and abiotic influence, and health benefits. Food Res. Int. 77: 92-108.

Turoli, D., G. Testolin, R. Zanini and R. Bellù. 2004. Determination of oxidative status in breast and formula milk. Acta Paediatr. Int. J. Paediatr. 93: 1569-1574.

Wanasundara, U. N. and F. Shahidi. 1998. Antioxidant and pro-oxidant activity of green tea extracts in marine oils. Food Chem. 63: 335-342.