Effect of *in vitro* gastrointestinal digestion on the total phenolic contents and antioxidant activity of wild Mediterranean edible plants extracts

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Abstract

The recent interest in wild edible plants is associated to their health benefits, which are mainly due to their richness in antioxidant compounds, particularly phenolics. Nevertheless, some of these compounds are metabolized after ingestion, being transformed into metabolites frequently with lower antioxidant activity. The aim of the present study was to evaluate the influence of the digestive process on the total phenolic contents and antioxidant activity of extracts from four wild edible plants used in the Mediterranean diet (*Beta maritima* L., *Plantago major* L., *Oxalis pes-caprae* L. and *Scolymus hispanicus* L.). HPLC-DAD analysis revealed that *S. hispanicus* is characterized by the presence of caffeoylquinic acids, dicaffeoylquinic acids and flavonol derivatives, *P. major* by high amounts of verbascoside, *B. maritima* possesses 2,4-dihydroxybenzoic acid, 5-*O*-caffeoylquinic acid, quercetin derivatives and kaempferol-3-*O*-rutinoside and *O. pes-caprae* extract contains hydroxycinnamic acids and flavone derivatives. Total phenolic contents were determined by Folin-Ciocalteu assay, and antioxidant activity by the ABTS, DPPH, ORAC and FRAP assays. Phenolic contents of *P. major* and *S. hispanicus* extracts were not affected by digestion, but they significantly decreased in *B. maritima* after both phases of digestion process and in *O. pes-caprae* after the gastric phase. The antioxidant activity results varied with the extract and the method used to evaluate the activity. Results showed that *P. major* extract has the highest total phenolic contents and antioxidant activity, with considerable values even after digestion, reinforcing the health benefits of this species.

Keywords: *Beta maritima* L.; gastric digestion; intestinal digestion; *Plantago major* L., *Oxalis pes-caprae* L.; *Scolymus hispanicus* L.

Introduction

Wild edible plants have represented an important food source for the communities of the Mediterranean basin, providing a relevant role in Mediterranean diet [1-3]. The habit of eating spontaneous plants is increasing nowadays because they are considered a healthy way of diversifying and enriching the modern diet with distinct colours and flavours [4-7]. Indeed, it is well recognized that the diversification of food habits with wild resources contributes to improve nutrition, health, livelihoods and also ecological sustainability [8]. Wild vegetables have been highly appreciated raw in salads or cooked in traditional recipes and the basis of human diets for centuries [5]. The knowledge about the bioactive properties of underutilized plants could provide feedback about their value and agro-industrial potential and could also be used by gastronomic companies interested in the exploitation of these plants as additives or natural ingredients [9, 10].

Some wild edible plants have been recently considered as interesting functional foods since they provide health benefits [11]. These plants are recognized as valuable sources of bioactive compounds like antioxidants [3, 7, 11]. The intake of food rich in antioxidants is correlated with the reduction of some chronic diseases in which oxidative stress may play a role, namely diabetes, cancer, cardiovascular diseases, etc. [12]. Antioxidants scavenge reactive oxygen species (ROS) and other reactive species involved in the progression of such diseases and, therefore, there is particular interest in the potential health benefits of plants with the greatest ROS scavenging activity [13].

Among plant bioactive compounds, phenolics are probably the most important candidates contributing to the claimed antioxidant properties of plants. Phenolics have strong antioxidant activity associated with their ability to scavenge free radicals, break

radical chain reactions, and chelate metals [14, 15]. However, phenolics, particularly flavonoids and phenolic acids, are metabolized after ingestion and gastrointestinal absorption, usually being transformed into plasma metabolites with lower antioxidant activity than the precursor molecules [16]. In this sense, the comparison of antioxidant activity of food products before and after *in vitro* digestion is important to evaluate their real therapeutic capabilities [17]. Although there has been extensive investigation on the evaluation of antioxidant activity of plant extracts and foods, research studying the effect of digestion on the activity is scarce. *In vitro* methods of simulated gastrointestinal digestion have proven to be useful in determining the stability of bioactive compounds under gastrointestinal conditions and the results can be well correlated with those from human studies and animal models [18, 19]. The present study focused on four wild edible plants (*Beta maritima* L., *Plantago major* L., *Oxalis pes-caprae* L. and *Scolymus hispanicus* L.) used in Mediterranean diet (Figure 1 and Table 1) with nutritional and health benefits [8, 20-27]. The aim of the present study was to assess, for the first time, if the total phenolic contents and antioxidant activity of extracts from these species are affected by simulated gastrointestinal digestion.

