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DOI: 10.1039/C9FO03000J

Simulated digestion of olive pomace water-soluble ingredient: Relationship between the compounds bioaccessibility and their potential health benefits

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Abstract

Olive pomace is a semisolid by-product with a great potential as a source of bioactive compounds. Using its soluble fraction, a liquid-enriched powder (LOPP) was obtained, exhibiting a rich composition in sugars, polyphenols and minerals, with potential antioxidant, antihypertensive and antidiabetic health benefits. To validate LOPP potential as a functional ingredient the effect of the gastrointestinal tract on its bioactive composition and bioactivities was examined. Polyphenols and minerals were the most affected compounds, however, a significant bioaccessibility of potassium and hydroxytyrosol was verified (≥ 57 %). As a consequence, the LOPP bioactivities were only moderately affected (losses around 50%). For example, 57.82 ± 1.27 % of the recovered antioxidant activity by ORAC was serum - available. From an initial α-glucosidase inhibition activity of 87.11 ± 1.04 %, at least 50% of the initial potential was kept (43.82 ± 1.14 %). Regarding initial ACE inhibitory activity (91.98 ± 3.24 %), after gastrointestinal tract losses, significant antihypertensive activity was retained in the serum-available fraction (43.4 ± 3.65 %). The colon-available fraction also exhibited an abundant composition in phenolics and minerals. LOPP showed to be a potential functional ingredient not only with potential benefits in preventing cardiovascular diseases but also in gut health.

Keywords: Bioeconomy, *In vitro* digestion, Minerals, Polyphenols, Antioxidant, Antihypertensive, Antidiabetic, Bioaccessibility index.

1. Introduction

Currently, sustainable treatment of olive pomace (OP) is one of the biggest challenges of the olive oil industry, despite its high nutritional value [1]. As a solution, the development of OP-based ingredients has been studied to add-value and reduce its environmental impact. Powdered ingredients have been

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proposed as a more feasible, stable and sustainable solution. A liquid-enriched olive pomace powder (LOPP) has been obtained (data not shown). Our previous study has demonstrated the great potential and safety of LOPP as a potential food ingredient to be applied in functional food formulation due to (1) its rich composition in sugars (mainly mannitol), minerals (mainly potassium) and polyphenols (mainly hydroxytyrosol and its derivatives); and (2) its antioxidant and antimicrobial properties (data not shown). Considering its polyphenol composition and antioxidant properties, LOPP could be a functional ingredient with health benefits in cardiovascular prevention, as an antihypertensive and antioxidant ingredient [2,3], and also as an antidiabetic agent [4–7]. These health benefits have been mostly associated with the phenolic compounds with high antioxidant capacity, however, sugars, more specifically mannitol [8–10], and minerals [11,12], could also have an important role.

Hydroxytyrosol and its derivatives (tyrosol, oleuropein, hydroxytyrosol glucoside, etc.) were the principal phenolics identified in LOPP. These phenolics have been associated with potent antioxidant, anti-inflammatory and antimicrobial activities [1]. Moreover, hydroxytyrosol and its derivatives have been approved with the claim “Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress” by the European Food Safety Authority (EFSA) for olive oil that contains at least 5 mg of hydroxytyrosol and its derivatives per 20 g of olive oil. Besides, bioaccessibility studies have shown that hydroxytyrosol from olive can be absorbed efficiently in humans [13].

Cardiovascular disease and diabetes mellitus are the most prevalent diseases of the 21st century, being hypertension a primary risk factor of cardiovascular disease [14], and hyperglycaemia one of the characteristics of diabetes mellitus with main impact in the disease [5]. Hypertension and hyperglycaemia are commonly treated with drugs to inhibit the enzymatic activity of angiotensin-converting (ACE) and α-glucosidase, respectively. The inhibition of the angiotensin-converting enzyme (ACE) may help reduce the formation of angiotensin II, a vasoconstrictor and a ROS initiator [15]. On the other hand, the inhibition of hydrolytic enzyme α-glucosidase may help minimise postprandial hyperglycaemia and hence delay the absorption of glucose [5].

Several studies have explored natural products in the search of ACE and α-glucosidase inhibitors. Peptides and triterpenes have been described as strong potential inhibitors of ACE. Recent studies have revealed that various phenolic compounds from different plants possess ACE [16] and α-glucosidase inhibitory activity [17], which varies significantly according to their chemical structures. As some studies showed, olive oil polyphenols were responsible for the anti-hypertensive effect in hypertensive rats [2], but, there are no previous reports showing ACE inhibitory activity of olive oil polyphenols[14]. Olive oil polyphenols have also been related to the anti-diabetic effect by inhibition of α-glucosidase [17]. Figueiredo-González *et al.* (2019), Figueiredo-González *et al.* (2018) and Nadour *et al.* (2015) demonstrated a close relationship between the higher amount of olive phenolic content with most effective α-glucosidase inhibition activity. Indeed, hydroxytyrosol has been suggested as an effective α-glucosidase inhibitor [5]. Other compounds from LOPP that could also act as ACE and α-glucosidase inhibitors are soluble sugars as mannitol [8,9]. Minerals, like calcium, naturally present in LOPP, have also been reported as potential inhibitors of α-glucosidase [11].

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LOPP composition and demonstration of its bioactive properties support the potential application of this ingredient as a functional ingredient with antioxidant capacity and health benefits such as antihypertensive and antidiabetic activities. Nevertheless, to guarantee LOPP health benefits, the digestion impact on polyphenols, minerals and sugars needs to be evaluated to guarantee bioactives bioaccessibility and related health benefits. Several studies have developed new functional ingredients from OP [18], namely bioactive extracts used as a source of health-protecting effects [19] or food preservatives [20] and powders, used on the formulation of bakery products [21], snacks [22] or pasta [23]. Besides, these OP functional ingredients were obtained applying more elaborate processes or using water/organic solvents, while LOPP was obtained using a simple fractionation process followed by a drying step. However, only a few of these studies considered the impact of the gastrointestinal tract on potential bioactivities of OP functional ingredients [24–26].

The *in vitro* simulation of gastrointestinal digestion (SGD) has been a valuable tool to analyse changes, bioaccessibility and digestibility of bioactive compounds from functional foods. Several differences are observed between *in vitro* models and *in vivo* studies. Nevertheless, SGD models remain a useful and less expensive alternative to animal and human models for a rapid screening of food ingredients; furthermore, *in vitro* techniques are ethically superior than *in vivo* techniques aligned with EU directives [27,28].

The present study attempted to investigate (1) the bioaccessibility of sugars, minerals, polyphenols and dietary fibre throughout SGD and (2) to demonstrate the potential antioxidant, antihypertensive and antidiabetic activities after SGD. This is the first study that demonstrates the stability and bioaccessibility of olive pomace liquid fraction (LOPP bioactive compounds) that constitute a critical information because only bioavailable compounds can exert their biological function.

2. Materials and methods

2.1 Chemicals and reagents

The ABTS diammonium salt (2, 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid), 2,2-diphenyl-1-picrylhydrazyl (DPPH), pancreatin, pepsin, formic acid, potassium sorbate, sodium carbonate, trifluoroacetic acid (TFA), peptidyl-dipeptidase, α- glucosidase and all reagents of ACE and α-glucosidase inhibitory activity assay were purchased from Sigma-Aldrich (Sintra, Portugal). Acetonitrile and methanol were purchased from Fischer Scientific (Oeiras, Portugal). Folin-Ciocalteu's reagent and potassium persulfate were purchased from Merck (Algés, Portugal). Bile salts are from Oxoid™ (Hampshire, UK). Standards of Trolox, gallic acid, *p*-coumaric acid, vanillin, protocatechuic acid, caffeic acid and quercetin were obtained from Sigma-Aldrich (Sintra, Portugal), whereas hydroxytyrosol, tyrosol, luteolin were purchased from Extrasynthese (Lyon, France).

2.2 Preparation of olive pomace water-soluble ingredient

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The OP was collected from an olive mill in Oliveira do Hospital, Portugal, being composed mainly by the olive cultivar *Galega Vulgar* (80% of the olive heritage). Various samples were taken, transported to the laboratory where they were mixed. The homogenous sample was packed in polyethene flasks and kept in a freezer at - 80 ^{0}C until use.

OP was fractionated by centrifugation (10,000 ×g for 10 min). The liquid fraction was freeze-dried (Telstar Lyo Quest HT 40) with 2% of mannitol (as a cryoprotectant and to prevent aggregation) and the powder obtained was denominated liquid-enriched olive pomace powder (LOPP).

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2.3 *In vitro* digestion

LOPP *in vitro* simulation of gastrointestinal digestion (SGD) (**Figure 1**) was performed according to the method described by Madureira *et al.* (2011) and Costa et al., (2019) with dialyses process to simulate the intestinal and serum absorption [31,32]. Mouth digestion was conducted with 0.6 mL of α–amylase solution (100 U/mL) and incubation took place for 1 min, at 37 °C and 200 rpm. For gastric digestion, the pH was adjusted to 2.0 with concentrated HCl (1 mol/L), and the mixture was incubated with pepsin (25 mg/mL) (from porcine stomach mucosa, pepsin A), at a rate of 0.05 mL/mL of sample, in a shaking bath, for 60 min at 37 °C. Small intestinal digestion was performed by adjusting pH to 6.0 with $NaHCO_3$ (1 mol/L), before the addition of pancreatin (from porcine pancreas, 2 g/L) and bile salts (12 g/L), at a ratio of 0.25 mL/mL of sample, and further incubation of the mixture for an additional 120 min at 37 °C.

To screen the release of individual polyphenols from LOPP (1 g) at different stages of digestion, samples were collected from the simulated mouth (ca. 4 mL), gastric digest (ca. 4 mL), and intestinal digest (ca. 4mL) and used to make extracts to further perform polyphenols analysis.

In the last phase of intestinal digestion, a segment (10 cm) of dialysis tubing (3.5 kDa molecular weight cut-off [30,31]) filled with $NaHCO_3$ (1 M) was placed inside of screw-topped bottles filled with digested samples and incubated for 2 h in a shaking water bath, at 37 °C and 50 rpm. The dialysis process applied had as a goal, to be closer to the biologic process [27] and simulate, at least, the passage by duodenum and jejunum[33].

At the end of the incubation process, the solution left outside the dialysis tubing (OUT) represented the non-absorbable sample (colon-available), and the solution that managed to diffuse into the dialysis tubing (IN) represents the sample that is available for absorption (serum-available).

All enzymes solutions were prepared fresh and filter-sterilised using a 0.22 µm-membrane filter. All solutions were maintained in an ice bath during the entire period of gastrointestinal digestion processes before gradual addition (when appropriate). Three replicas of the SGD were made. Two replicas of blanks were prepared without sample and underwent using the same conditions. All stages of digestion were lyophilised and stored for analysis of the compound's composition and potential bioactivities. Blanks are used to correct the concentration of the compounds of the digested samples analysed.

