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Quercus ilex leaf as a functional ingredient: Polyphenolic profile and antioxidant activity throughout simulated gastrointestinal digestion and antimicrobial activity

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ABSTRACT

Quercus ilex leaf constitute a rich source of bioactive compounds, especially phenolic compounds, with health and technological properties. In this study, the influence of *in vitro* gastrointestinal digestion on the bioaccessibility and bioactivity of phenolic compounds from ground leaf (GL) and leaf powder extract (PE) was evaluated for the first time. The GL showed an increase in antioxidant capacity, total phenolic content and individual phenolic compounds identified in the oral and gastric phase, decreasing in the intestinal and absorption (dialysis) phases, while PE showed a slight decrease in the oral and gastric phase and a more pronounced reduction in the last phases. Although the content of phenolic compounds and the antioxidant capacity were higher in the initial PE, the bioaccessibility (55.27% in GL vs. 34.23% in PE) and the recovery in the last step of the gastrointestinal tract in the colon-available fraction (29.24% in GL vs. 27.90% in PE) were higher in GL. The PE showed high antimicrobial activity against *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Yersinia enterocolitica* and *Staphylococcus aureus*, showing minimum inhibitory concentration values between 1 and 5 mg/mL. These results showed the complexity and richness of *Q. ilex* PE and GL in phenolics with high antioxidant and antimicrobial activity, underlining their use as a source of biofunctional compounds for the development of novel food additives and nutraceuticals.

1. Introduction

In recent decades there has been an increasing interest in advancing a more sustainable and environmentally friendly production, based on a circular economy. This approach is an alternative to the current model of linear production and is based on the need for innovative solutions to waste reduction and recycling (Bascón-Villegas et al., 2020). Agriculture and agri-food industry are the sectors generating the largest volume of by-products and residues with great diversity in composition and high bioactivity potential. Currently, the limited valorisation of such waste streams contrasts with its promising applications in the food, pharmaceutical or nutraceutical industries (Cádiz-Gurrea, Pinto, Delerue-Matos, & Rodrigues, 2021; Costa et al., 2019).

In this context, where the bioeconomy is emerging as a new production model that addresses the main challenges of today's society, forest biomass should be positioned not only as a renewable source of energy, but also as a source of added-value compounds. In this sense, the by-products resulting from forest harvesting in the *dehesa* include wood, branches and leaves from the pruning of *Q. ilex*, which represent a risk factor in the spread of plagues and forest fires. Therefore, these *Q. ilex* by-products must be collected/employed in an efficient way in terms of low energy consumption to decrease economic costs due to its high annually production of about 2.4 million tonnes in Andalusia and also because its current application as firewood for heating is not sustainable, opening scientific and industrial opportunities to exploit all their nutritional and bioactive value, which could increase economic income

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in the region (Agencia Andaluza de la Energía, 2018).

Dehesa is a silvopastoral ecosystem of Mediterranean forest in the Iberian Peninsula that in the Andalusian region covers a total area of about one million hectares (Consejería de Medio Ambiente de la Junta de Andalucía, 2006) and is comprised of pastures and tree species, mainly from the genus *Quercus*, being *Quercus ilex* subsp. *ballota* and *Quercus suber* the most representative species in this region (Maya-Manzano et al., 2016). The Iberian Peninsula *Dehesa* integrates not only forestry activity, but also agricultural and livestock activities, highlighting the extensive free-range production system known as "*montanera*", where Iberian pigs graze in the final phase of fattening in the *dehesa*, and eat the fruit of *Q. ilex*, i.e., acorns (Horcada, Valera, Juárez, & Fernández-Cabanás, 2020). Acorns contain monounsaturated fatty acids, numerous compounds with antioxidant properties particularly phenolic compounds that would result in meat and dry-cured meat products with a good content of beneficial compounds for human health (Rey & López-Bote, 2014).

For long, different parts of the *Quercus* tree have been used as natural remedy for the treatment of gastrointestinal inflammations and disorders. Extracts from leaves, bark and wood have shown antioxidant, antimicrobial, anti-inflammatory, antitumor and gastroprotective properties, attributed to their high content of phenolic compounds (Karioti, Bilia, Gabbiani, Messori, & Skaltsa, 2009). Also, the intake of polyphenols is associated with their beneficial bioactivity related to cardiovascular protection, inhibiting the oxidation of low-density lipoprotein cholesterol, and to glucose metabolism, promoting absorption and helping to prevent hyperglycaemia (Sánchez-Gutiérrez et al., 2021). All this beneficial bioactive potential has led some authors to study the polyphenolic profile of *Q. ilex* leaf (QIL), being ellagic acid, catechin, epicatechin, quercetin, gallic acid and kaempferol the main compounds (Hadidi et al., 2017).

Recent studies have demonstrated the potential applications of polyphenols in the food technology field such as their use as antioxidant (Estévez, 2021). On the other hand, previous investigations have attributed to catechins, the main phenolic compound in QIL, an inhibitory capacity of the growth of several microorganisms (Rashidinejad et al., 2021; Zhou, Tang, Zou, & Wang, 2022), including foodborne pathogenic bacterial strains, such as *E. coli* and *Salmonella* (Ma et al., 2019). In this respect, this type of natural substances could constitute a safe, cheap and sustainable alternative to conventional antimicrobial drugs, reducing antibiotic resistance, and helping to control cross-contamination by foodborne pathogens (Olszewska, Gędas, & Simões, 2020; Pires, Dias, Barros, Calhelha, Alves, Oliveira, & Ferreira, 2018).

The rich composition of *Q. ilex* leaf in phenolic compounds, offer unlimited opportunities to obtain new high added-value products given their high bioactive potential, with different applications. In the food sector, despite the widely accepted use of polyphenols as functional ingredients, the gastrointestinal tract (GIT) constitutes a barrier which should be evaluated to predict the potential of biological active levels after GIT digestion. In fact, it has been demonstrated that polyphenols bioaccessibility can be reduced or increased after their passage throughout the GIT, and thus, their bioactive effect varies (de Paulo Farias et al., 2021; Ribeiro, Bonifácio-Lopes, Morais, Miranda, Nunes, Vicente, & Pintado, 2021a; Ribeiro, Campos, Oliveira, Nunes, Vicente, & Pintado, 2021b; Santana Andrade, Chagas Barros, Pereira, Nogueira, Gualberto, Santos de Oliveira, & Narain, 2022; Silva & de Queiroga, 2022). To assess the health benefits of QIL polyphenols, the effects of digestion on the bioaccessibility and stability of polyphenols should be studied (Chait, Gunenc, Bendali, & Hosseinian, 2020). As far as we are concerned, no previous studies have addressed any new functional ingredients from QIL and the impact of the GIT on their bioactivity.

In this context, the main objectives of the present study were to evaluate for the first time the effects of simulated gastrointestinal conditions on the stability of phenolics and antioxidant activity from *Q. ilex* leaf extract (PE) and ground leaf (GL), as well as antibacterial properties of PE, with a view for its application as a potential functional food and natural additive.

2. Materials and methods

2.1. Chemicals

AAPH (2,2-azo-bis-(2-methylpropionamidine) dihydrochloride), $ABTS^{\bullet+}$ diammonium salt (2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)), anhydrous sodium carbonate (Na_2CO_3), fluorescein (F-6377), Folin-Ciocalteu's reagent, hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Merck (Algés, Portugal). α-amylase, bile salts pancreatin, pepsin and sodium hydrogen carbonate ($NaHCO_3$) and standards of gallic acid, *p*-coumaric acid, quercetin, resveratrol, rutin and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were acquired from Sigma-Aldrich (St. Louis, MA, USA), whereas catechin, (-) epicatechin, epicatechin gallate, epigallocatechin gallate, epicatechin-3-*O*-gallate, hydroxytyrosol, kaempferol and luteolin-7-glycoside were purchased from Extrasynthese (Lyon, France). Formic acid and methanol were purchased from Fischer Scientific (Oeiras, Portugal).