Materials and methods

Chemicals and reagents

2,2'-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) tablets, ethanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol, pepsin from porcine gastric mucosa (CAS: 9001-75-6), pancreatin from porcine pancreas (CAS: 8049-47-6), bovine bile extract, porcine bile extract, trichloroacetic acid (TCA) and $K_2S_2O_8$, were purchased from Sigma–Aldrich (Steinheim, Germany). Folin-Ciocalteu reagent (F-C reagent),

gallic acid, Na_2CO_3, CH_3COONa and $FeCl_3$ were acquired from VWR (Leuven, Belgium). $K_3[Fe(CN)_6]$, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Acros Organics (Geel, Germany). Fluorescein was acquired from Panreac (Barcelona, Spain). Formic acid was obtained from Merck (Darmstadt, Germany). 3-*O*-Caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid were from Chengdu Biopurity Phytochemicals Ltd. (Schuan, China), 2,4-dihydroxybenzoic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA) and 5-*O*-caffeoylquinic acid, verbascoside, luteolin-8-*C*-glucoside, luteolin-6-*C*-glucoside, apigenin-7-O-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside and isorhamnetin-3-*O*-rutinoside were purchased from Extrasynthèse (Genay, France).

Plant material and extraction procedure

Leaves of *B. maritima*, *O. pes-caprae*, *P. major* and *S. hispanicus* were collected from plants growing wild in the Algarve region (South Portugal). A representative sample of each plant was authenticated by JM Rosa Pinto from the herbarium of the University of Algarve (Faro, Portugal). The plant material was dried at 40ºC until constant weight and powdered in a blender (< 2 mm particle size). Dried plant material was extracted twice by maceration with 80% methanol (1:20, w/v) during 24 h at room temperature. After filtration, the extracts were concentrated to dryness in a rotary evaporator at 40ºC and under reduced pressure, and stored at -20ºC.

High-performance liquid chromatography–diode array detection (HPLC–DAD) analysis

The extracts were analyzed on an analytical HPLC unit (Gilson), using a Spherisorb ODS2 column (4.6 × 250 mm, 5 μm, particle size). The solvent system used was a gradient of water-formic acid (19:1) (A) and methanol (B), starting with 5% methanol and installing a gradient to obtain 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 45% B at 39 min, 45% B at 42 min, 55% B at 47 min, 75% B at 56 min, 100% B at 60 min, at a solvent flow rate of 0.9 ml/min. Detection was achieved with a Gilson Diode Array Detector (DAD). Spectral data from all peaks were accumulated in the range 200–400 nm. Chromatograms were recorded at 280 nm for hydroxybenzoic acids, at 320 nm for hydroxycinnamic acids and at 350 nm for flavonoids. The data were processed on an Unipoint® System software (Gilson Medical Electronics, Villiers le Bel, France). The compounds in each extract were identified by comparing their retention times and UV–Vis spectra in the 200–400 nm range with authentic standards injected in the same conditions. Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards, by using the following equation:

$$C(c) = \frac{A\,(c)}{A\,(st)} \times C(st)$$

Where, C(c) and A(c) are the concentration and the area of the compound in the sample, respectively, and C(st) and A(st) are the concentration and the area of the standard, respectively.

In vitro digestion procedure

The *in vitro* digestion model was preformed as described by Ryan et al. [28] with some modifications. The extracts were mixed with saline solution in a final volume of 20 ml. The resulting solutions were acidified to pH 2.0 with 1 ml of porcine pepsin preparation (0.04 g/ml in 0.1 M HCl) and were incubated for 1 h at 37ºC in a shaking bath. After gastric digestion, the pH was increased to 5.3 with 0.9 M sodium bicarbonate solution, followed by the addition of 200 µL of bovine and porcine bile extract solution (0.1 g/ml in saline), and 100 µl of pancreatin solution (0.08 g/ml in saline). The pH was increased to 7.4 with 1 M NaOH and then the samples were incubated again at 37ºC for 2.5 h to complete the intestinal phase of the *in vitro* digestion process. Samples were stored at -20ºC and were analyzed within 2 weeks.