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Several protocols of static *in vitro* simulation of digestion can be found in the literature[34]. All these protocols exhibit different conditions (e.g., pH, duration of each step, ratio of enzymes to substrate), making the comparison between studies very difficult. Recently, it was implemented a standardized static digestion model suitable for foods under the INFOGEST Cost Action[28,34]. This INFOGEST protocol, as the method adopted in this work, is also divided in oral phase, gastric phase and intestinal phase. The main differences between the two methods are in the: execution of the enzyme activity assays, composition of the simulated fluids for each digestion phase (use of a electrolyte stock solutions), a longer time of gastric digestion (1h more), different pH on intestinal phase (pH 6 instead pH 7), higher ratio of sample to digestive fluids (1:10 instead 1:1) and ratio of enzymes to sample. The different digestion conditions the adopted protocol in this work compared to the INFOGEST protocol could lead to different results. In the future works, the INFOGEST protocol should be applied to harmonize the experimental conditions of the static *in vitro* simulation of gastrointestinal food digestion.

2.4 Stability and bioaccessibility of compounds through in vitro gastrointestinal digestion

2.4.1. Recovery and bioaccessibility Index

The results of each extract determination (on the sample, after mouth, gastric and intestinal digestion) were reported to the 100 g of dry weight (DW) of LOPP.

Recovery index (RI %) and bioaccessibility index (BI %) were studied to evaluate the effect of the matrix composition on the digestion of the main nutritional/bioactive components of LOPP compounds and not only of phenolic groups. The values for LOPP before digestion were assumed as 100% of all bioactive compounds and bioactivities of sample [31,32]. At this point, it is essential to define the terms “bioavailability” and “bioaccessibility carefully”. Bioavailability expresses the fraction of ingested bioactive compound or nutrient that reaches the systemic circulation and finally utilised. Before becoming bioavailable, bioactives must be released from the food matrix and modified in the gastrointestinal tract. Thus, bioavailability includes the term bioaccessibility. Bioaccessibility is described as the amount of a compound that is released from its matrix in the digestive tract, becoming available for bloodstream absorption [35].

The percentage of recovery allows the determination of the amount of each main compound, on the tested food, after oral, gastric and intestinal digestion, according to:

$$\textit{Recovery index (\%)} = (BC_{DF}/BC_{TF}) \times 100$$

Where: BC_{DF} is the bioactive content (mg) in the digested and BC_{TF} is the bioactive content (mg) quantified in the test matrix.

The bioaccessibility is defined as the percentage of the bioactive compound that is solubilised after intestinal dialysis step. Thus, this index defines the proportion of the bioactive compound that could become available for absorption into the blood system:

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$$Bioaccessibility\ index\ (\%) = (BC_S/BC_{DFE}) \times 100$$

where: BC_S is the bioactive content (mg) in the digested sample after the duodenal dialysis step (IN) and BC_{DF} is the bioactive content (mg) in the digested sample after the duodenal step (IN + OUT) – end of digestion.

2.4.2. Sugars and organic acids

Free sugar and organic acid profiles were determined using the lyophilised samples dissolved in water at a concentration comprised between 50 and 70 mg/mL. The analyses were performed using a Beckman Coulter System Gold HPLC (Knauer, Berlin, Germany) coupled to IR and UV detector using Aminex 37-H column (Bio-rad, Berkeley, USA) at 55 °C and 35 mM H_2SO_4 as mobile phase (flow rate: 0.5 mL/min). Both identification and quantification were achieved by comparison of the relative retention times of sample peaks with standards, using a calibration curve in the range of concentrations of 0.2-2.0 mg/mL.

2.4.3. Minerals

Mineral concentration was carried out following the methodology of Amorim et al. (2016), using an optical emission spectrometer Model Optima 7000 DV™ ICP-OES (Dual View, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) with a radial configuration and an amount of lyophilised digested samples of 250 mg.

2.4.4. Polyphenolic compounds

Lyophilised samples were dissolved in methanol (between 20 and 200 mg/mL) to perform phenolics analysis. Total phenolic content (TPC) of FPC and BPC extracts was determined according to the Folin-Ciocalteu method [36]. Results were expressed as mg gallic acid equivalents (GAE)/100 g DW.

The chromatographic analysis was performed using a Waters e2695 separation module system interfaced with a Photodiode array UV/Vis detector (PDA 190-600 nm). The separation was conducted in a reverse-phase column (COSMOSIL 5C1 8-AR-II Packed Column – 4.6 mm I.D. × 250 mm; Dartford, UK). The flow rate, gradient program and mobile phases used following the Oliveira *et al.* (2015). Compound detection was performed at specific wavelengths: 280, 320 and 360 nm, while data acquisition and analysis were accomplished using Software Empower 3. Identification of main phenolic compounds in methanol was performed by comparison of retention times, spectra and peak areas at maximum absorption wavelength.

The complete profile of phenolic compounds and its derivatives trough SGD was also analysed in an LC-ESI-UHR-QqTOF-MS following the methodology of Monforte *et al.* (2018) with some modifications in the gradient elution program to obtain a good separation of phenolic compounds. Mobile phases were 0.1% aqueous formic acid (A) and acetonitrile with 0.1% formic acid (B).The gradient elution program used was the following: 0–5 min (5 % B); 5–25 min (15 % B); 25-35 min (30 %); 35-40 min (95 % B), 40-41 (5 % B) and 41–42 min (0 % B). Identification of main phenolic compounds was based on the retention time, UV-Vis and mass spectra with those obtained from the standard

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solutions, when available. The other peaks were tentatively identified comparing the information with available data reported in the literature. The elemental composition for these compounds was confirmed according to accurate mass (5 mDa), and isotope rate calculations designated mSigma (<20) (Bruker Daltonics).

The UV-visible absorption spectra acquired with DAD detector was used to identify and classify the phenolic compounds. DAD is currently the most widely available and commonly used technique for routine qualitative and quantitative analyses of these metabolites. The combination of these data with mass spectra (MS) data and information from the respective literature or comparison with standard compounds can be used for tentative identification of each peak in a chromatogram.

The main phenolic compounds identified (3-hydroxytyrosol, protocatechuic acid, tyrosol, vanillin, caffeic and *p*-coumaric acid) were quantified by HPLC using external calibration curves created based on their maximum UV signal. The results were expressed as mg/100 g DW. Some compounds were expressed as equivalents of its the basic constituent: hydroxytyrosol glucoside was expressed as hydroxytyrosol, tyrosol glucoside was expressed as tyrosol, verbascoside and caffeoyl-6'-secologanoside were expressed as caffeic acid equivalents [39].

2.4.5. Fibre composition

The digested sample was filtered using a sintered glass crucible (no. 2). The insoluble dietary fibre (IDF) was retained in the crucible, and the supernatant was saved for soluble dietary fibre (SDF) analysis. IDF was washed twice with 10 mL of distilled water, and the supernatants were combined for determining SDF. IDF residue was washed once with 20 mL of 96 % ethanol, followed by 20 mL of acetone twice, and then dried overnight at 100 °C (ash and protein were corrected during this step). The filtrate was purified by dialysis (dialysis tube with a molecular weight cut-off of 3.5 kDa) to obtain the SDF. The dialysis was applied to avoid the error caused by the precipitation of dietary fibre with ethanol [40,41]. Afterwards, SDF was recovered by dialysate freeze-drying. The results were expressed as total dietary fibre (TDF), IDF and SDF g/100 g DW.

Sugar composition of SDF was achieved by acid hydrolysis (6 % sulfuric acid) at 121 ◦C for 1 h (29). The hydrolysate is used for quantifying uronic acids (UA) and neutral sugars (NS). NS were determined by HPLC (micro guard column: Aminex Carbo-P, Bio-Rad; carbohydrate analysis column: Aminex HPX-87P heavy metal, 300–7.8 mm, Bio-Rad; flow rate: 0.6 mL/min; detector: refractive index), and UA was determined colourimetrically by adapting the 3-hydroxydiphenyl method of Blumenkrantz & Asboe-Hansen (1973) with *d*-galacturonic acid as standard. The sum of NS and UA was taken as the amount of SDF in LOPP after the SGD.

Polyphenol compounds were released from SDF using a hydrolysis process with 4 mol/L NaOH at room temperature, according to Xie *et al.* (2015). The extract obtained was dissolved in methanol and named "SDF bound polyphenol compounds" (SDF-BPC). Aliquots of the hydrolysates were used for spectrophotometric measurement using Folin-Ciocalteu, antioxidant activity and to identify the phenolic compounds by HPLC and LC-ESI-UHR-QqTOF-MS.

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2.5 Effect of *in vitro* gastrointestinal digestion on bioactivities

2.5.1. Antioxidant activity: ABTS, DPPH e ORAC

The antioxidant activity of LOPP during the SGD was achieved according to the methods of $DPPH^{\bullet}$ [44], $ABTS^{\bullet+}$ [45] and ORAC [46] using a multi-detection plate reader (Synergy H1, Vermont, USA). The radical stock solutions were freshly prepared. Lyophilised samples were dissolved in methanol to obtain a concentration comprised between 20 and 200 mg/mL. All analyses were performed in triplicate and expressed in mM of Trolox-equivalents (TE)/ g DW.

2.5.2. Antihypertensive activity (ACE-inhibitory activity)

The *in vitro* ACE-inhibitory activity was measured using the fluorimetric assay of Sentandreu and Toldrá (2006) [47] with some modifications. A total of 40 µL of ultrapure water or ACE working solution was added to each microplate well, then adjusted to 80 µL by adding ultrapure water to a blank (BLK), control (CTL) or samples (SPL). The enzyme reaction was started with the addition of 160 µL of the substrate solution, and the mixture was incubated at 37 °C. The fluorescence generated was measured during 30 min using a multi-detection plate reader (Synergy H1, Vermont, USA). The assay was performed in a black 96-well microplate (Nunc, Denmark). Excitation and emission wavelengths used were 350 and 420 nm, respectively.

The inhibitory activity was expressed as inhibited % of the maximum ACE activity. The equation applied to calculate de percentage of ACE-inhibitory was the following:

$$iACE\ inhibition\ (\%) = [(F_{CTL} - F_{BLK}) - (F_{SPL} - F_{SPLB})] \times [100/(F_{CTL} - F_{BLK})]$$

2.5.3. Antidiabetic activity (α-glucosidase inhibitory activity)

The α-glucosidase inhibitory activity was determined in 96 well plates according to the method described by Kwon *et al.* (2006). The sample (50 µL), was mixed with 100 µL of 0.1 M phosphate buffer (pH 6.9) containing α-Glucosidase solution (1.0 U/mL) and pre-incubated at 25 ºC for 10 min. Then, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each line and absorbance was read. Afterwards, substrate was added, and the absorbance was reread. The reaction mixtures were incubated at 25 ºC for 5 min, and the absorbance readings were recorded at 405 nm by a multi-detection plate reader (Synergy H1, Vermont, USA). As a negative control, 50 µL of buffer solution was used to replace the sample. Acarbose was used as a positive control at the concentration of 10 mg/mL. A blank without the enzyme (used 100 µL of 0.1 mol/L phosphate buffer instead) was performed for each sample.