2.2. Bacterial strains

The microorganism tested in this study were *Escherichia coli* (CECT 8295), *Listeria monocytogenes* (CECT 4032), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (CECT 704), *Staphylococcus aureus* (CECT 5193) and *Yersinia enterocolitica* (CECT 754).

All strains were acquired from the Spanish Collection of Type Culture (Valencia, Spain), all culture media from Oxoid™ (Hampshire, UK) whereas cations supplements, magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$) and calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$), were obtained from Sigma-Aldrich (St. Louis, MA, USA).

2.3. Raw material and samples preparation

The *Q. ilex* leaves were randomly collected from the *dehesa* area, located in Los Pedroches Valley (Córdoba, southern Spain, Latitude 38.2967, Longitude −4.4539) in mid-March 2019, during the period authorized for pruning and after the end of "*montanera*" season. Each sample contained approximately 250 g of leaves from at least ten different trees.

The leaves were hand-washed with tap water and left to dry in open air and darkness. Then, they were ground and sieved into powder with a diameter of <2 mm and stored at room temperature (around 25 °C) in a dark dry room until analysis.

In this study, two different samples were prepared (Fig. 1): (i) ground leaves (GL); and (ii) powder extract (PE) from GL. PE was obtained by a microwave-assisted extraction (MAE) procedure by using an ETHOS Microwave Extraction System (Milestone, Sorisole, Italy). The extraction was performed at 800 W using magnetic stirring at a level of 90% (2970 rpm), at 80 °C for 10 min. The extraction ratio was 1:10, and distilled water was used as solvent. After the process, the extract was collected and then filtered through a Whatman No. 1 paper. Finally, the extract was freeze-dried for preservation and kept in the dark at room temperature for two weeks until analysis (Sánchez-Gutiérrez et al., 2021).

2.4. In vitro simulated gastrointestinal digestion

To study the effect of simulated GIT upon the stability of GL and PE, *in vitro* simulation of GIT (Fig. 2) was performed according to the method previously described by Madureira, Amorim, Gomes, Pintado, & Malcata, 2011 with some modifications. Samples were prepared in three independent experiments by adding 2 g of GL and 0.350 g of lyophilized PE in 20 mL of ultra-pure water (the yield of the ground leaf extraction was 17.5%). Three independent experiments were set up. The antioxidant activity was measured before and after exposure to the simulated

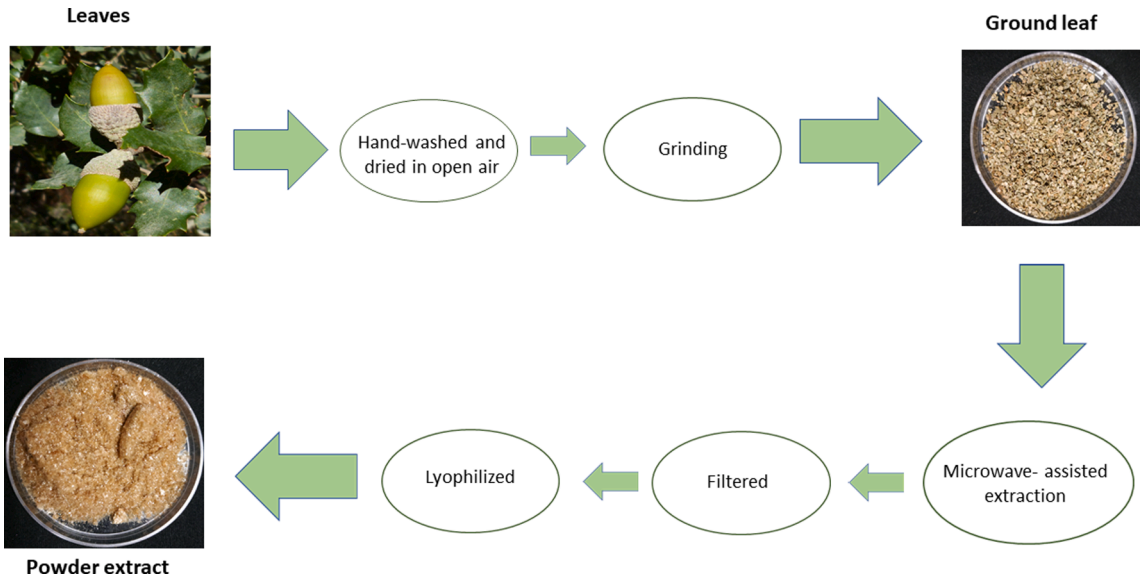

Fig. 1. Flowchart of the process to obtain ground leaf (GL) and powder extract (PE) from *Quercus ilex* leaves co-product.

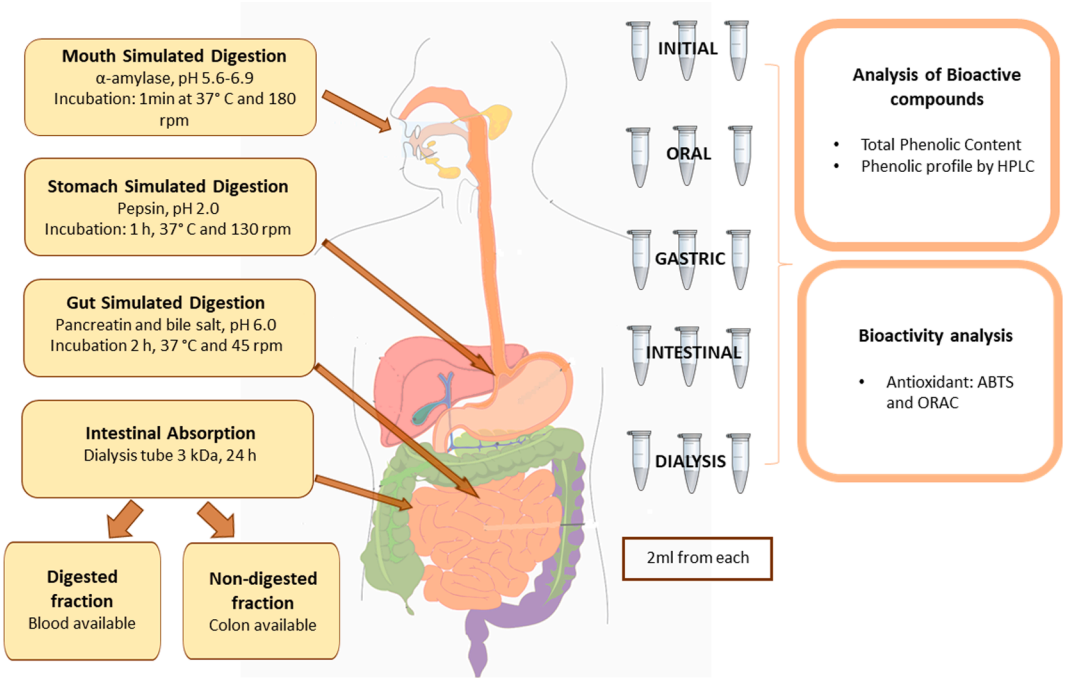

Fig. 2. Graphic representation of the *in vitro* gastrointestinal digestion procedure carried out with ground leaf (GL) and powder extract (PE) form *Quercus ilex* leaves.

digestion conditions using $ABTS^{\bullet+}$ and ORAC assay

2.4.1. Mouth simulated digestion

The initial pH of the samples was adjusted between 5.6 and 6.9 using 0.1 M NaOH. The oral digestion was performed adding 0.6 mL of α-amylase solution (100 U/mL) to samples with subsequent incubation at 37 ℃ and 180 rpm for 1 min.