Determination of the total phenolic contents

The total phenolic contents of undigested extracts, and after gastric and pancreatic digestion were measured using a colorimetric method [29]. Briefly, 200 µl of 10% (v/v) F-C reagent was mixed with 100 µl of each extract in phosphate buffer (75 mM, pH 7.0) and 800 µl of 700 mM Na_2CO_3. After an incubation period of 2 h at room temperature, 200 µl of each reaction mixture were transferred to a clear 96-well microplate and the absorbance was measured at 765 nm. Instead of the plant extracts, gallic acid was used as a positive control and phosphate buffer as a negative control. A standard curve was calculated using several gallic acid dilutions and the results were expressed as gallic acid equivalents per gram of extract ($\mu mol_{GAE}/g_{extract}$).

Antioxidant activity

The ABTS, DPPH and peroxyl radicals scavenging capacity, and ferric reducing antioxidant power (FRAP) of the extracts from the four plant species was evaluated before, and after gastric and intestinal digestion.

$ABTS^{\bullet+}$ radical cation decoloration assay

The ABTS free radical-scavenging activity of each sample was determined as described by Re et al. [30]. A stock solution of 7 mM $ABTS^{\bullet+}$ prepared using potassium persulfate as the oxidizing agent was diluted to an absorbance of 0.700 at 734 nm to form the test reagent. Then, 190 µl of this reagent were mixed with 10 µl of each extract and the absorbance was determined at 734 nm. The extract dilution that achieved 20–80% inhibition of the blank was used for the calculations and the results were expressed as trolox equivalents per gram of extract ($\mu mol_{TE}/g_{extract}$).

DPPH free radical scavenging assay

The ability of the extracts to scavenge DPPH radicals was determined using the procedure described by Soler-Rivas et al. [31] with some modifications. One hundred microliters of 90 µM DPPH methanolic solution was added to 10 µl of extract at different concentrations, and the mixture was diluted with 190 µl of methanol in a clear 96-well microplate. Methanol was used as negative control and Trolox as positive control. After 30 min, the reduction of DPPH radicals was measured at 515 nm. The extract dilution that achieved 20–80% inhibition of the blank was used for the calculations and the results were expressed as trolox equivalents per gram of extract ($\mu mol_{TE}/g_{extract}$).

180

181 ***Oxygen radical absorbance capacity (ORAC) assay***

182 ORAC assay was performed as described by Gillespie et al. [32] using fluorescein as
183 the fluorescent probe and AAPH as peroxyl radical generator. A black microplate was
184 loaded with 150 µl of 0.08 µM fluorescein and 25 µl of plant extract. Trolox was used
185 as standard and phosphate buffer (75 mM, pH 7) as a negative control. The reaction was
186 initiated with the addition of 25 µl 150 mM AAPH to each well after incubating for 10
187 min at 37 ºC. The reduction in fluorescence was determined by reading fluorescein
188 excitation at 485 nm and emission at 530 nm every minute for 90 min. The ORAC
189 values were calculated using the area under the curve (AUC) and the regression
190 equation between the TE and the net AUC, and the results were expressed as TE per
191 gram of extract.

192

193 ***Ferric reducing antioxidant power (FRAP)***

194 The reducing properties of the extracts were determined as described by Pulido et al.
195 [33] with some modifications using $FeCl_3$. In brief, 100 µl of plant extract was mixed
196 with 250 µl sodium phosphate buffer (200 mM, pH 6.6) and 250 µl 1% $K_3[Fe(CN)_6]$.
197 The mixture was incubated at 50°C for 20 min followed by the addition of 250 µl 10%
198 TCA. After centrifugation at 650 rpm for 10 min, 100 µl of the supernatant were mixed
199 with 100 µl of water and 20 µl 0.1% $FeCl_3$ in a 96-well microplate. Instead of the plant
200 extracts, ascorbic acid was used as a positive control and phosphate buffer as a negative
201 control. Reducing activity was measured by determining the absorbance at 700 nm and
202 the results were expressed as ascorbic acid equivalents ($\mu mol_{AAE}/g_{extract}$).

Statistical analysis

The data were presented as the mean ± standard error of three replicates of each experiment. Data were analyzed by one-way analysis of variance (ANOVA) ($p < 0.05$). Statistical analysis was carried out using the SPSS statistical package for Windows (release 18.0; SPSS Inc., Chicago, IL, USA).