The α-Glucosidase inhibitory activity was expressed as inhibition (%) and was calculated as follows:

$$\alpha\text{- Glucosidase inhibition (\%)} = [(\Delta Abs\ control - \Delta Abs\ sample) / \Delta Abs\ control] \times 100$$

Where ΔAbs control is the variation of absorbance of the control and ΔAbs sample is the variation of absorbance of the samples.

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2.6 Statistical analysis

R software was used to carry out statistical analyses. All experiments were carried out in triplicates and data was reported as mean ± standard deviation. Shapiro - Wilk test tested the normality of data distribution. The differences of mean values among a concentration of bioactive compounds or bioactivities obtained in the different steps of the *in vitro* SGD were analysed by one-way analysis of variance (ANOVA). The Tukey's *post hoc* test was applied for comparisons of means, and differences were considered significant at $p < 0.05$. Correlation analysis was performed between bioactive compounds contents and bioactivities of LOPP using Pearson correlation analysis.

3. Results and Discussion

3.1 Stability and bioaccessibility of compounds throughout *in vitro* gastrointestinal digestion

3.1.1. Soluble sugars and organic acids

Soluble sugars and organic acids concentration through SGD are described in **Figure 2**. Observing this figure is it possible to see that one of the main components detected in LOPP were soluble sugars. Sugars identified in LOPP were mannitol > glucose > fructose. Lactic and acetic acid were detected in LOPP, but the most significant organic acid identified was formic acid.

The predominant sugar present in LOPP, mannitol, is a polyol widely used as a reduced-calorie sweetener (Acceptable Dietary Intake of 0–50 mg/kg), but can also exert other technological functions in food, such as: a preservative preventing oxidative damage of food components, and increasing the food shelf life by reducing sugar crystallisation [49]. Regarding health benefits, mannitol requires no insulin for its metabolism, which makes of it a potential glycaemic control agent, especially for diabetic individuals. Recently, mannitol was suggested as a dietary supplement in controlling postprandial blood glucose by inhibition of α-glucosidase activity, α-amylase activity, glucose absorption, etc. [9].

Formic acid as an organic acid reduces pH value in the stomach, enhancing pepsin activity and consequently, increasing digestibility of nitrogen, phosphorus and minerals [50]. Besides that, formic acid has a key role in the production of colonic acetate by acetogenic bacteria through the Wood–Ljungdahl pathway [51]. Therefore, assessing sugar and formic acid bioaccessibility during physiological stages allows the identification of valuable indicators for the evaluation of their potential health properties.

The recovery index (RI) of soluble sugars and organic acids through SGD is described in **Table 1**. The recovery of soluble sugars and formic acid from LOPP showed similar behaviour throughout the SGD. In the mouth, RI was between 59 % and 62 %, then in stomach ranged between 36-47 % and in the intestine the values remained similar (RI = 39-47 %). As an effect of SGD, the concentration of sugars and organic acids was reduced to half in the mouth and decreased even more in the stomach. On the mouth, the surface area increases and multiple interactions can occur between sugars,

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minerals and polyphenol due to LOPP composition [32,52]. The highest reduction of sugars and organic acids had place in the stomach as a result of acidic conditions (pH=2) and improvement of pepsin activity by formic acid [50].

A significant increase in RI values was observed after the simulation of intestinal absorption phase. This sugar increase might be related to the breakdown of α-(1→4)-glycosidic bonds and other linkages by action of amylase present in pancreatin enzyme and the consequent liberation of monosaccharides [53]. The isomerisation of some of the glucose into fructose could justify the higher RI of fructose. On the other hand, the occurrence of oxidation processes might explain the increase of formic acid during this SGD phase. Formic acid is commonly produced by oxidation processes in olive oil [54].

Regarding the accessibility of the serum available fraction (IN), the uptake was between 14-18 % for sugars and organic acids, and bioaccessibility indexes (BI) exhibited slightly higher values (Table 1). Formic acid revealed the highest BI (23.88 ± 2.51 %) and fructose the lowest BI (15.92 ± 3.93 %). After SGD, formic acid was the most bioaccessible compound. On the other hand, mannitol was less affected by SGD digestion until intestine (RI = 46.40 ± 0.99 %) and was the most bioaccessible sugar (BI = 19.86 ± 2.37 %). At the end of digestion, 52.42 ± 1.63 mg/g LOPP DW of mannitol (**Figure 2**) were available to exert its functional benefits.

Higher recovery index values of soluble sugars and organic acids were attained in colon available fraction (OUT). Fructose (89.32 ± 11.42 %) and mannitol (71.43 ± 8.72 %) exhibited higher RI values, followed by glucose (61.52 ± 7.56 %) and formic acid (43.11 ± 2.32 %). Despite the higher RI of fructose, mannitol (214.08 ± 26.13 mg/g LOPP DW) and glucose (75.55 ± 9.28 mg/g LOPP DW) were the predominant sugars on the colon (**Figure 2**). Mannitol has been used in medicine to promote digestive and urinary excretion of toxins (orally in combination with activated charcoal by gastroclysis), as an enema to prepare the bowel for gastrointestinal procedures and also to reduce hepatic encephalopathy. Intestinal mannitol could increase the molarity of intestinal lumen, decrease hyperammonemia (hepatic inability to remove nitrogenous products generated by protein metabolism of intestinal microbiota) and reduce oxidative stress (excessive production of reactive oxygen species) [55]. These potential effects could also be expected with the ingestion of LOPP, due to the higher amount of mannitol available in colon.

Regarding the high amount of formic acid retained in the colon, formic acid could be used by gut microbiota to produce colonic acetate through the Wood–Ljungdahl pathway [51], which could have potential health benefits in several organs [56].

3.1.2. Minerals

The concentration of minerals (phosphorus, magnesium, calcium and potassium) throughout the SGD is presented in **Figure 2**. Minerals have several potential health benefits. Potassium was the

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predominant mineral in LOPP (5.431 ± 0.11 g/100 g DW). Other minerals, like phosphorus, magnesium, calcium and sodium, were also detected. Potassium is well-known to have a positive effect on the maintenance of normal blood pressure, and consequently, associated with the prevention of cardiovascular disease [57]. The EFSA considers that a potassium intake of 3.5 g/day has beneficial effects on blood pressure in adults [57]. Therefore, the high amount of potassium makes of LOPP a potential agent for cardiovascular diseases. Besides that, minerals have been correlated positively with antioxidant proprieties [12]. Still, to ensure the potential health benefits of minerals from LOPP, it is essential to assess its bioaccessibility after SGD.

Minerals were considerably affected by the SGD in all phases and, as a result, the intestinal RI ranged between 13-17 % (calcium < magnesium < phosphorus < potassium) (**Table 1**). The amount of minerals that reached the gut was significantly lower than the amount present in the initial LOPP ($p < 0.05$), as observed in **Figure 2**. A similar negative effect of *in vitro* digestion on minerals was reported by Costa *et al*., (2019) in grape pomace extract.

The bioaccessibility of all minerals was also negatively affected by SGD. Calcium was not bioaccessible, phosphorus and magnesium exhibited similar low BI values (≈35-36 %). Only potassium was detected in higher amounts in the serum available fraction (IN) than in the colon available fraction (OUT), presenting a BI of 57.42 ± 3.18 %. The bioaccessibility of minerals is influenced by many factors, which may enhance or inhibit their absorption, of the human diet. Generally, polyphenols and dietary fibres are the main inhibitors of the absorption, while organic acids act as enhancers. Therefore, the low recovery and bioaccessibility of minerals in LOPP could be linked to its high amount of polyphenols. Generally, polyphenols reduce the absorption of minerals by chelation mechanisms leading to the sequestration of minerals. For example, calcium and magnesium bioaccessibility has been negatively correlated with polyphenol content in whole grain tea-biscuits [58].

Potassium has been significantly affected by SGD. Only 6.91 ± 0.09 mg of potassium was delivered by 1 g of LOPP. This amount of potassium represents only ≈ 0.2 % of the dietary reference value established (3.5 g of potassium/day) by the EFSA to have beneficial effects on blood pressure [57,59]. However, when integrated int an equilibrated diet, LOPP could help reach the potassium intake of 3.5 g (90 mmol)/day which has beneficial effects on blood pressure and reduces the risk of stroke in adults, without adverse effects on heart function or undesirable gastrointestinal symptoms [57,59]. However, future *in vivo* studies need to be done to validate the uptake of potassium that is regulated by several mechanisms [60].

3.1.3. Polyphenolic compounds

Total phenolic compound (TPC) content of LOPP was determined using spectrophotometric Folin-Ciocalteu method (**Figure 3**). Individual phenolic compounds of LOPP before and after *in vitro*

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digestion were identified by using HPLC-DAD and LC-ESI-UHR-QqTOF-MS detection (Table 2). The main individual phenolics were quantified by HPLC-DAD (**Figure 3**).

TPC amount of LOPP decreased significantly after the SGD. An evident decrease of TPC occurred in the mouth phase (RI 6 %) followed by a slight increase in the stomach (RI 13 %) and a decrease in the intestine (RI 4 %). This pronounced negative effect of SGD on olive polyphenols has also been reported to olive tables [61].

The TPC increment on stomach could be explained by the increase of hydroxytyrosol, *p*-coumaric and caffeic acid. In this phase of the digestion there was also the detection of other derivative compounds by LC-ESI-UHR-QqTOF-MS. One example of this was the ion at TR = 7.5 with m/z 315.1085 which may have the formula $C_{14}H_{20}O_8$ (error = -0.2 mDa, mSigma = 7.3), corresponding to an isomer of hydroxytyrosol glucoside. Furthermore, during SGD, pH has a vital role as a protector of polyphenols against degradation in the stomach (acidic conditions) and as a promotor of degradation in the small intestine (mild alkaline conditions) [62].

During the intestinal absorption phase, an increase of TPC was observed principally in the serum available fraction (IN), which could be explained by the additional contact time of the material with the intestinal fluids and intestinal digestive enzymes (plus 2 h after the small intestine phase). This additional contact time facilitates the release of polyphenols linked to the matrix by the action of enzymes present in pancreatin (extract from porcine pancreas composed by proteolytic, lipolytic, amylolytic, and nucleic acid splitting enzymes). A similar effect was also observed for apple polyphenols [63].

Regarding LOPP individual polyphenols, the results obtained by HPLC were in line with TPC results. High correlation coefficients ($r^2 \geq 0.9$) between TPC and individual phenolics throughout SGD were obtained. Hydroxytyrosol and caffeic acids exhibited better correlation with TPC ($r^2 = 0.99$), followed by hydroxytyrosol glucoside and caffeoyl-6'-secologanoside ($r^2 = 0.98$). Tyrosol ($r^2 = 0.90$) and tyrosol glucoside ($r^2 = 0.93$) revealed a lower correlation with TPC.