2.4.2. Stomach simulated digestion

For gastric digestion, the pH of the samples was adjusted to 2.0 using 1 M HCl. Pepsin at 25 mg/mL was added at a rate of 0.05 mL/mL of sample, and the mixture was incubated in a shaking water bath at 37 °C and 130 rpm for 1 h.

2.4.3. Gut simulated digestion

Small intestinal digestion was performed by adjusting the pH to 6.0 with 1 M $NaHCO_3$. The intestinal juice was simulated by the addition of a solution of pancreatin (2 g/L) and bile salts (12 g/L) at a concentration of 0.25 mL/mL of sample. The solution mixture was incubated at 37 ℃ and 45 rpm for 2 h, mimicking a long intestine digestion.

2.4.4. Small intestine absorption – dialysis

In the last phase of intestinal digestion, samples were transferred into a cellulose acetate dialysis tube with a molecular weight cut-off of 3 kDa (Spectra/Pro, Spectrum Lab, Breda, Netherlands) to reproduce the natural absorption step in the small intestine. Then, the membranes were immersed into a regularly renewed water bath stirred at 1000 rpm at room temperature during 24 h (Campos, Ribeiro, Teixeira, & Pastrana,

2020). At the end of the process, the solution that managed to diffuse the dialysis tubing represents the fraction that has the potential to be absorbed, while the remaining solution inside the dialysis tubing represented the non-absorbable sample (colon-available).

2.5. Bioaccessibility and stability of polyphenols from Quercus ilex GL and PE through in vitro gastrointestinal digestion

2.5.1. Recovery and bioaccessibility index

To analyse the effect of the type of matrix (GL vs PE) on the *in vitro* digestion of phenolic compounds, two different percentage indexes were studied: the recovery index (RI) and the bioaccessibility index (BI) (Ribeiro et al., 2021b). The RI measures the percentage of phenolic compounds present in the digested food material after mouth, gastric, intestinal or dialysis steps, according to the equation (1):

$$Recovery\ \ Index\ \ (RI,\%) = \frac{PC_{DS}}{PC_{TS}} \times 100 \quad (1)$$

where PC_{DS} is the total phenolic content (mg/mL) in the digested sample (i.e. those corresponding to the fractions after digestion in the mouth, stomach and intestine and after the absorption phase of dialysis) and PC_{TS} is the total phenolic content (mg/mL) quantified in the test sample (undigested).

The BI measures the percentage of polyphenols recovered after the intestinal dialysis phase, which could be available for absorption into the bloodstream (Chait et al., 2020):

$$Bioaccessibility\ \ Index\ \ (BI,\%) = \frac{PC_{A}}{PC_{IDS}} \times 100 \quad (2)$$

where PC_A is the total phenolic content (mg/mL) absorbed after dialysis from PC_{IDS}, the phenolic content (mg/mL) in the intestinal digested sample.

2.5.2. Total phenolic content (TPC)

The total phenolic content of GL and PE obtained during the different phases of *in vitro* gastrointestinal digestion was determined by the Folin-Ciocalteu method with some modifications (Ramos et al., 2019). Gallic acid was the reference standard. Results were expressed as mg gallic acid equivalents (GAE)/g of dry weight (DW). All measurements were performed in triplicate for each experiment.

2.5.3. Identification and quantification of phenolics by HPLC

Polyphenolic profile of all samples (GL and PE) obtained in each phase of the GIT were determined by High Performance Liquid Chromatography, using a diode-array detector (HPLC-DAD), according to the method described by Campos et al. (2020) with some modifications. Samples were injected into Waters Series e2695 Separation Module System (Mildford MA, USA) interfaced with the UV/Vis photodiode array detector (PDA 190–600 nm). Separation was performed in a reverse-phase column (COSMOSIL 5C1 8-AR-II Packed Column – 4.6 mm I.D. × 250 mm: Dartford, UK), using two mobile phases composed by mobile phase A – water/methanol/formic acid (92.5:5:2.5, %v/v/v) – and B – methanol/water/formic acid (92.5:5:2.5, %v/v/v) with the following gradient and conditions: injection volume of 50 μL; continuous flow of 0.5 mL/min; gradient elution starting at 100% mobile phase A for 50 min, then gradient reset at 45% A and 55% B between 50 and 55 min; return of mobile phase A to 100%, remaining at this percentage for 4 min (until 59 min). Data acquisition and analysis were carried out using Software Empower 3. Detection was carried out at wavelengths ranging from 200 to 600 nm. The peaks were searched at different wavelengths to identify catechins or procyanidins (280 nm), phenolic acids (320 nm) and flavanols (330 nm). Compounds were identified and quantified through external calibration curve by comparison with pure standards in terms of retention times, UV absorption spectra and peak areas at maximum absorption wavelength. All determinations were made in triplicate. Results were expressed as mg of phenolic compounds per gram of DW.

2.6. Antioxidant activity determination

2.6.1. $ABTS^{\bullet+}$ assay

The $ABTS^{\bullet+}$ scavenging activity assay of GL and PE samples obtained during the different phases of the *in vitro* gastrointestinal digestion was determined as described by Ribeiro et al. (2020). A calibration curve was built with Trolox standards, and results obtained were expressed as mmol of Trolox equivalents (TE) per gram of DW. All assays were performed in triplicate for each experiment.

2.6.2. Oxygen radical absorbance capacity (ORAC)

ORAC assay was performed according to the method described by Ribeiro et al. (2020). The final ORAC values were expressed as mmol of Trolox equivalent (TE) per gram of DW. All assays were performed in duplicate.

2.7. Antimicrobial activity of the powder extract (PE): Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The antimicrobial activity of PE was tested against the following foodborne pathogen strains: *E. coli* (CECT 8295), *L. monocytogenes* (CECT 4032), *S.* Typhimurium (CECT 704), *S. aureus* (CECT 5193) and *Y. enterocolitica* (CECT 754).

2.7.1. Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The MIC was determined using a broth microdilution assay, following the standards for antimicrobial susceptibility testing provided by the Clinical and Laboratory Standards Institute (2015). The PE samples were prepared at concentrations ranging from 0.25 to 20 mg/mL in CAMHB (cation adjusted MHB) and sterilized by filtration through a 0.22 μm filter (Filter-Lab, Barcelona, Spain). Each well was inoculated with approximately 5×10^5 CFU/mL of the test bacteria, and variable concentrations of the PE were added. Microplate were incubated at 37 °C for 24 h, with the exception of *Yersinia enterocolitica* cultures, which were incubated for 48 h. The MIC value corresponded to the lowest extract concentration that inhibited visible bacterial growth. Bacterial cells viability was read every hour in a microplate reader (Bioscreen C Microbiology Reader, Oy Growth Curves Ab Ltd, Helsinki) at a wavelength of 600 nm. All assays were performed in triplicate. The MBC is defined as the lowest concentration of extract that results in killing 99.9% of the bacteria being tested and was determined by surface plating the culture from each non-growth well from the MIC assay on an appropriate growth medium.

2.8. Statistical analysis

Statistical analysis was carried out through IBM® SPSS® Statistics software Version 25 (IBM Corporation, New York, NY, USA). Data were reported as mean ± standard deviation of the three trials. Differences in data means for TPC and antioxidant activity between the different phases of the *in vitro* gastrointestinal digestion, were analysed by one-way analysis of variance (ANOVA), with the application of the Tukey's post hoc test for pairwise multiple comparison. Significant differences were considered at a level of $p < 0.05$. Also, correlation analysis between TPC and the antioxidant activity of the two types of samples (GL and PE) was performed through the calculation of the Pearson's correlation coefficient r.