Results and discussion

Phenolic composition of the plant extracts

Phenolic compounds are considered the major contributors to the antioxidant capacity of many plants and an important part of human diet [34, 35]. Hence, the phenolic composition of the extracts studied in this work was analysed by HPLC–DAD (Table 2 and Figure 2). *S. hispanicus* extract contained the highest amount of the identified phenolic compounds, 75.33 mg/g of dry extract. Among the identified compounds, the most abundant in this extract were 5-*O*-caffeoylquinic acid (chlorogenic acid) (33.30 mg/g of dry extract) and kaempferol-3-*O*-glucoside (29.34 mg/g of dry extract). Another caffeoylquinic acid derivative (4-*O*-caffeoylquinic acid) and three dicaffeoylquinic acids (3,4-, 3,5- and 4,5-di-*O*-caffeoylquinic acids) were also identified (Table 2). Caffeoylquinic and dicaffeoylquinic acids are frequent in several Asteraceae species [36, 37]. In addition, *S. hispanicus* extract also contained four other flavonoids: quercetin 3-*O*-galactoside, quercetin 3-*O*-rutinoside, kaempferol-3-*O*-rutinoside and isorhamnetin-3-*O*-rutinoside. In a chemotaxonomic study with several Asteraceae

225 species, Sareedenchai and Zidorn [38] also identified several flavonoids in *S.*
226 *hispanicus*, namely quercetin, kaempferol and isorhamnetin derivatives.

227 Phenylethanoid glycosides are key metabolites in *Plantago* species [39], therefore it is
228 not surprising that *P. major* extract contains large amount of verbascoside (32.37 mg/g
229 of dry extract). This compound was previously identified in *P. major*, as well as in
230 several other *Plantago* species [24, 39, 40].

231 Five compounds were identified in *B. maritima* extract: 2,4-dihydroxybenzoic acid
232 (1.53 mg/g of dry extract), 5-*O*-caffeoylquinic acid (0.73 mg/g of dry extract), quercetin
233 3-*O*-galactoside (3.62 mg/g of dry extract), quercetin 3-*O*-rutinoside (5.47 mg/g of dry
234 extract) and kaempferol-3-*O*-rutinoside (3.85 mg/g of dry extract). *O. pes-caprae*
235 extract contained 3-*O*-caffeoylquinic acid (4.25 mg/g of dry extract), 4-*O*-
236 caffeoylquinic acid (0.75 mg/g of dry extract), luteolin-8-*C*-glucoside (0.74 mg/g of dry
237 extract), luteolin-6-*C*-glucoside (2.92 mg/g of dry extract) and apigenin 7-*O*-glucoside
238 (0.21 mg/g of dry extract). Recently, three luteolin derivatives and three apigenin
239 derivatives were identified in an extract from this species [20]. Additionally, Güçlütürk
240 et al. [21] reported the presence of chlorogenic acid in an *O. pes-caprae* methanol
241 extract. In our study we did not find chlorogenic acid (5-*O*-caffeoylquinic acid), but 3-
242 *O*-caffeoylquinic and 4-*O*-caffeoylquinic acids. This incoherency may be due to the
243 confusion in the literature about the nomenclature of caffeoylquinic acids. According to
244 IUPAC recommendations (IUPAC Commission on the Nomenclature of Organic
245 Chemistry and IUPAC-IUB Commission in Biochemical Nomenclature, 1976) [41] 3-
246 *O*-caffeoylquinic acid is now designated as 5-*O*-caffeoylquinic acid [42], however, there
247 are still papers where 3-*O*-caffeoylquinic acid isomer is called chlorogenic acid.

248

Total phenolic contents and antioxidant activity

The total phenolic contents of the extracts from the four species studied before and after *in vitro* digestion are shown in Fig. 2. Total phenolic contents of undigested extracts varied between 121.66 ± 2.71 $\mu mol_{GAE}/g_{extract}$ in *O. pes-caprae* and 431.89 ± 14.54 $\mu mol_{GAE}/g_{extract}$ in *P. major*. The total phenolic content of a methanol extract from *B. maritima* (61.91 $mg_{GAE}/g_{extract}$) was previously evaluated by Morales et al. [25] and the results were similar to the obtained in this study (53.70 $mg_{GAE}/g_{extract}$). The total phenolic contents in different *O. pes-caprae* extracts were also previously reported; however, since those results are expressed in a fresh weight basis it is difficult to compare them with the obtained in this work [8, 21]. Recently, Mazzutti et al. [24, 43] reported the total phenolic contents in *P. major* extracts obtained by different extraction techniques, solvents and extraction conditions. The values obtained ranged from 2.1 to 132.20 $mg_{GAE}/g_{extract}$ and the value found in this work was 68.81 $mg_{GAE}/g_{extract}$. The total phenolic content found in *S. hispanicus* (52.52 $mg_{GAE}/g_{extract}$) was higher than that found in an ethanol extract of the same species obtained by soxhlet extraction (18.24 $mg_{GAE}/g_{extract}$) [23].