The high correlation of some individual phenolics with TPC was evident when the recovery indexes of individual phenolics was analysed (**Table 1**). For example, hydroxytyrosol content decreased in the mouth (RI = 7.52 %) followed by an increase in the stomach (RI = 10.27 %), and a considerable decrease in the small intestine phase (RI = 0.73 %) and a slight increase after the intestinal absorption phase in the serum bioaccessible fraction (IN) (RI =1.47 %). As shown above, TPC and hydroxytyrosol had a similar behaviour throughout SGD simulation. On the other hand, tyrosol glucoside, tyrosol and *p*-coumaric acid were not so highly correlated with TPC, mainly due to its high amount in the serum-bioaccessible fraction (IN) which led to higher RI values (36-48%).

An essential class of phenolic compounds belonging to elenolic-acid derivatives was also identified by LC-ESI-UHR-QqTOF-MS. Elenolic acid is formed, together with the hydroxytyrosol, tyrosol, and glucose, by hydrolysis of oleuropein [64]. Among these, hydrated and hydroxylated products of dialdehydic form of decarboxymethyl-elenolic acid and aldehydic form of decarboxymethyl-elenolic

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acid were found in all LOPP digestion phases. All elenolic-acid derivatives have been previously reported in olive-oil wastes [65]. Elenolic acid derivatives are the most potent antimicrobial compounds in olive and may exert potential health benefits in the gut [64]. Another compound with potential antioxidant and antimicrobial action identified by LC-ESI-UHR-QqTOF-MS was quinic acid. Quinic acid has demonstrated to be an antioxidant agent and, together with other acids, to act as an inhibitor of virulence traits of oral pathogens [66].

Besides the negative effect of SGD on hydroxytyrosol and derivatives, the phenolic compounds mentioned above exhibited the highest bioaccessibility indexes (**Table 1**), being hydroxytyrosol the most bioaccessible compound (BI 82.10 ± 2.59 %) followed by tyrosol (77.79 ± 2.03 %). Some studies have studied the effect of the gastrointestinal tract on olive polyphenols, but most of these studies have been carried out with olive oil [67] or with table olives [61]. Seiquer *et al.*, (2015) reported that, in olive oil, the most bioaccessible and stable compounds after *in vitro* digestion were also tyrosol and hydroxytyrosol. Other studies explored the bioaccessibility, and potential bioavailability of polyphenols from olive leaf extracts [68], but OP extracts bioaccessibility has been less examined. Rubió *et al.*, (2014) assessed the bioaccessibility of phenolic compounds using the gastrointestinal *in vitro* model and cell models from an OP extract, a thyme extract and a conjugation of both. In this study, the bioaccessibility of hydroxytyrosol and secoiridoids from OP extract and the conjugation of both extracts was 13.3% and 23.0%, respectively. Results also indicated that the bioaccessibility of hydroxytyrosol was enhanced by thyme and no significant differences were observed in hydroxytyrosol transport after Caco-2 cells exposure.

Taking into account the positive health claim approved by EFSA, the recommended consumption for hydroxytyrosol and derivatives is 5 mg/day [57]. Based on the results of the present study, the levels of LOPP ingested directly as nutraceutical allowed to estimate that a daily dose of 5.2 g was enough to deliver 5 mg to human blood. However, as a food ingredient, the food matrix may play a crucial role in LOPP phenolics' bioaccessibility, affecting its absorption positively or negatively. Consequently, it is needed to study the effect of SGD using cellular or *in vivo* models and after LOPP incorporation in different food matrixes to clarify LOPP phenolics' bioaccessibility.

3.1.4. Dietary fibre

The amount of dietary fibre (DF) present in LOPP and its composition (neutral sugars, uronic acids and phenolics) throughout SGD are showed in **Table 3**. In the LOPP fraction, soluble dietary fibre (SDF) represents 9% of its composition. SDF has an essential role in health. As a relevant component of LOPP, SDF could confer intestinal health benefits like, the capacity to slow intestinal transit, to delay gastric emptying and to slow glucose/sterol absorption [69]. Nonetheless, the beneficial effects of DF depend not only of its solubility in water but also on (i) fibre composition, (ii) the bioactive compounds associated with it, mainly polyphenolic compounds which are directly related to the antioxidant properties [70] and (iii) the changes produced during gastrointestinal digestion.

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The effect of the SGD on dietary fibre had a meaningful impact on SDF and all its fibre components. The simulated mastication and stomach digestion lead, probably, to a higher vegetable cell rupture, and compounds release [71] which reflected in lower SDF amount after the SGD. Uronic acids and neutral sugars decreased significantly after the SGD, except for glucose that increased significantly. In the SGD, glucose was the predominant neutral sugar (37.73 ± 3.44 mg/g fibre DW*)* followed by galactose and arabinose. The action of the tract enzymes of SGD probably led to the release of polysaccharides rich in glucose by the cleavage of glycosidic bonds [51].

The amount of bound phenolic compounds was also affected by the SGD, being the decrease in TPC amount linked to fibre (**Table 3**), in comparison to the AOAC method. Nonetheless, the amount of total phenolic compounds related to fibre was considerable when expressed per 100 g of LOPP (33.60 ± 3.15 mg GAE / 100 g DW). The identification, by LC-ESI-UHR-QqTOF-MS (**Table 2**) and quantification by HPLC (**Figure 4**), of bound phenolics in SDF obtained by AOAC and after SGD allowed to explain the differences in TPC. Regarding hydroxytyrosol and its derivatives, only tyrosol glucoside was identified in SDF after SGD. On the other hand, 4-hydroxybenzoic acid was only detected in SDF after SGD. The higher release of compounds after SGD hypothesised could explain the no detection of hydroxytyrosol and its higher liberation from fibre and consecutive decarboxylation (α-oxidation dihydroxylation) into protocatechuic acid followed by dihydroxylation into 4-hydroxybenzoic acid [72]. A higher concentration of *p*-coumaric was also exhibited by SDF after SGD, which might be related to SDF higher glucose composition. The linkage between *p*-coumaric and glucose-rich polysaccharides has been verified in several *p*-coumaric extraction studies[73].

Concerning the antioxidant activity of bound phenolics in SDF (**Figure 4**), as for the TPC, the values were higher when using the AOAC method than after the SGD. Only the antioxidant potential by DPPH was not negatively affected by the gastrointestinal tract.

The significant amount of phenolics and antioxidant potential of SDF, although not bioaccessible, could exert antioxidant and antimicrobial activity to improve the gut health [74,75].

3.2 Effect of *in vitro* gastrointestinal digestion on bioactivities

3.2.1. Antioxidant activity: ABTS, DPPH e ORAC

The results obtained in the *in vitro* ABTS, DPPH and ORAC assays showed evidence that LOPP antioxidant activity was negatively affected by SGD (**Figure 5**). In all tests, non-digested LOPP presented higher antioxidant capacity than the digested LOPP available, to be assimilated, in the small intestine, (p<0.05).

Correlation of antioxidant results from ABTS and DPPH (r^2 = 0.97) and from ABTS and ORAC (r^2 =0.97) were higher than with DPPH and ORAC (r^2 =0.96)]. On the other hand, ABTS values were slightly higher than DPPH, and ORAC values were much higher than the values obtained by DPPH and ABTS. Similar differences were also observed in previous works reporting antioxidant assays for olives [76] and OP [77]. These differences probably arise from different mechanisms to measure the

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antioxidant capacity of each methodology. According to the chemical reaction used, methods can be mainly grouped into 2 classes: electron transfer (ET) and hydrogen atom transfer (HAT) based methods. ET methods measure the ability of a potent antioxidant to transfer 1 electron to reduce radicals and HAT methods measure the ability of an antioxidant to quench free radicals by hydrogen donation. DPPH/ABTS assays are examples of the ET methods and ORAC is a HAT method. DPPH and ABTS capture are based on oxide-reduction reactions, so it is normal the similarity of its results. However, ABTS allows the measure of antioxidant activity of hydrophilic and lipophilic compounds, but DPPH can only be dissolved in organic media (especially in alcoholic media), not in aqueous media, which is an important limitation when interpreting the role of hydrophilic antioxidants [78]. The limitation of the antioxidant evaluation of the hydrophilic compounds using DPPH could explain its lower results. On the other hand, ORAC assay is based on the reaction of water and lipid-soluble substances with peroxyl free radical from ROS generator AAPH ((2,2′-azobis(2-methylpropionamidine) dihydrochloride)) [79], which can explain the higher correlation of ORAC/ABTS. To interpret the antioxidant activity results, it is imperative to understand the mechanism of action and limitations of each antioxidant activity.

A higher loss of antioxidant capacity occurred in the small intestinal phase, which was more evident in ABTS (RI 7%), followed by DPPH (RI 13%) and lastly on ORAC (26 %). However, after the simulated intestinal absorption, ORAC values (57.82 ± 1.27 %) remained at higher proportion in the serum-available fraction (IN) than in the colon-available fraction (OUT). However, in ABTS (37.02 ± 1.56 %) and DPPH (18.23 ± 2.12 %) only a small proportion of the total antioxidant activity at the end of the digestion (IN + OUT) was detected in the serum-available fraction. The compounds evaluated by ABTS or ORAC were more bioaccessible, which could be linked to the higher hydrophilic nature of the antioxidants present in LOPP serum available fraction [80]. Despite the loss of a significant amount of antioxidant potential after SGD, the bioaccessible LOPP fraction represents a good source of antioxidant compounds (ABTS: 12.25 ± 0.76 mM TE/g DW; DPPH: 2.75 ± 0.15 mM TE/g DW; ORAC: 147.60 ± 10.99 mM TE/g DW).

Several works reported that phenolic compounds largely contribute to the antioxidant properties presenting the occurrence of good correlations between these compounds and antioxidant activity [31]. ORAC exhibited a better correlation with TPC (r^2 = 0.98) followed by ABTS (r^2 = 0.97) and DPPH (r^2 = 0.97). The good correlations of antioxidant activity methodologies with TPC demonstrate that phenolic compounds are the main contributors of the antioxidant properties of LOPP. In addition, it was demonstrated that hydroxytyrosol ($r^2 \geq 0.97$), caffeic acid ($r^2 \geq 0.96$), caffeoyl-6'-secologanoside ($r^2 \geq 0.96$), hydroxytyrosol glucoside ($r^2 \geq 0.93$) and *p*-coumaric acid ($r^2 \geq 0.91$) were highly correlated with antioxidant capacity. However, tyrosol glucoside ($r^2 \geq 0.82$) and tyrosol ($r^2 \geq 0.73$) exhibited good correlations with antioxidant methodologies, but not as strong.