3. Results and discussion

3.1. Recovery index (RI) and bioaccessibility index (BI) of phenolic compounds

The RI of total phenolics of powder extract (PE) and ground leaf (GL) from *Q. ilex* obtained after the different phases of the *in vitro* gastrointestinal digestion is shown in Fig. 3.

The values for non-digested samples were assumed as the 100% of TPC. In the case of PE after oral digestion, the RI of total phenolic did not change ($p > 0.05$) with respect to the non-digested sample since the percentage of polyphenols recuperated was 100%, whereas in the case of GL, the RI value had a slight but significant increase over the non-digested sample with a RI % value of 111.81%. In this phase, minimal modifications were observed in the concentration of the phenolic compounds due to the short exposure time and marginal effects of amylase.

The gastric digestion of PE had a slight significant decrease on the RI with a value of 90.29%. On the contrary, in GL, a significant increase with respect to both the non-digested sample and the oral phase, was observed, with a recovery value of 129.17%. In PE, the free phenolic acids present would be more labile because of their solubilization in the solution and thus, their direct and rapid exposure to gastric conditions from the beginning, decreasing during this phase (Lucas-González et al., 2016). With reference to the GL samples, with high fibre content, this may have interacted with the polyphenols physically trapped in the sample matrix, being the acidic pH and the enzymatic activity of the gastric environment the responsible for the extractability of phenolic compounds by breaking the bonds between these compounds and proteins, fibre or sugar residues, thus increasing the release of these compounds (Ribeiro et al., 2021b). In this sense, Lucas-González, Viuda-Martos, Álvarez, and Fernández-López (2018) reported that the RI of phenolic compounds from persimmon flours after the oral phase did not change with respect to the undigested sample, and increased after the gastric phase.

In the intestinal phase, the RI in both PE and GL was strongly affected, with values of 42.67 and 65.23% respectively, compared to the non-digested samples. This difference between both types of samples could be caused by the composition of the matrix, as discussed above. The free phenolic acids present in the PE matrix were presumably more unstable, as they were not protected by the fibre, opposite to the GL matrix, with a high fibre content. Nevertheless, the difference in RI between PE and GL observed after the gastric digestion diminished after intestinal digestion. The noticeable negative effect of the intestinal digestion on the phenolic compounds has also been observed by other authors with RI values in date pit flour of 46% (Gullon et al., 2015) and in pomegranate peel flour of 43% (Gullon, Pintado, Fernández-López, Pérez-Álvarez, & Viuda-Martos, 2015).

Similar behaviour was observed during the intestinal absorption phase, i.e., dialysis, where the RI of TPC in both PE and GL slightly decreased, with values of 27.89 and 29.23% of RI, respectively. In this phase, practically no differences in RI were observed between both matrices. These values are the result of a balance between increment and degradation of polyphenols. On the one hand, increments could be attributed due to the prolonged contact time (24 h) between the sample and intestinal fluids and intestinal digestive enzymes (lipase and pancreatin, with the latter also having amylase and protease activity), which facilitates the release of polysaccharides-linked polyphenols. On the other hand, the reduced availability of phenolic compounds after intestinal digestion is probably influenced by different factors. Firstly, the pH value of the small intestine enhances the degradation of polyphenols. The acidic medium in the gastric phase promotes the break of bonds between bioactive compounds and other compounds like fibre, protein and carbohydrates, whereas the mild alkaline conditions present in the small intestine, where these compounds are highly sensitive, favour the degradation or transformation the dietary polyphenols into other compounds (Ribeiro et al., 2020). In the second place, some interactions of polyphenols with other dietary components, such as protein, carbohydrate or minerals, may remain, making them (partially) unavailable for absorption. Thirdly, chemical reactions taking place in the small intestine, mainly oxidation and polymerization, would lead to the formation of other phenolic by-products. Lastly, variations in molecular structures due to enzymatic action can affect the solubility of polyphenols (Lucas-González et al., 2018), thus reducing their absorption.

In order to exert their bioactive effect, polyphenols must be released from the food matrix and solubilized to be bioaccessible, where bioaccessibility is the amount of ingested polyphenols available for absorption in the intestine after digestion (Martínez-Las Heras, Pinazo, Heredia, & Andrés, 2017). The BI of phenolic compounds present in PE and GL were 34.23 ± 6.11 and 55.27 ± 5.91% respectively. These values suggest that several changes in phenolic compounds such as modification of chemical structure, changes in solubility or interaction with other compounds, might have occurred during the gastrointestinal digestion of PE and GL samples, which influenced their bioaccessibility

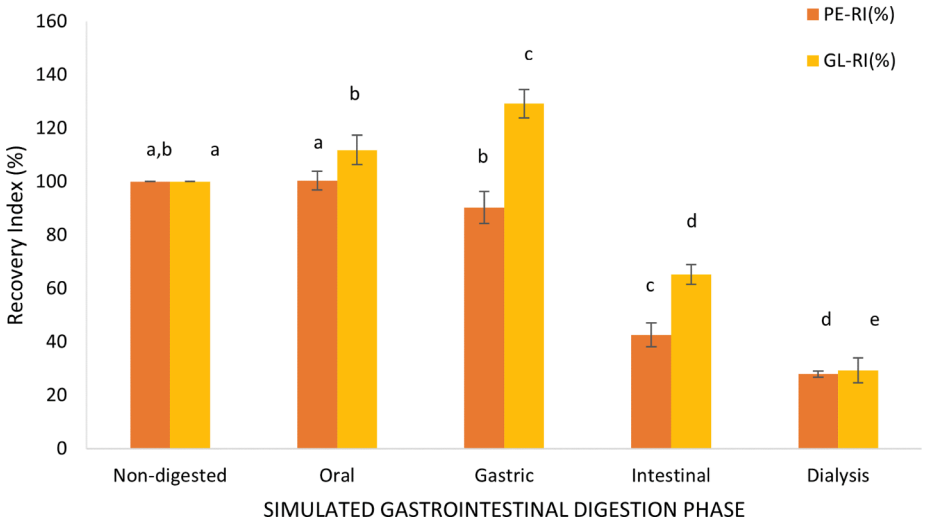

Fig. 3. Recovery index of total phenolic compounds throughout the *in vitro* gastrointestinal digestion (initial, oral, gastric, intestinal, after dialysis phase) of powder extract (PE) and ground leaf (GL) in the different digestion phases. Results are the mean of three determination ± standard deviation. Different letters represent different homogeneous groups according to the Tukey's test ($p < 0.05$).

(Dou, Chen, Huang, & Fu, 2022). These results are in accordance with the work of other authors who reported a BI of 35.90% in pomegranate peel flour (Gullon et al., 2015), 33.91% in grape juice (He et al., 2016) and 65.52% in olive pomace (Ribeiro et al., 2020). The higher values in GL and olive pomace would probably be due to the fibre content and other compounds, that may have trapped or protected polyphenols during the whole digestion process, providing in the dialysis phase an important concentration of polyphenols in an adequate state for absorption.

3.2. Stability of polyphenolic compounds during simulated in vitro gastrointestinal digestion

The evolution of TPC in PE and GL samples followed different trends, as can be seen in Fig. 4, with a remarkably more intense decrease in PE than in GL. Nevertheless, TPC was maintained at higher levels in PE in all digestion phases than in GL, with a ratio of TPC_{PE}/TPC_{GL} of around 7 in the initial and final phases of the digestion.

In the oral phase, no significant TPC losses were observed in PE, while in the gastric phase a significant decrease of 10% was found. In contrast, in the case of GL, successive short increments in TPC were observed until the gastric phase, showing a 29% increase.