The benefits of phenolic compounds for human health are incontestable, however most of these compounds are considered as xenobiotics by the human body and their bioavailability is relatively low in comparison to other nutrients [44]. Thus, studies reporting the effect of *in vitro* digestion on the bioactivity of phenolic extracts are important. In this study, the effect of *in vitro* digestion on total phenolic contents and antioxidant activity of extracts from the four species studied was evaluated using an *in vitro* digestion protocol to simulate digestion. The total phenolic content of *P. major* and *S. hispanicus* was not affected by *in vitro* digestion. On the other hand, the total phenolic content of *B. maritima* and *O. pes-caprae* extracts obtained after the two

phases of the process and after the gastric phase, respectively, significantly decreased ($p < 0.05$) (Figure 3). Significant decreases in the total phenolic content of extracts from various plants after gastrointestinal simulation were also reported by several authors [45-47]. In the literature different results can be found concerning the effect of gastrointestinal digestion on total phenolic contents. Jayawardena et al. [48] observed no decreases on total phenolic contents of extracts from 10 edible plants after digestion process. Chen et al. [49] studied the effect of digestion on total phenolic contents of extracts from 23 edible flowers and observed that the results varied considerably between species. Phenolics are sensitive to various factors, such as pH and enzymatic reactions, and different changes in total phenolic contents after digestion could be due to the stability of each type of phenolic compound present in the food matrix [18, 50]. Thus, the differences in the phenolic behaviour observed between extracts can be related to the different qualitative and quantitative phenolic profile of extracts from the different plants.

The antioxidant activity of the extracts studied was measured before and after *in vitro* simulated digestion by using DPPH, ABTS, ORAC and FRAP assays (Figure 4). All the extracts were capable of scavenging DPPH radicals with values ranging from 24.70 ± 0.44 to 404.47 ± 10.35 $\mu mol_{TE}/g_{extract}$ before digestion. Although *P. major* extract showed a significant decrease in the activity after both phases of digestion it showed the strongest scavenging capacity of DPPH radicals (404.47, 239.06 and 345.98 $\mu mol_{TE}/g_{extract}$, before digestion, after gastric phase and intestinal phase, respectively). The DPPH scavenging capacity also significantly decreased after both phases of digestion of *B. maritima* extract and after gastric digestion of *S. hispanicus* extract. On the other hand, the DPPH scavenging capacity of *O. pes-caprae* extract significantly increased after intestinal digestion. The ABTS scavenging capacities of undigested

299 extracts varied between 88.45 ± 33.36 $\mu mol_{TE}/g_{extract}$ for *O. pes-caprae* and 355.47 ±
300 29.19 $\mu mol_{TE}/g_{extract}$ for *S. hispanicus*. After digestion process, values significantly
301 decreased after the gastric phase for all the extracts and after intestinal phase only for *P.*
302 *major* extract. The capacities of the extracts to neutralize peroxyl radicals was evaluated
303 by the ORAC assay, an hydrogen atom transfer based method that uses a fluorescent
304 probe to compete with antioxidants for peroxyl radicals generated by the decomposition
305 of AAPH. The ORAC values of undigested extracts varied between 354.75 ± 4.85
306 $\mu mol_{TE}/g_{extract}$ for *O. pes-caprae* and 1344.87 ± 18.15 $\mu mol_{TE}/g_{extract}$ for *P. major*. The
307 digestion process did not significantly affect the ORAC values in *B. maritima* and *O.*
308 *pes-caprae* extracts ($p \geq 0.05$). Otherwise the ORAC values significantly decreased
309 after both phases of digestion process in *P. major* and *S. hispanicus* (Figure 4).
310 Similarly to the observed in the other assays, the initial FRAP values of the tested
311 extracts varied considerably among species, between 78.22 ± 1.91 for *O. pes-caprae*
312 and 497.67 ± 10.74 $\mu mol_{AAE}/g_{extract}$ for *P. major*. In this method, the values obtained
313 after both phases of the digestion process of all the extracts did not differ significantly
314 ($p \geq 0.05$) from the initial (Figure 4).