The different correlations coefficients between individual polyphenols and the antioxidant assays used were related to polyphenol chemical structure. For example, the antioxidant action of tyrosol is only as hydroxyl radical scavenger or at most α-tocopherol regenerator [81]. None of the mechanism of

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the action of tyrosol was individually evaluated by antioxidant assays used. So, the lower correlation of tyrosol and tyrosol glucoside with TPC ($r^2 \geq 0.90$) compared to the other LOPP phenolics ($r^2 \geq 0.97$) might be related to the limited antioxidant effect of these compounds (only carry one hydroxyl group in *para*-position) [81]. Folin–Ciocalteu procedure could also be interpreted as an alternative way to measure the total reducing capacity of the sample as the reagent reacts with any reducing substance [82].

Not only polyphenol compounds influence the antioxidant activity trough gastrointestinal tract, but also, minerals could have an important role in antioxidant activity of digested LOPP [12]. Minerals were strongly correlated with $ABTS^{\cdot+}$, DPPH and ORAC ($r^2 \geq 0.97$). This correlation between antioxidant activity and metals is related to the ability of metals to react with phenolic compounds to form complex compounds that can increase or decrease antioxidant properties [83].

Despite the negative effect of the SGD in LOPP antioxidant activity (until the small intestine phase) and in the bioaccessibility, an increase of the antioxidant activity was revealed during the intestinal absorption phase by ABTS and ORAC methods, mainly on the non-bioaccessible fraction (OUT). As emphasised before, the 2 h extra of contact of LOPP with intestinal fluids and intestinal digestive enzymes during the dialysis process, may have facilitated the release of antioxidant components in the colon fraction. Besides that, the possibility of interaction between phenolics and minerals could explain the high antioxidant activity by ABTS and DPPH on the OUT fraction. This higher antioxidant activity of LOPP in the colon after the whole digestion may play a significant role in the decrease of local oxidative stress and improving microbiota composition, consequently improving gut permeability and boosting anti-inflammatory/immunity mechanisms [74].

The variation in LOPP antioxidant activity (by different assays) throughout the SGD allowed an understanding that phenolic compounds bioaccessibility has an essential role in antioxidant activity. However, the possible formation of metal-polyphenol complexes causes changes in the molecular size and in the electronic charge distribution in the ligand molecule and consequently alters its bioaccessibility and antioxidant functions [84]. Thus, these chelating roles observed in LOPP besides having a negative effect on the bioaccessibility might be of potential interest as dietary antioxidants [84]. Future studies to establish a better correlation between minerals and polyphenols regarding its antioxidant activity and bioaccessibility will be needed to design a more effective functional ingredient from LOPP.

3.2.2. Antihypertensive and antidiabetic activity

LOPP exhibited a higher ACE inhibitory activity (91.98 ± 3.24 %) and a higher α-glucosidase inhibitory activity (87.77± 1.04 %), using a tested dose (50 mg/mL) (**Figure 5**). As an effect of the SGD, ACE (RI 31 %) and α-glucosidase (RI 50 %) inhibitory activity decreased significantly in small intestine phase (p<0.05). In the case of ACE inhibitory activity, a considerable increase occurred during the dialysis process. The highest release of bioactive compounds during the dialysis process allowed the

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recovery of 57% of LOPP initial ACE inhibitory activity in the serum available fraction and the retention of 71% of that initial potential in the OUT fraction. Even though, after SGD, more or less of 50% ACE inhibitory activity was measured in the serum-available fraction (IN). Regarding α-glucosidase inhibitory activity, at least 50 % of the initial potential of LOPP was verified in the small intestine, where α-glucosidase hydrolyses the disaccharides into simple sugars, facilitating sugar intestinal absorption [85]. Using the tested dose (50 mg/mL) of LOPP, at least 43 % of the activity of α-glucosidase could be inhibited. As a conclusion, at least 50 % of both inhibitory activities of LOPP were maintained after SGD.

As reported by several studies, plants polyphenols have potential ACE [16], and α-glucosidase inhibition capacity [84]. TPC was significantly correlated with α-glucosidase (r^2 = 0.69). Indeed, the high degradation of LOPP phenolic compounds verified after gastrointestinal tract seems to influence the α-glucosidase inhibitory activity negatively. Between the LOPP phenolics, *p*-coumaric acid was highly correlated with α-glucosidase inhibitory activity (r^2 = 0.70), but substantial correlation was also found between hydroxytyrosol (r^2 = 0.62), caffeic acid (r^2 = 0.64) and caffeoyl-6'-secologanoside (r^2 = 0.74) with the α-glucosidase inhibitory activity. However, tyrosol and its glucoside were weakly correlated with α-glucosidase inhibitory activity (r^2 < 0.30). Similar interactions have been reported for olive oils [4,5,7,86].

ACE inhibitory activity was also relatively influenced by the loss and low bioaccessibility of phenolic compounds (TPC) throughout the SGD (r^2 = 0.65). In previous studies, ACE inhibitory activity of different polyphenols was variable and dependent of its chemical structure [16]. For example, phenolic acids as hydroxytyrosol (r^2 = 0.62), tyrosol (r^2 = 0.61) and its derivatives, hydroxytyrosol glucoside (r^2 = 0.62) and tyrosol glucoside (r^2 = 0.68), were probably key compounds to explain ACE inhibitory activity of the bioaccessible fraction (IN). Similar results were observed in previous works in olive oil [14]. On the other hand, the presence of caffeic acid [16] in the non-bioaccessible fraction (OUT) explains, in part, its high ACE inhibitory activity (r^2 = 0.73).

Nevertheless, not only phenolic compounds inhibit α-glucosidase and ACE activity, mannitol [9,10] and glucose [8] from LOPP could also have a substantial role as reported in previous works. A high correlation between α-glucosidase inhibitory activity and mannitol or glucose was attained ($r^2 \geq 0.86$). But, regarding ACE inhibitory activity, a lower correlation with mannitol and glucose was observed ($r^2 \geq 0.58$). Therefore, other compounds besides polyphenols and sugars could be present in LOPP and influence ACE inhibitory activity. Among ACE inhibitor compounds not evaluated in the present study were triterpenoids and small peptides with attested activity in olive oil [14].

Minerals have been reported to possess a significant α-glucosidase inhibitory activity [11]. In LOPP, the α-glucosidase inhibitory activity was strongly correlated with minerals content ($r^2 \geq 0.70$). The considerable loss of polyphenols, but also minerals, explained the loss of 50% of the initial α-glucosidase inhibitory activity until small intestine.

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In the future, the addition of ascorbic acid to LOPP as a protective agent of polyphenols [87] and enhancer of minerals absorption [88] throughout gastrointestinal tract might be explored to improve, not only LOPP antioxidant activity, but also, its α-glucosidase and ACE inhibitory activity. Other solutions as the conjugation with other phenolic extracts [26] or the development of micro and nano delivery systems [89] could be considered in the future for the improvement of the bioaccessibility of LOPP bioactive compounds.

4. Conclusion

The bioaccessibility/stability of all liquid-enriched olive pomace powder (LOPP) bioactive compounds (soluble sugars, organic acids, minerals and polyphenolic compounds) and not only polyphenols have been analysed throughout the different phases of gastrointestinal digestion. This study demonstrates that the gastrointestinal tract has a substantial effect in all LOPP bioactive compounds and that all these changes affected LOPP potential health benefits. Polyphenols and minerals were the most affected LOPP compounds by the gastrointestinal tract. Its low stability was reflected in the significantly lower values of LOPP antioxidant activity throughout the gastrointestinal tract. Even though, notable bioaccessibility indexes (> 50 %) were obtained in potassium and hydroxytyrosol derivatives, which could exert its potential cardiovascular health benefits. Furthermore, substantial antihypertensive activity and α-glucosidase inhibitory activity were maintained (50% of the initial inhibitory activities).

Additionally, a significant amount of soluble sugars and formic acid, and a considerable content of polyphenols were retained in the non-bioaccessible fraction (OUT), which could be interesting to study in the future to understand its possible benefits in the gut health.

In the future, new strategies to improve the bioaccessibility of LOPP bioactive compounds and to assess the bioaccessibility and potential health benefits from LOPP may be studied. *In vitro* experiments represent a consistent approach to evaluate the health effect of new functional ingredients, but future studies in cell lines and *in vivo* studies are needed before drawing final conclusions. Besides that, more research into *in vitro–in vivo* correlations are required to achieve more realistic *in vitro* models and therefore to screen the bioaccessibility and digestibility of foods.

Finally, the valorisation of biocompounds in olive pomace bioresidue to increase the sustainability and circular bioeconomy is a key option for the olive oil industry, taking into account the high nutritional value identified in this study.

Abbreviations

OP - olive pomace; **LOPP -** liquid-enriched olive pomace powder; **DW –** dry weight; **DF-** dietary fibre; **ADF -** antioxidant dietary fibre; **TPC -** Total phenolic compounds; **FPC -** free phenolics compounds;

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BPC - bound phenolics compounds; **DF** – dietary fibre; **TDF -** total dietary fibre; **IDF-** insoluble dietary fibre; **SDF -** soluble dietary fibre; **TE -** Trolox-equivalents; **UA -** uronic acids.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgements

Tânia I. B. Ribeiro thanks the Fundação para a Ciência e Tecnologia (FCT), Portugal and *Association BLC3 – Technology and Innovation Campus,* Centre Bio R&D Unit for the PhD Grant SFRH/BDE/108271/2015. This work was supported by the project "Biological tools for adding and defending value in key agro-food chains (bio – n2 – value)", nº NORTE-01-0145-FEDER-000030, funded by Fundo Europeu de Desenvolvimento Regional (FEDER), under Programa Operacional Regional do Norte - Norte2020. We would also like to thank the scientific collaboration of the project UID/Multi/50016/2019.