After the intestinal phases, i.e., digestion and dialysis (colon available fraction), the TPC values of PE and GL decreased significantly, being more prominent in PE (138.55 and 90.85 mg GAE/g DW after digestion and dialysis, respectively) than in GL (28.21 and 12.62 mg GAE/g DW after digestion and dialysis, respectively). This decrease in TPC after *in vitro* gastrointestinal digestion was also reported by other authors that addressed this issue in lettuce (Ketnawa, Suwannachot, & Ogawa, 2020) and burdock root flour (Herrera-Balandrano et al., 2021). According to these works, phenolics are highly sensitive to pH changes, which affect the stability of bioactive compounds after gastrointestinal digestion. Similarly, other factors discussed previously also contributed to the loss of bioactive compounds after the intestinal digestion. After dialysis, the compounds remaining in the colon-available fraction could be metabolized by bacteria in the colon, transforming dietary polyphenols into simple phenolic compounds, which may result in more biologically active metabolites (Santana Andrade et al., 2022). In turn, the phenolic compounds that end up reaching the bloodstream are those which can exert their bioactive beneficial effect on the organism, being PE the matrix with major contribution of TPC (47.70 GAE/g DW) in comparison with GL (15.58 GAE/g DW), despite the superior BI of GL, as described in previous section.

In order to evaluate the stability of individual phenolic compounds during the *in vitro* gastrointestinal digestion, 11 compounds were identified, from which 8 were quantified by HPLC. Table 1 shows these compounds, indeed, three phenolic acids (gallic acid, *p*-coumaric acid and hydroxytyrosol), three flavanols (catechin, epicatechin and epicatechin gallate), two flavonols (kaempferol and rutin), one flavone (luteolin), one flavanone (naranginin) and one stilbene (resveratrol). The polyphenols identified have been previously detected in different *Q. ilex* by-products (acorns and leaves), with a wide range of total phenolics (from 2.39 to 45.80 mg/g) quantified by HPLC as the sum of individuals (Hadidi et al., 2017).

In all digestion phases, the TPC determined by the Folin-Ciocalteu method showed higher values than the sum of phenolic compounds quantified by HPLC (Table 1). Some factors may account for this difference: (i) the Folin-Ciocalteu reagent can react with other non-phenolic substances like ascorbic acid, sugars, aromatic amines, organic acids and proteins present in the samples, causing interference with the measurement, and thus leading to an overestimation of the TPC; (ii) the quantification limit of certain phenolic compounds may become an issue, hindering the accurate estimation of the polyphenols content (Gómez-García, Campos, Oliveira, Aguilar, Madureira, & Pintado, 2021); and (iii) it should be noted that PE and GL certainly contain other phenolic compounds which were not quantified by HPLC (kaempferol, naranginin and resveratrol). Despite these facts, a high correlation was found between TPC and the sum of individuals phenolic compounds along the *in vitro* gastrointestinal digestion, showing a high correlation in both GL ($r^2 = 0.952$) and PE ($r^2 = 0.922$).

The different samples, PE and GL, and the digestion phases affected the stability and release of polyphenols from the sample matrix. As can be seen in Table 1, the *p*-coumaric acid, catechin and rutin in PE and the *p*-coumaric acid, gallic acid and catechin in GL exhibited the highest contents in the non-digested samples. With respect to PE, in the oral phase, four phenolic compounds (gallic acid, catechin, rutin and luteolin-7-glycoside) remained unchanged from the non-digested sample, whereas hydroxytyrosol, *p*-coumaric acid and epicatechin decreased. In contrast, in the case of GL, in the oral phase, a slight increase was observed in gallic acid, *p*-coumaric acid, epicatechin gallate and luteolin-7-glycoside, whereas catechin and hydroxytyrosol showed no changes compared to the non-digested sample. This would suggest that during the oral phase most phenolic compounds were less effectively released probably due to the low contact time with enzymes

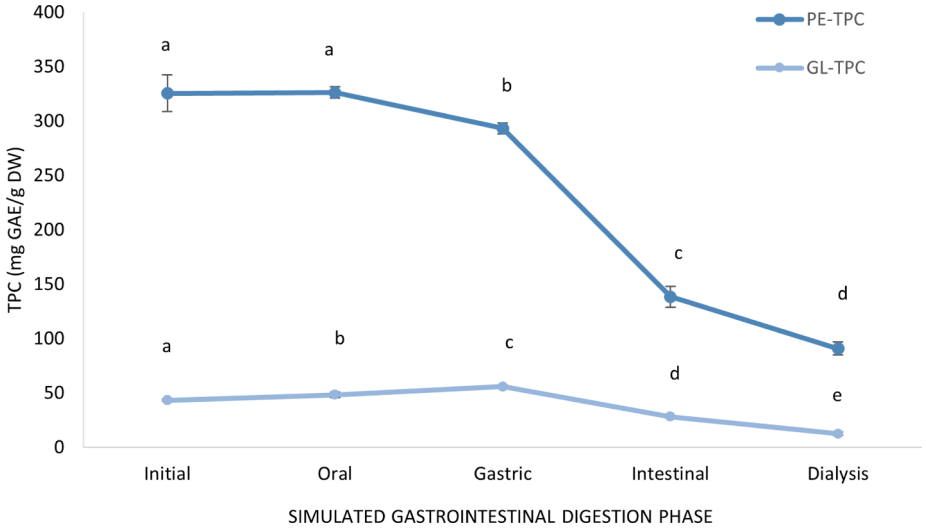

Fig. 4. Total phenolic compounds (TPC; mg GAE/g DW) throughout the *in vitro* gastrointestinal digestion (initial, oral, gastric, intestinal, after dialysis phase) of powder extract (PE) and ground leaf (GL). Results are the mean of three determinations ± standard deviation. Different letters represent different homogeneous groups according to the Tukey's test ($p < 0.05$).

Table 1
Concentrations (mg/g dry weight) of individual phenolic compounds identified in powder extract and ground leaf before (initial phase; non-digested samples) and after *in vitro* digestion (oral, gastric, intestinal and dialysis).

	Powder extract					Ground Leaf				
	Initial	Oral	Gastric	Intestinal	Dialysis	Initial	Oral	Gastric	Intestinal	Dialysis
Phenolic acids										
Gallic acid	2.32 ± 0.33^{a,b}	2.42 ± 0.26^{a}	1.40 ± 0.32^{b,c}	1.22 ± 0.07^{c}	UD	0.32 ± 0.01^{a}	0.46 ± 0.01^{b}	0.97 ± 0.03^{c}	ND	ND
Hydroxytyrosol	0.06 ± 0.01^{a}	0.03 ± 0.00^{b}	ND	ND	ND	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	UD	ND	ND
p-Coumaric acid	6.45 ± 0.50^{a}	4.33 ± 0.34^{b}	3.00 ± 0.24^{c}	1.90 ± 0.38^{c}	ND	1.26 ± 0.02^{a}	1.59 ± 0.03^{b}	0.74 ± 0.02^{c}	ND	ND
Flavanols										
Catechin	11.54 ± 0.86^{a}	11.10 ± 0.82^{a}	9.79 ± 0.73^{a}	2.71 ± 0.20^{b}	UD	1.49 ± 0.02^{a}	1.51 ± 0.03^{a}	1.61 ± 0.05^{b}	ND	ND
(-) Epicatechin	2.25 ± 0.08^{a}	1.73 ± 0.10^{b}	1.22 ± 0.03^{c}	0.24 ± 0.01^{d}	ND	ND	ND	0.09 ± 0.00^{a}	0.08 ± 0.00^{a}	ND
Epicatechin gallate	ND	ND	ND	ND	ND	0.31 ± 0.00^{a}	0.33 ± 0.01^{b}	ND	ND	ND
Flavonols										
Kaempferol	NQ	NQ	NQ	NQ	UD	NQ	NQ	NQ	NQ	NQ
Rutin	10.04 ± 0.38^{a,b}	9.05 ± 0.34^{a,b}	7.77 ± 0.29^{b}	11.54 ± 0.43^{a}	ND	ND	ND	0.36 ± 0.01^{a}	0.44 ± 0.02^{b}	ND
Flavones										
Luteolin-7-Glycoside	4.61 ± 0.34^{a}	3.99 ± 0.39^{a}	1.96 ± 0.15^{b}	1.42 ± 0.11^{b}	UD	0.23 ± 0.01^{a}	0.44 ± 0.01^{b}	0.19 ± 0.01^{a}	0.12 ± 0.00^{c}	0.03 ± 0.00^{d}
Flavanones										
Naranginin	NQ	NQ	NQ	NQ	UD	ND	ND	NQ	ND	ND
Stilbenes										
Resveratrol	NQ	NQ	NQ	NQ	ND	NQ	NQ	NQ	ND	ND
Total phenolic compounds										
Sum of individuals	37.26 ± 0.33^{a}	32.65 ± 0.30^{b}	25.14 ± 0.23^{c}	19.03 ± 0.23^{d}	ND	3.32 ± 0.01^{a}	4.36 ± 0.02^{b}	3.96 ± 0.02^{c}	0.65 ± 0.01^{d}	0.03 ± 0.00^{e}
Folin-Ciocalteu method	325. 65 ± 16.73^{a}	326.34 ± 5.44^{a}	293. 38 ± 4.97^{b}	138.55 ± 9.51^{c}	90.85 ± 5.81^{d}	43.27 ± 0.89^{a}	48.39 ± 2.61^{b}	55.87 ± 1.44^{c}	28.21 ± 1.21^{d}	12.62 ± 1.78^{e}