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826 **Table 1. Recovery index (RI %) and Bioaccessibility index (%) of bioactive compounds simulated gastrointestinal**
827 **digestion (SGD) from LOPP.**

	Recovery Index (%)					Bioaccessibility Index (%)
	Oral	Gastric	Intestinal	IN (Serum available)	OUT (Colon available)	
			Sugars & Organic acids			
Glucose	51.28 ± 0.88 ^{b}	41.92 ± 0.60 ^{c}	41.37 ± 0.80^{c}	15.19 ± 0.53 ^{d}	61.52 ± 7.56 ^{a}	19.95 ± 1.66 ^{g}
Fructose	60.54 ± 1.53 ^{b}	47.04 ± 3.65 ^{b}	47.43 ± 2.81 ^{b}	16.58 ± 1.63 ^{c}	89.32 ± 11.42 ^{a}	15.92 ± 3.93 ^{g}
Mannitol	59.07 ± 1.39 ^{b}	43.21 ± 1.41 ^{c}	46.40 ± 0.99 ^{c}	17.49 ± 0.55 ^{d}	71.43 ± 8.72 ^{a}	19.86 ± 2.37 ^{g}
Formic acid	59.69 ± 2.66 ^{a}	35.84 ± 1.31 ^{b}	38.74 ± 6.57 ^{b}	13.51 ± 1.37 ^{c}	43.11 ± 2.32 ^{b}	23.88 ± 2.51 ^{fg}
			Minerals			
Phosphorus	19.43 ± 2.53 ^{a}	17.24 ± 0.61 ^{ab}	16.15 ± 0.51 ^{ab}	5.23 ± 0.05 ^{c}	11.79 ± 1.68 ^{b}	36.22 ± 3.25 ^{e}
Magnesium	18.94 ± 1.13 ^{a}	18.39 ± 0.57 ^{a}	15.59 ± 0.81 ^{a}	5.10 ± 0.16 ^{c}	11.90 ± 1.19 ^{b}	35.36 ± 2.40 ^{ef}
Calcium	13.29 ± 1.00 ^{a}	12.89 ± 0.87 ^{a}	13.00 ± 1.52 ^{a}	nd	8.83 ± 1.88 ^{a}	nq
Potassium	19.57 ± 1.04 ^{a}	19.59 ± 0.88 ^{a}	16.95 ± 1.52 ^{a}	12.71 ± 0.19 ^{b}	12.00 ± 1.24 ^{b}	57.42 ± 3.18 ^{d}
			Phenolic compounds			
TPC	6.03 ± 1.14 ^{b}	13.35 ± 2.76 ^{a}	3.92 ± 0.44 ^{bc}	5.63 ± 0.76 ^{bc}	2.77 ± 0.67 ^{c}	65.52 ± 6.82 ^{cd}
Hydroxytyrosol glucoside	8.46 ± 5.61 ^{b}	5.57 ± 3.05 ^{c}	6.07 ± 2.65 ^{bc}	11.61 ± 0.17 ^{a}	3.76 ± 2.53 ^{c}	75.47 ± 2.84 ^{ab}
Hydroxytyrosol	7.52 ± 1.48 ^{b}	10.27 ± 2.21 ^{a}	0.73 ± 0.12 ^{c}	1.47 ± 0.11 ^{c}	0.32 ± 0.05 ^{c}	82.10 ± 2.59 ^{a}
Tyrosol glucoside	14.65 ± 3.21 ^{b}	12.37 ± 2.76 ^{b}	5.45 ± 0.89 ^{c}	35.87 ± 4.26 ^{a}	13.88 ± 1.16 ^{b}	71.93 ± 3.58 ^{bc}
Tyrosol	17.62 ± 4.72 ^{b}	14.32 ± 3.63 ^{b}	5.42 ± 0.90 ^{c}	48.00 ± 1.60 ^{a}	13.78 ± 2.02 ^{b}	77.79 ± 2.03 ^{ab}
Caffeic acid	5.32 ± 0.72 ^{b}	13.52 ± 2.11 ^{a}	0.84 ± 0.22 ^{c}	nd	0.89 ± 0.11 ^{c}	0.00 ± 0.00 ^{h}
Verbascoside	17.14 ± 2.75 ^{a}	12.82 ± 2.36 ^{a}	nd	nd	nd	nq
p-coumaric acid	24.21 ± 4.48 ^{b}	34.62 ± 7.44 ^{a}	9.03 ± 1.69 ^{cd}	4.84 ± 1.08 ^{d}	15.40 ± 0.79 ^{bc}	24.51 ± 3.81 ^{fg}
Caffeoyl-6'-secologanoside	13.76 ± 2.89 ^{b}	22.22 ± 2.82 ^{a}	2.83 ± 0.07 ^{cd}	nd	4.45 ± 0.69 ^{c}	nq

828 *nd – not detected; nq – not quantified. Results are the means of three determinations ± standard deviation. Values with different*
829 *letters in the same line to RI (%) and the same column to BI (%) are significantly different, as determined by one-way ANOVA*
830 *test (*$p < 0.05$*), respectively.*

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Table 2. Retention time, maximum wavelength absorbance and MSn fragmentations profiles of phenolic compounds identified in LOPP obtained before (initial) and after each step of in vitro gastrointestinal digestion (oral, gastric, intestinal, after dialysis IN and OUT) and the bound phenolics of dietary fibre achieved by AOAC and after the tract.

Compound	Molecular formula	RT (min)	λ_{max}	m/z calcd	m/z exptl	Err [mDa]	mSigma	Major fragments ESI negative MS/MS ions	*In vitro* gastrointestinal digestion step
Quinic acid [*2]	$C_7H_{12}O_6$	1.6	269	191.0561	191.0565	-0.4	12.2	191.0563; 127.0398; 93.0398; 87.0089; 85.097	Initial, Oral, Gastric, Intestinal, IN, OUT
Protocathecuic acid[*3]	$C_7H_6O_4$	6.8	203	153.0197	153.0193	-0.4	7	153.0197; 109.0298	SDF Tract
Hydroxytyrosol glucoside[*2]	$C_{14}H_{20}O_8$	7.1	279	315.1088	315.1085	-0.2	4	315.1092; 153.0556; 123.0451	Initial, Oral, Gastric, Intestinal, IN, OUT, SDF AOAC
Hydrated product of the dialdehydic form of decarboxymethyl-elenolic acid[*2]	$C_9H_{14}O_5$	7.3	197/281	201.077	201.0768	-0.2	4	201.0405; 153.0553; 123.0449; 95.0502	Initial, Oral, Intestinal, IN, OUT, SDF AOAC
Hydroxytyrosol [*1]	$C_8H_{10}O_3$	7.4	280	153.0556	153.0557	0.1	5.7	153.0452; 123.0457	Initial, Oral, Intestinal, IN, OUT, SDF AOAC, SDF Tract
Hydroxytyrosol glucoside isomer[*2]	$C_{14}H_{20}O_8$	7.5	279	315.1087	315.1085	-0.2	7.3	315.1089; 151.0398; 123.0449	Gastric
Loganin[*2]	$C_{17}H_{26}O_{10}$	7.6	281	389.1455	389.1453	-0.2	8.9	151.0763; 113.0244; 101.0244	Initial, Oral
Oleoside derivative isomer[*2]	$C_{17}H_{28}O_{11}$	7.7	228	407.1557	407.1559	-0.5	11.7	151.0760; 119.0346	Initial, Oral, Gastric, Intestinal, IN, OUT, SDF AOAC
Decarboxylated form of hydroxy-elenolic acid[*2]	$C_{10}H_{14}O_5$	7.9	197/280	213.0765	213.0768	0.1	5.6	213.0920; 137.0601; 121.0665; 111.0085	Initial, Oral, Gastric, Intestinal, OUT
Tyrosol glucoside[*3]	$C_{14}H_{20}O_7$	8.1	227/276	299.1139	299.1136	-0.3	3.2	119.0505; 137.0244; 135.0299	Initial, Oral, Gastric, Intestinal, IN, OUT, SDF AOAC, SDF Tract
4-Hydroxybenzoic acid[*4]	$C_7H_6O_3$	8.2	204	137.0241	137.0244	0.4	4	137.0241; 138.0280	SDF Tract
Oleoside[*2]	$C_{16}H_{22}O_{11}$	8.4	277	389.1093	389.1089	-0.4	4.3	389.1088; 183.0664; 165.0557	Initial, Oral, Gastric, Intestinal, IN, OUT, SDF AOAC, SDF Tract
p-Coumaroyl-D-glucose[*3]	$C_{15}H_{18}O_8$	8.7	195/280	325.0925	325.0929	0.4	0.9	326.0976; 163.0402; 119.0501	Initial, Oral, Gastric, Intestinal, IN, OUT
Caffeic acid [*1]	$C_9H_8O_4$	9.3	323	179.0350	179.0350	-0.1	7.9	179.0350; 135.0448	Initial, Oral, Gastric, Intestinal, OUT, SDF Tract
10-Hydroxyloganin [*2]	$C_{17}H_{26}O_{11}$	9.5	197 /278	405.1402	405.1406	-0.4	5.7	405.1402; 165.0557; 139.0035; 123.045	Initial, Oral, Gastric
Tyrosol [*1]	$C_8H_{10}O_2$	9.8	277	137.0608	137.0608	0.5	4.5	111.0084; 95.0510	Initial, Oral, Gastric, Intestinal, IN, OUT, SDF AOAC
p - Coumaric acid [*1]	$C_9H_8O_3$	10.6	309	163.0403	163.0401	-0.2	10.5	163.0397; 119.0499	Initial, Oral, Gastric, Intestinal, IN, OUT,SDF Tract
Verbascoside [*3]	$C_{29}H_{36}O_{15}$	10.7	245/330	623.1988	623.1981	-0.7	2.4	623.1983; 161.0244; 461.1665	Initial, Oral, Gastric
Caffeoyl-6-secologanoside[*3]	$C_{25}H_{28}O_{14}$	10.8	221/326	551.1407	551.1406	0	18.1	551.1416; 161.0245; 507.1504	Initial, Oral, Gastric, Intestinal, OUT
Hydroxylated form of elenolic acid[*2]	$C_{11}H_{14}O_7$	10.9	280/320	257.0666	257.0667	0.1	2.7	257.1271; 181.0505; 137.0603; 109.0655; 95.0498	Initial, Oral, Gastric, Intestinal, IN, OUT, SDF AOAC; SDF Tract
Elenolic acid [*2]	$C_{11}H_{14}O_6$	12.5	197/288	241.0720	241.0718	-0.2	0.5	241.0737; 139.0035; 127.0398; 111.0086; 95.0551	Initial, Oral, Gastric, Intestinal, IN, OUT, SDF AOAC
Comsegoloside[*3]	$C_{25}H_{28}O_{13}$	12.6	223/311	535.1462	535.1457	-0.5	6.4	535.1465; 145.0296; 491.1558	Initial, Oral, Gastric, Intestinal, OUT
Luteolin [*1]	$C_{15}H_{10}O_6$	15.9	268/349	285.0406	285.0405	-0.1	0.8	285.0414; 151.0037	Initial, Oral, Gastric, Intestinal, OUT, SDF AOAC, SDF Tract
Quercetin [*1]	$C_{15}H_{10}O_7$	15.0		301.0357	301.0354	-0.3	3.4	301.0359; 151.0035; 178.9988	Initial
Apigenin [*1]	$C_{15}H_{10}O_5$	18.0	268/338	269.0460	269.0455	-0.5	4.8	269.0461; 151.0035	Initial

[*1] *Hydroxytyrosol, tyrosol, oleuropein, rutin, luteolin-7-O-glucoside, luteolin, quercetin, apigenin, vanillin, caffeic acid, p-coumaric acid and ferulic acid were identified by comparison with the standards. The other compounds were tentatively identified by LC-ESI-UHR-QqTOF-MS based on accurate mass, isotope rate calculations designated mSigma and literature.* [*2]Lozano-Sánchez *et al*., (2013), [*3]Jerman Klen & Mozetič Vodopivec(2012), [*4][90].

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Table 3. Dietary composition (g/100 g DW) and profile (mg/g fibre DW) of LOPP by using modified AOAC dietary fibre analysis method and simulated gastrointestinal system.

	SDF g/100 g DW	**Monosaccharide composition** (mg/g fibre DW)				***UA*[*2]**	***BPC*[*3]**
		Glucose	*Galactose*	*Arabinose*	*NS*		
AOAC method [*1]	9.20 ± 1.18	25.04 ± 1.77	36.00 ± 6.00	28.75 ± 3.97	89.79	16.47 ± 2.46	14.56 ± 1.74
SGD	4.01 ± 0.53	37.73 ± *3.44*	22.59 ± 1.65	12.20 ± 0.79	72.52	12.93 ± 1.49	8.38± 0.79

NS – Neutral sugars; UA – Uronic acids; RP – Resistant protein; KL – Klason lignin; BPC: Bound phenolic compounds; TDF – Total dietary fibre; IDF – insoluble dietary fibre; SDF – Soluble dietary fibre. [*1] *– Data from previous paper Tânia Ribeiro et al. (2019);* [*2] *– mg* GUAE / g fibre dry weight; [*3] - mg GAE/ g fibre DW. *Results are the means of three determinations ± standard deviation. Different letters in the same column are significantly different, as determined by ANOVA (*$p < 0.05$*).*

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Figure 1. Graphic representation of the static in vitro gastrointestinal digestion procedure carried out with LOPP.

Figure 2. The concentration of LOPP soluble sugars/organic acids (A) and minerals (B) (g/100 g DW) obtained after each phase (oral, gastric, intestinal, after dialysis IN and OUT) of simulated gastrointestinal digestion (SGD). *Results are the means of three determinations ± standard deviation. Values with different letters are significantly different, as determined by one-way ANOVA test (*$p < 0.05$*).*

Note: The initial amount before digestion (BC_{TF}) and the amounts detected in the digested sample for each digestion step (BC_{DF}) expressed in this figure were used to calculate the Recovery Index (RI %) for each sugar, organic acid and mineral enunciated in Table 1. On the other hand, to calculate the Bioaccessibility Index (BI %) of each sugar, organic acid and mineral, the BC_S amount detected in the digested sample after the duodenal dialysis step (IN) and BC_{DFE} content which is the sum of the amounts after the duodenal step (IN + OUT) detected in this figure were used.

Figure 3. Stability and bioaccessibility of phenolic compounds throughout *in vitro* gastrointestinal digestion. (A)Total phenolic compounds (mg GAE/g DW) and (B) (C) (D) the concentration of the main individual phenolic compounds (mg/100 g DW) obtained after each step of *simulated* gastrointestinal digestion (oral, gastric, intestinal, after dialysis IN and OUT) of LOPP. *Results are the means of three determinations ± standard deviation. Values with different letters above are significantly different, as determined by one-way ANOVA test (*$p < 0.05$*).*

Note: The initial amount before digestion (BC_{TF}) and the amounts detected in the digested sample for each digestion step (BC_{DF}) expressed in this figure were used to calculate the Recovery Index (RI %) of total polyphenol compounds and each polyphenol enunciated in Table 1. On the other hand, to calculate the Bioaccessibility Index (BI %) of total polyphenol compounds and each individual polyphenol compound, the BC_S amount detected in the digested sample after the duodenal dialysis step (IN) and BC_{DFE} content which is the sum of the amounts after the duodenal step (IN + OUT) detected in this figure were used.

Figure 4. The concentration of main polyphenolics (mg/100 g sample dry weight) present in fibre fraction of LOPP and respective antioxidant activity using ABTS, DPPH and ORAC methods of soluble fibre from by AOAC method and SGD system. *Results are the means of three determinations ± standard deviation. Different letters in the same column are significantly different, as determined by the t-Student test (*$p < 0.05$*).*

Figure 5. Effect of *in vitro* gastrointestinal digestion on LOPP bioactivities. (A) Antioxidant properties of LOPP measured by ABTS, DPPH and ORAC assays after each step of *in vitro* gastrointestinal digestion (oral, gastric, intestinal, after dialysis IN and OUT) of LOPP. (B) ACE and α - glucosidase inhibitory activity of LOPP before and after simulated gastrointestinal digestion (intestinal, after dialysis IN and OUT) of LOPP. *Results are the means of three determinations ± standard deviation. Values with different letters above are significantly different, as determined by one-way ANOVA test (*$p < 0.05$*).*

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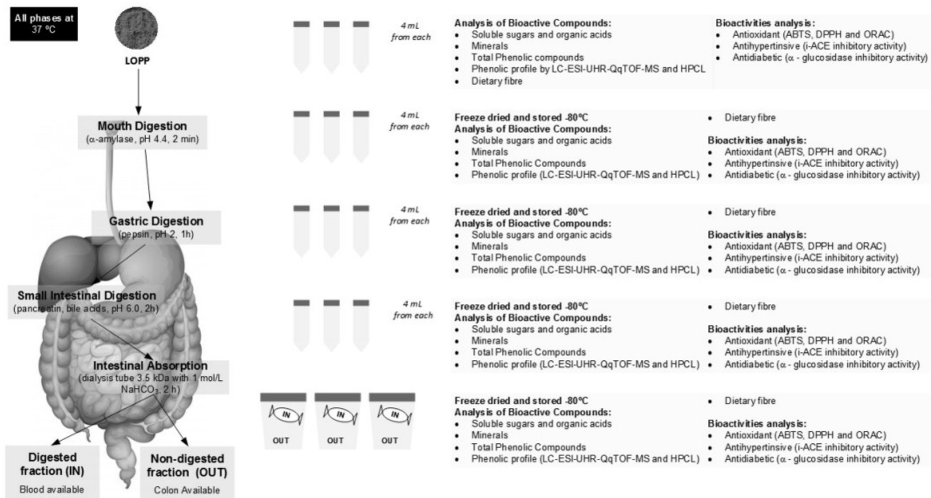

Graphic representation of the static in vitro gastrointestinal digestion procedure carried out with LOPP.

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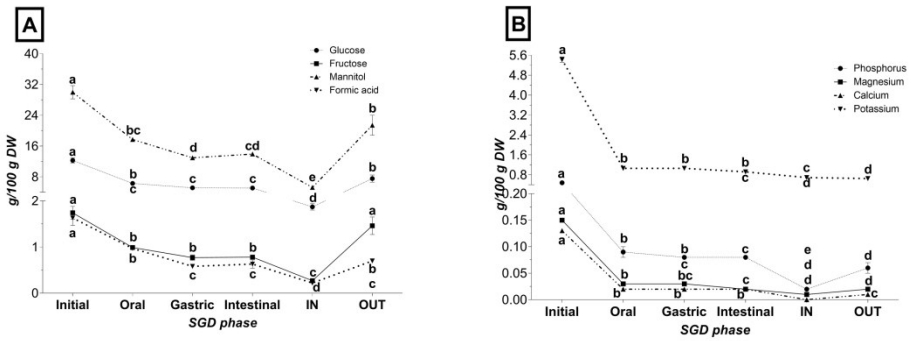

Figure 2. The concentration of LOPP soluble sugars/organic acids (A) and minerals (B) (g/100 g DW) obtained after each phase (oral, gastric, intestinal, after dialysis IN and OUT) of simulated gastrointestinal digestion (SGD).
Results are the means of three determinations ± standard deviation. Values with different letters are significantly different, as determined by one-way ANOVA test ($p < 0.05$).
Note: The initial amount before digestion (BCTF) and the amounts detected in the digested sample for each digestion step (BCDF) expressed in this figure were used to calculate the Recovery Index (RI %) for each sugar, organic acid and mineral enunciated in Table 1. On the other hand, to calculate the Bioaccessibility Index (BI %) of each sugar, organic acid and mineral, the BCS amount detected in the digested sample after the duodenal dialysis step (IN) and BCDFE content which is the sum of the amounts after the duodenal step (IN + OUT) detected in this figure were used.

265x96mm (300 x 300 DPI)

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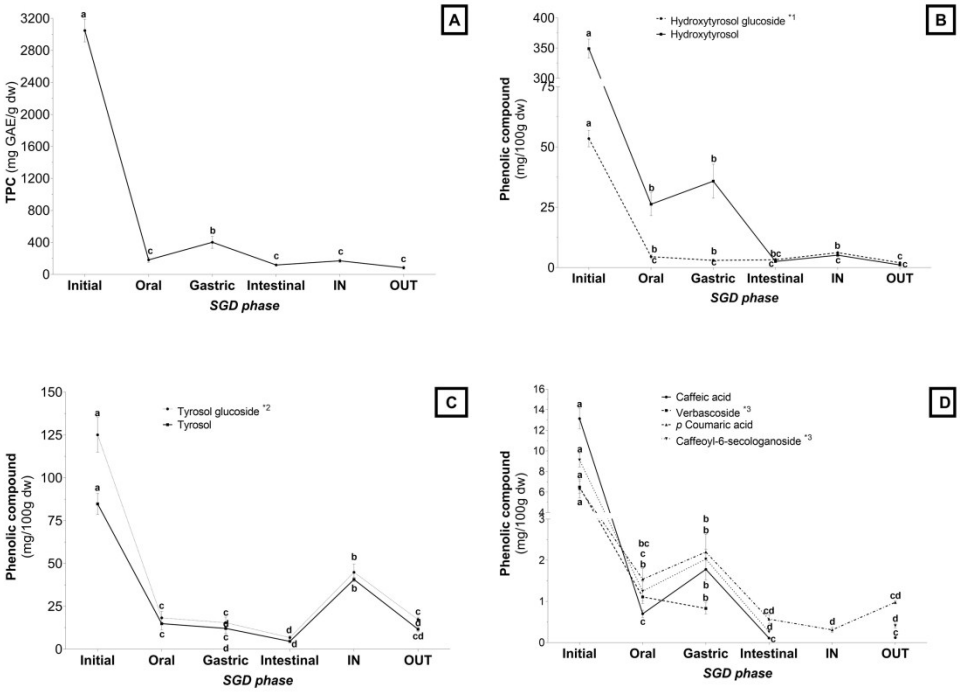

Figure 3. Stability and bioaccessibility of phenolic compounds throughout in vitro gastrointestinal digestion. (A)Total phenolic compounds (mg GAE/g DW) and (B) (C) (D) the concentration of the main individual phenolic compounds (mg/100 g DW) obtained after each step of simulated gastrointestinal digestion (oral, gastric, intestinal, after dialysis IN and OUT) of LOPP. Results are the means of three determinations ± standard deviation. Values with different letters above are significantly different, as determined by one-way ANOVA test ($p < 0.05$).

Note: The initial amount before digestion (BCTF) and the amounts detected in the digested sample for each digestion step (BCDF) expressed in this figure were used to calculate the Recovery Index (RI %) of total polyphenol compounds and each polyphenol enunciated in Table 1. On the other hand, to calculate the Bioaccessibility Index (BI %) of total polyphenol compounds and each individual polyphenol compound, the BCS amount detected in the digested sample after the duodenal dialysis step (IN) and BCDFE content which is the sum of the amounts after the duodenal step (IN + OUT) detected in this figure were used.