Values are expressed as mean of three determinations ± standard deviation. For each polyphenol, values in the same row followed by different superscript letters indicate significantly different homogeneous groups ($p < 0.05$) between initial, oral, gastric, intestinal or dialysis phase, according to the Tukey's Multiple Range Test. NQ: not quantified. ND: not detected. UD: under the limit of detection.

(Lucas-González et al., 2016).

In the gastric phase of digestion of PE, gallic acid, *p*-coumaric acid, epicatechin and luteolin-7-glycoside content decreased, while catechin and rutin content did not change significantly from the initial and oral phases. On the contrary, in the case of GL, gallic acid, catechin, epicatechin and rutin increased, while *p*-coumaric acid decreased with respect to the initial and oral phases. Luteolin-7-glycoside content did not significantly change from the non-digested sample whereas it decreased in comparison with the oral phase. The decrease in the concentration of PE phenolic acids in the gastric phase could be explained by the interaction with other components of the sample, causing changes in their molecular weight, solubility and chemical structure, as well as by the gastric conditions (pH value and enzymatic activity), which would affect the release and stability of these compounds (Lucas-González et al., 2016). However, GL results indicate that gastric digestion environment would improve the release of phenolic compounds, breaking the bonds with dietary components like proteins or fibre (Lucas-González et al., 2018).

The lowest TPC values were observed in the intestinal phase. At this stage, a significant decrease in the concentration of all phenolic compounds from the non-digested PE sample was found, with the exception of rutin, in which there was no significant difference. In the case of GL, only three compounds were detected at the end of the intestinal phase; rutin content increased, luteolin content decreased and epicatechin content did not change. At the end of the dialysis phase, a drastic reduction in polyphenolic concentrations was found. In PE, no polyphenols were detected, while in GL, only luteolin-7-glycoside was detected at a very low level. All these significative reductions on phenolic acids in both intestinal digestion and dialysis phases could be due to the instability of these compounds under alkaline conditions, the formation of complexes between these compounds and other dietary compounds (metal ions, proteins and/or fibre) and/or the interaction with bile salts (Lucas-González et al., 2018). On the contrary, the increased concentration of rutin after intestinal digestion might be explained by the enzymatic hydrolysis of bonds previously formed between rutin and proteins or fibre in the original matrix, enhancing the release of rutin (Gullon et al., 2015). The results presented in this section are very relevant since, to the best of our knowledge, no previous studies have been found on the effect of simulated GIT on the individual phenolic compounds of GL and PE of QIL, showing high stability and highlighting that most of the phenolic compounds present in the intestinal digested sample were not detected in the fraction available in the colon.

3.3. Effect of in vitro gastrointestinal digestion on the antioxidant activity

Antioxidant activity is the most studied bioactivity in plant extracts and has been attributed to the presence of certain bioactive compounds, mainly polyphenols. The passage of polyphenols through the GIT affects their integrity, as demonstrated above, so it is expected that the antioxidant activity of both PE and GL also changes. In this work, two different methods were applied, based on different chemical mechanisms: electron transfer ($ABTS^{\bullet+}$) and hydrogen atom transfer (ORAC). Fig. 5 shows the antioxidant capacity of PE and GL before and after the simulated gastrointestinal digestion phases.

$ABTS^{\bullet+}$ and ORAC assays evidenced that the antioxidant activity of PE and GL was negatively affected by the simulated gastrointestinal digestion, showing the same trend as the TPC. Likewise, the antioxidant activity of PE was higher than GL in all phases, although the ratio between them was more pronounced at the beginning (ca. 8.9) than at the end (c.a. 5.5). $ABTS^{\bullet+}$ and ORAC values presented an important decrease from the undigested initial sample until dialysis, with final values of 555.44 and 458.93 mM TE/g DW in PE and 84.63 and 102.70 mM TE/g DW in GL. Similarly, a loss of antioxidant activity after gastrointestinal digestion, has been previously reported in other matrices such as *Moringa oleifera* leaves (Mendoza, 2019), jaboticaba peel powder (Quatrin et al., 2020) or espresso coffee (Vilas-boas, Oliveira, Jesus, Rodrigues, Figueira, & Gomes, 2020).

The antioxidant capacity of PE and GL samples changed differently throughout the digestion process (Fig. 3a, b). No significant differences of $ABTS^{\bullet+}$ and ORAC were observed in PE after the oral (2685.87 and 3525.01 mM TE/g DW) and gastric phases (2665.77 and 3498.35 mM TE/g DW), whereas a pronounced decrease was detected after intestinal (917.88 and 1396.61 mM TE/g DW) and dialysis phase (555.43 and 458.93 mM TE/g DW), compared to the undigested initial fraction. In the case of GL, firstly, an increase in $ABTS^{\bullet+}$ and ORAC values were found after oral (451.27 and 602.90 mM TE/g DW) and gastric phases (472.98 and 610.46 mM TE/g DW) from the initial sample, while after the intestinal and dialysis phases, a marked reduction was observed (191.68 and 384.17 mM TE/g DW, and 84.63 and 102.70 mM TE/g DW, respectively). It can be seen that $ABTS^{\bullet+}$ assay provided lower equivalent trolox values than ORAC assay, as shown in Fig. 3a and b. This variation may be due to the different reaction mechanisms behind the methods. Namely, the $ABTS^{\bullet+}$ radical has a higher molecular weight than the ORAC atom transfer molecules, which may reduce the reaction rate of this assay, and consequently, the ORAC assay would provide a more accurate estimation of the antioxidant activity (Campos et al., 2020).