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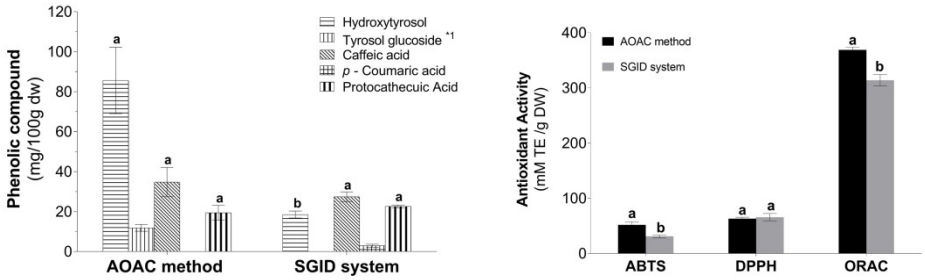

The concentration of main polyphenolics (mg/100 g sample dry weight) present in fibre fraction of LOPP and respective antioxidant activity using ABTS, DPPH and ORAC methods of soluble fibre from by AOAC method and SGD system. Results are the means of three determinations ± standard deviation. Different letters in the same column are significantly different, as determined by the t-Student test ($p < 0.05$).

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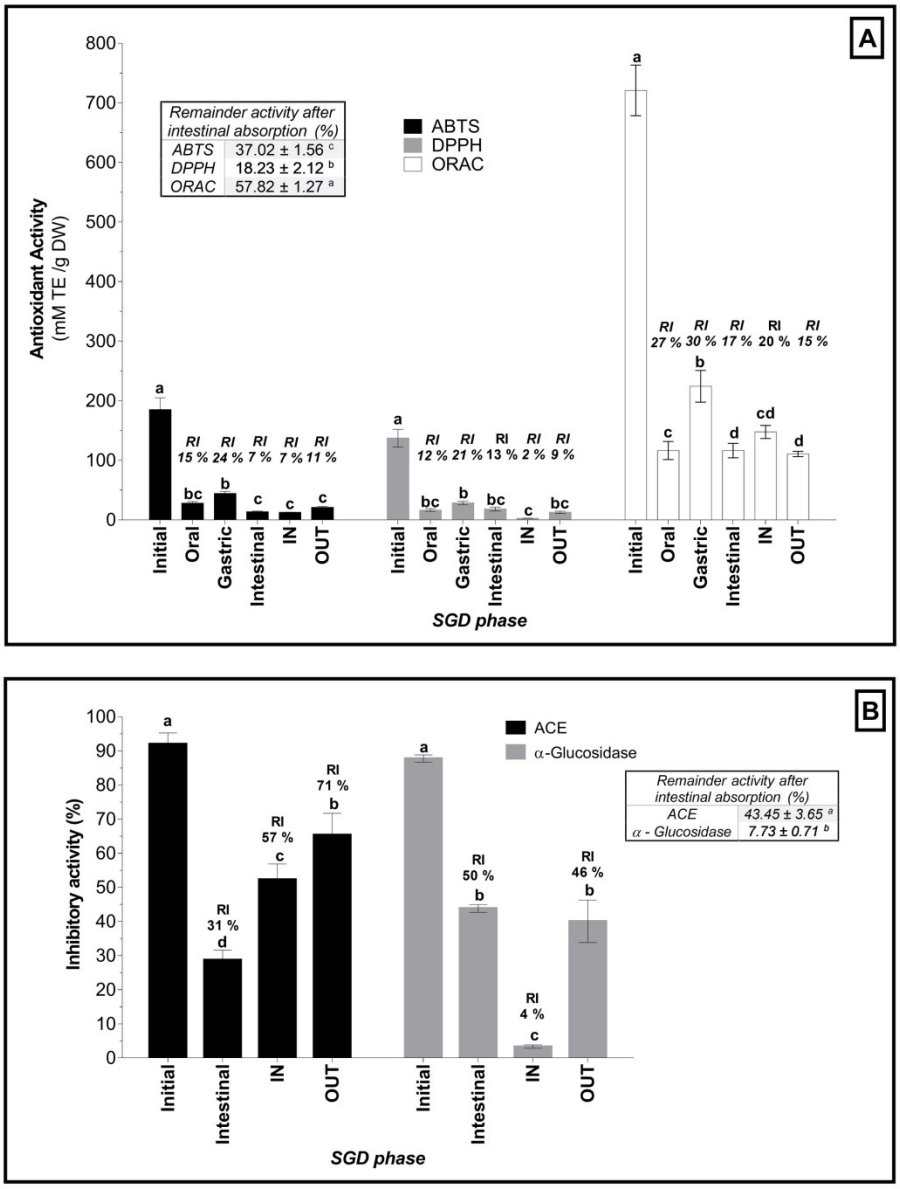

Figure 5. Effect of in vitro gastrointestinal digestion on LOPP bioactivities. (A) Antioxidant properties of LOPP measured by ABTS, DPPH and ORAC assays after each step of in vitro gastrointestinal digestion (oral, gastric, intestinal, after dialysis IN and OUT) of LOPP. (B) ACE and ☐ - glucosidase inhibitory activity of LOPP before and after simulated gastrointestinal digestion (intestinal, after dialysis IN and OUT) of LOPP. Results are the means of three determinations ± standard deviation. Values with different letters above are significantly different, as determined by one-way ANOVA test ($p < 0.05$).

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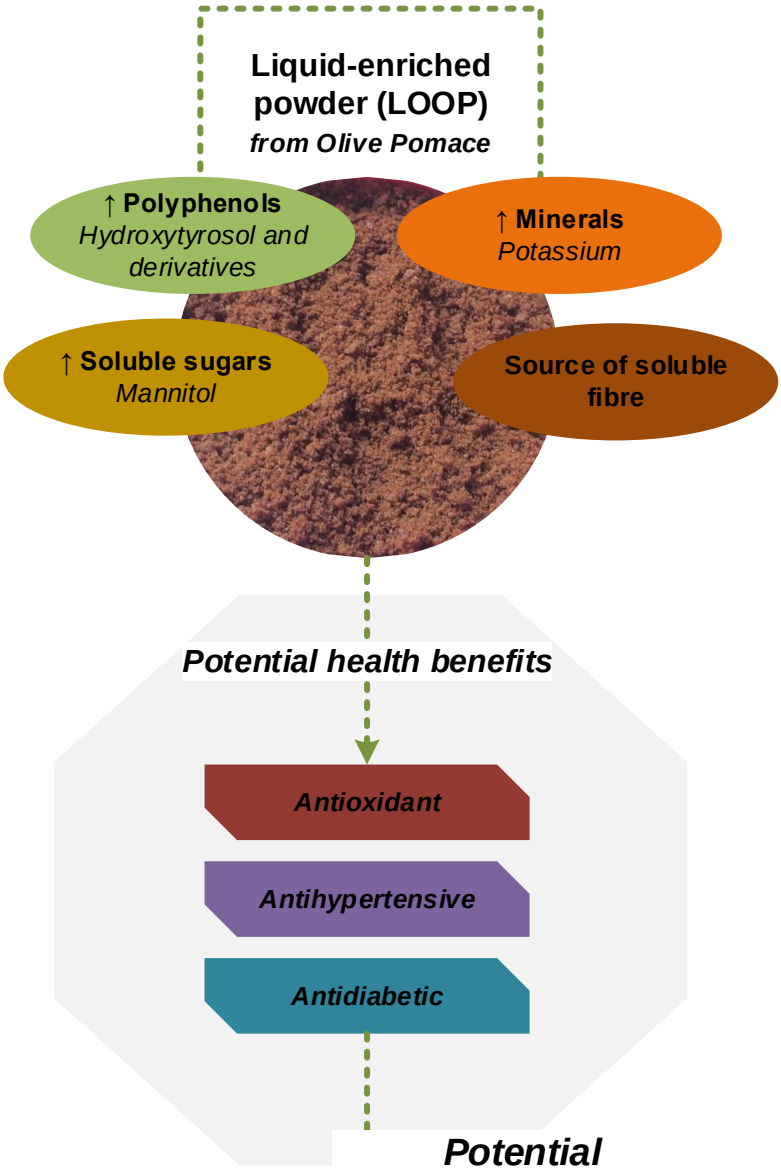

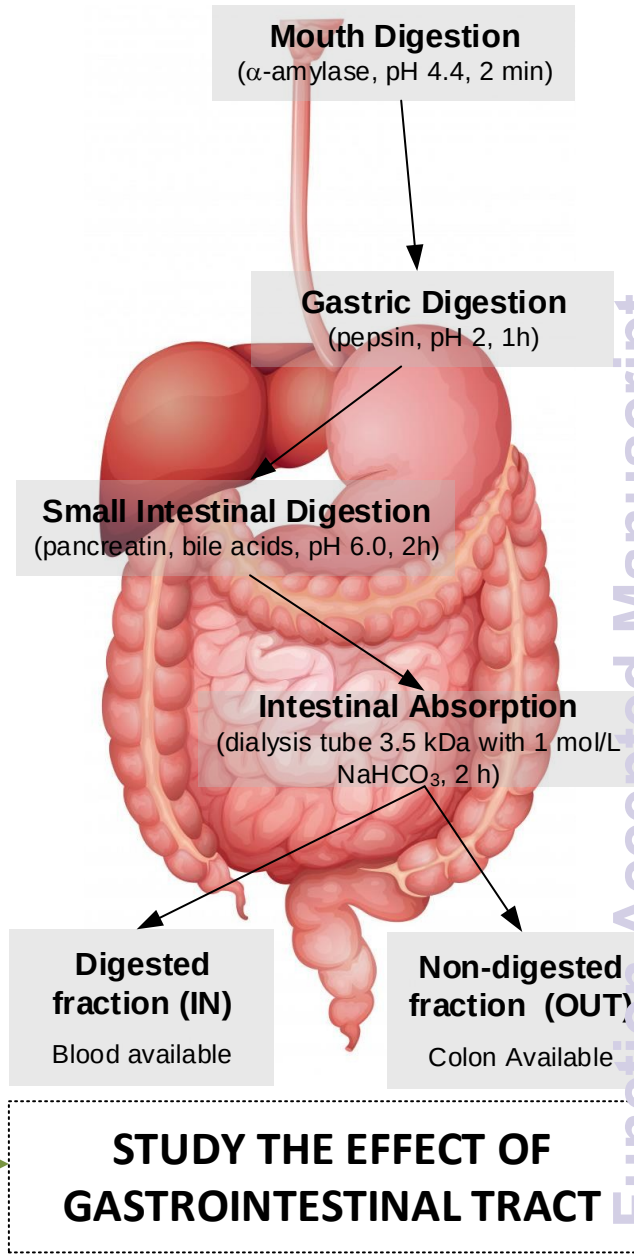

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