As mentioned above, the antioxidant activity is related to the presence of phenolic compounds, suggesting that there should be a significant correlation between polyphenols and the antioxidant capacity (Chait et al., 2020). A significant positive correlation was found between TPC and the antioxidant activity obtained with $ABTS^{\bullet+}$ assay in PE ($r^2 = 0.972$) and GL ($r^2 = 0.963$), as well with ORAC assay in PE ($r^2 = 0.978$) and GL ($r^2 = 0.918$). These results point out the great contribution of polyphenols to the antioxidant activity in both matrices. The correlation values obtained are in concordance with several studies that reported a high correlation between polyphenolic compounds and antioxidant activity (Chait et al., 2020; Lucas-González et al., 2016; Ribeiro et al., 2020). Additionally, a significant correlation was observed between

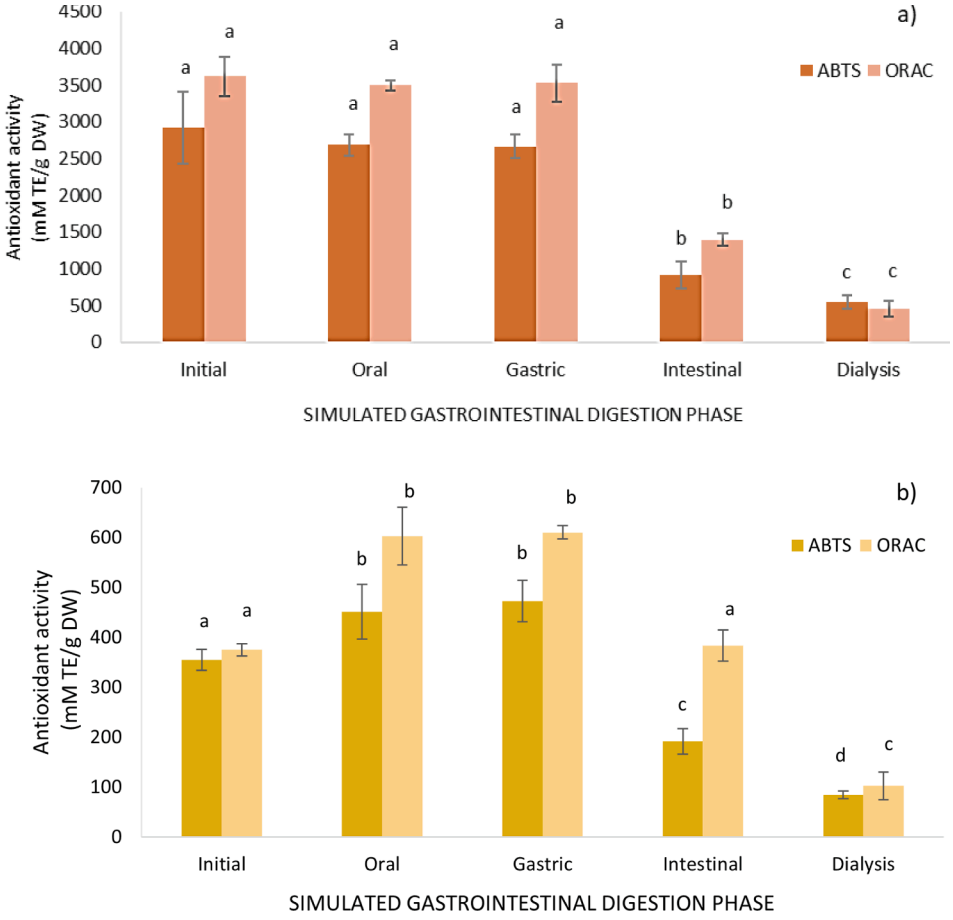

Fig. 5. Antioxidant activity of power extract (a) and ground leaf (b) measured by $ABTS^{\bullet+}$ and ORAC assays throughout the *in vitro* gastrointestinal digestion (initial, oral, gastric, intestinal and after dialysis phases). Results are the means of three determinations ± standard deviation. Different letters represent different homogeneous groups according to the Tukey's test ($p < 0.05$).

ABTS$^{\bullet+}$ and ORAC assays in PE ($r^2 = 0.939$) and GL ($r^2 = 0.919$).

3.4. Antimicrobial activity of Quercus ilex leaf extract (PE)

Polyphenols have been also studied for their attributed antimicrobial properties. In this work, the antimicrobial activity of PE was evaluated as a potential natural antimicrobial agent, being this capacity expressed as minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC). Different concentrations of the extract in a range of 0.25–20 mg/mL, were tested against three Gram negative (*E. coli*, *S.* Typhimurium *and Y. enterocolitica)* and two Gram positive *(L. monocytogenes* and *S. aureus)* foodborne pathogens (Table 2). Our results revealed that the extract was able to inhibit the growth of all the microorganisms studied. MIC values varied between 1 and 5 mg/mL. According to the MICs and MBCs established, the sensitivity of the bacteria tested to PE was, in decreasing order: *S. aureus* and *Y. enterocolitica* > *E. coli* and *S.* Typhimurium > *L. monocytogenes*. As can be observed in Table 2, MBCs equalled MICs values for every microorganism tested, confirming that the same concentration was required to kill and visibly inhibit bacterial their growth. The MICs established were confirmed by the interpretation of the growth/inhibition curves of the target microorganisms in the presence of PE at concentrations ranging from 0.25 to 5 mg/mL (Fig. 6).

In general, it has been found that MIC and MBC values are usually lower for Gram-positive bacteria than for Gram-negative, which may be explained by the higher sensitivity of the former, related to the presence of a lipopolysaccharide layer in the wall of Gram-positive bacteria (Gullón et al., 2018). However, investigations are controversial on this issue, with other works showing MIC values for Gram-positive bacteria such as *L. monocytogenes*, similar or higher than the MICs found for Gram-negative bacteria such as *E. coli* or *Salmonella* spp. (Maria et al., 2016). In addition, there may be other factors that may influence the inhibitory effect of the extract like the strain used, as reported by Gullon, Pintado, Pérez-Álvarez, and Viuda-Martos (2016) in their study on pomegranate peel flour, in which *L. monocytogenes* showed lower sensitivity (50 mg/mL MIC and 60 mg/mL MBC) than *L. innocua* (20 mg/mL MIC and 30 mg/mL). In our case, it is not possible to withdraw definite conclusions, as the two Gram-positive microorganisms studied, i.e., *S. aureus* and *L. monocytogenes*, represented the most and the less sensitive bacteria, respectively, which means that can be strain dependent.

The results of this work are very relevant, as the microorganisms studied have been involved in foodborne outbreaks worldwide (Sofia et al., 2021). Foodborne infectious diseases have been estimated to affect 600 million persons around the world and cause 420,000 deaths per year, with 40% of the disease burden occurring among children under 5 years of age, and 125,000 deaths per year in this age group (WHO, 2015). Likewise, it is estimated that the emergence of multi-drug resistant strains of foodborne pathogens could lead to high morbidity and mortality rates, emphasizing the need for new natural antimicrobial compounds (Aguirre-Becerra et al., 2020; Ali et al., 2021). In this regard, the market volume for novel natural antimicrobials is expected to grow at an annual growth rate of 7.3 % over the 2019–2027 period (MRFR, 2021). In this study, PE induced a strong antimicrobial action against *S.* Typhimurium, *E. coli*, *L. monocytogenes, S. aureus* and *Y. enterocolitica*, which are some of the most common agents implicated in bacterial foodborne diseases (WHO, 2015).

Table 2
Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Quercus ilex* leaf powder extract (PE) against five foodborne pathogens strains. Results are the means of three determinations.

Bacterial strains	MIC (mg/mL)	MBC (mg/mL)
Escherichia coli (CECT 8295)	2.5	2.5
Listeria monocytogenes (CECT 4032)	5	5
Salmonella Typhimurium (CECT 704)	2.5	2.5
Staphylococcus aureus (CECT 5193)	1	1
Yersinia enterocolitica (CECT 754)	1	1

Results observed in the present work are in agreement with those previously reported by Karioti et al. (2011), who attributed to a QIL methanolic extract antimicrobial activity against several microorganisms such as *L. monocytogenes*, *S. aureus*, *S.* Typhimurium and *E. coli* (17–34 μmol/mL). Similarly, Berahou et al. (2007) observed that a *Q. ilex* bark methanolic extract showed antimicrobial activity with MIC values between 0.25 and 0.5 mg/mL against *E. coli*, *S.* Typhimurium and *S. aureus*. On the other hand, some differences were observed with the work of Güllüce and Adıgüzel (2004) in a QIL methanolic extract. These authors found a MIC value of 0.25 mg/mL against *E. coli* but were not able to determine a MIC against *S.* Typhimurium nor *S. aureus*, opposite to our results. The differences observed in MIC values could be explained by variations in the strains sensitivity, differences in the phenolic composition of *Quercus* spp. extracts, the extraction procedure (Gullon et al., 2016) and the heterogeneity of leaves and harvest conditions (Hadidi et al., 2017). As can be seen, our results show, in general, higher values of MIC of PE against foodborne pathogens than those reported by the aforementioned authors. However, this could be far compensated by the green extraction method (MAE) applied in this work, with a short extraction time (10 min), low energy consumption and the use of water as a solvent, which is environmentally friendly and low cost.

The antimicrobial activity of PE could be attributed to the presence of some phenolic compounds with antimicrobial activity, such as catechin and rutin, the main phenolic compounds present in *Q. ilex* extracts. Nevertheless, it is difficult to attribute the antimicrobial activity to a specific bioactive compound of a mixture. In fact, it has been reported that the antibacterial effects of a PE could be due to the combined action of several bioactive compounds through various action mechanisms (Olszewska et al., 2020). In any case, these mechanisms are reported to be associated with the inhibition of the enzymatic reactions required for bacterial growth (Gómez-García, Campos, Aguilar, Madureira, & Pintado, 2020) and with an increase of the cell membrane permeability. In this way, flavonoids, such as catechin and rutin, are capable of interfering with the structural and functional properties of bacterial membranes by interacting with the membrane lipids, becoming more permeable, and thus, inducing the disruption of the cell integrity with leakage of cytoplasmic content (Maria et al., 2016).

4. Conclusions

In this work, the recovery, bioaccessibility and stability of polyphenolic compounds and changes in the antioxidant activity of QIL, have been studied for the first time throughout the different phases of the GIT digestion. Additionally, the antimicrobial activity of the PE has also been evaluated. This study demonstrates that the GIT had a substantial effect on bioactive compounds, affecting polyphenols stability. *In vitro* GIT digestion of PE and GL revealed that phenolic compounds mainly decreased in the intestinal and dialysis phases of digestion. However, the bioaccessibility of phenolic compounds (34.23 and 55.27%, respectively) demonstrates the stability of these compounds and the possibility to exert their bioactivity after absorption, mainly as antioxidant compounds for the prevention of oxidative stress diseases. In addition PE showed high antimicrobial activity against five foodborne pathogens, with low concentrations required to inhibit the growth of *Y. enterocolitica* and *S. aureus*. All in all, these results suggest that QIL, rich in flavonoids, has promising potential applications as a functional food ingredient with high antioxidant activity, and as antimicrobial agent against foodborne pathogens. Although the digestion process results in a reduction of phenolic compound and antioxidant properties of PE and GL, the final concentrations of polyphenols were significant, which demonstrates their suitability to exert their beneficial effect on health.

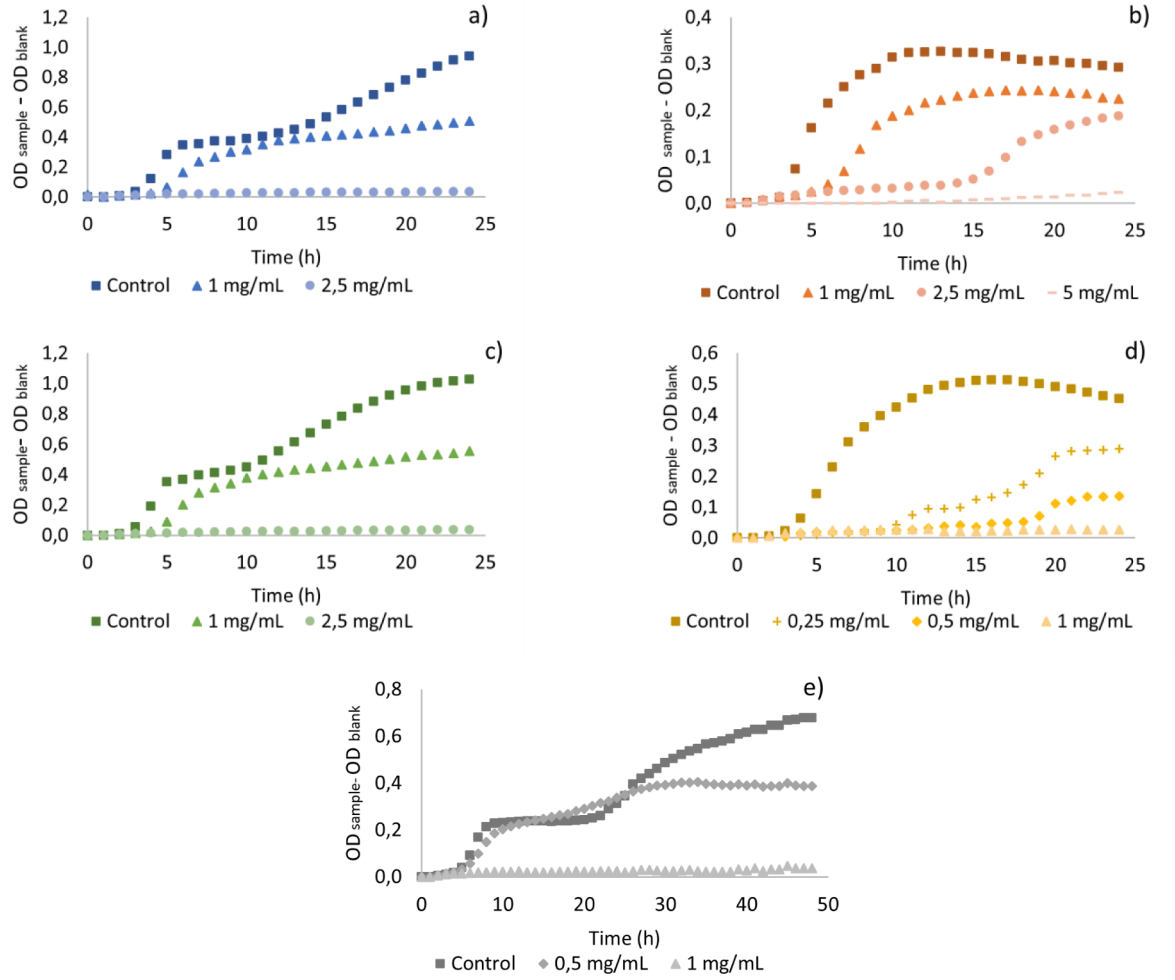

Fig. 6. Growth/inhibition curves of (a) *E. coli*, (b) *L. monocytogenes*, (c) *S. Typhimurium*, (d) *S. aureus* and (e) *Y. enterocolitica* under different concentrations of *Quercus ilex* leaf powder extract. Results are the means of three determinations.

Ethical statement

This research did not include any human subjects and animal experiments.

CRediT authorship contribution statement

Mónica Sánchez-Gutiérrez: Writing – original draft, Investigation, Data curation, Formal analysis. **Ricardo Gómez-García:** Investigation, Methodology, Formal analysis. **Elena Carrasco:** Supervision, Validation, Visualization. **Isabel Bascón-Villegas:** Investigation, Methodology. **Alejandro Rodríguez:** Resources, Supervision. **Manuela Pintado:** Conceptualization, Supervision, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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