315 There are some contradictory data in literature about the effect of gastrointestinal
316 digestion on the antioxidant activity of plant matrices. Interactions with other
317 components of the extract and pH variations also cause changes in antioxidant activity
318 [51]. For example, pH affects the racemization of molecules, leading to two chiral
319 enantiomers with different bioavailability, and, as a result, different bioactivity [52].
320 Phenolic compounds can interact with other dietary components released during
321 digestion (e.g., minerals, proteins, dietary fibers, volatile compounds), which also play
322 an important role in bioactivity [18, 53, 54]. The assay employed could also affect the
323 assessment of antioxidant activity since pH modifications may alter the structure of

phenolic compounds and, consequently, the antioxidant activity [55]. For instance, assays carried out at pH 7, such as ABTS and ORAC, are proposed as more appropriate to evaluate the activity of intestinal digests [18, 56].

Despite some exceptions, in this study it was noticed a trend for the radical scavenging capacity to be more affected by the gastric conditions than by the intestinal ones (Figure 4). Results from Jayawardena and co-authors [48, 57] showed significant increases in the antioxidant activity, particularly when measured with ORAC and FRAP assays, of extracts from some edible plants and fruit juices after the intestinal phase. According to Bouayed et al. [18], the change from acidic to alkaline environment improves the antioxidant activity of phenolics by causing deprotonation of the hydroxyl moieties present on their aromatic rings. Furthermore, the results vary with the extract and the method used to analyze the antioxidant activity. The activity measured by DPPH assay increased after intestinal phase for *O. pes-caprae* extract, although total phenolic content decreased ($p < 0.05$). In other cases, no differences in the total phenolic contents were observed after *in vitro* digestion but the antioxidant activity decreased ($p < 0.05$). These results suggest that the studied extracts probably also contain non-phenolic substances, such as peptides, that could be involved in this activity [50].

Comparing the results of the different antioxidant assays, *P. major* extract appears to be the most potent among the studied extracts. The antioxidant activity of this extract was probably related with the high content in verbascoside (Table 2). This compound is a phenylethanoid glycoside present in several *Plantago* species, which possesses beneficial activities for human health, namely antioxidant, anti-inflammatory, antimicrobial, wound-healing and neuroprotective properties [58]. Some investigations suggest that the four hydroxyls at the *ortho* position in the two aromatic rings of verbascoside contribute to its remarkable antioxidant activity [59]. In addition to its

food uses (Table 1), *P. major* is certainly one of the most commonly used medicinal herb in the world [27]. The leaves are employed in many countries for the treatment of skin infections and other infectious diseases, digestive and respiratory disorders, to enhance the circulation and reproduction, for pain and fever relief, and to prevent cancer [27].

Overall, the results of the present study demonstrate the importance of evaluating the bioactivity of plant extracts after digestion. Moreover, this study highlights the importance of analyzing the antioxidant activity by different methods with distinct mechanisms. Although antioxidant activity is affected by digestion conditions, in some cases the results obtained indicate the wild plants studied as sources of natural antioxidants. In future studies it is important to study the phenolic profile of the extracts after gastrointestinal digestion, and to analyze the bioaccessibility, bioavailability and bioactivity of the extracts in other systems, to accurately assess the health promoting benefits of these species. Knowledge about the biological potential of these spontaneous plants make them especially attractive, given the increasing awareness of people to consume natural healthy products, as well as interest in rediscover local traditions and food habits.

Acknowledgements

This work received financial support from the European Union (FEDER funds through COMPETE). This work also received financial support from National Funds (FCT/MEC, Fundação para a Ciência e Tecnologia/Ministério da Educação e Ciência) through project UID/QUI/50006/2013, co-financed by European Union (FEDER under the Partnership Agreement PT2020), from Programa de Cooperación Interreg V-A España – Portugal (POCTEP) 2014-2020 (project 0377_IBERPHENOL_6_E) and from

374 the project INTERREG - MD.Net: When Brand Meets People. S. Gonçalves
375 acknowledges the financial support from FCT.

376

377 **Compliance with ethical standards**

378 **Conflict of interest** The authors declare no conflict of interest.

379 **Research involving human and/or animal participants** This article does not contain
380 any studies with human or animal subjects.

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382 **References**

383 1. Hadjichambis AC, Paraskeva-Hadjichambi D, Della A, Giusti ME, De Pasquale C,
384 Lenzarini C, Censorii E, González-Tejero MR, Sanchez-Rojas CP, Ramiro-Gutiérrez
385 JM, Skoula M, Johnson C, Sarpaki A, Hmamouchi M, Jorhi S, El-DemerdashM, El-
386 Zayat, M, Pieroni A (2008) Wild and semi-domesticated food plant consumption in
387 seven circum-Mediterranean areas. Int J Food Sci. Nutr 59: 383–414

388 2. Heinrich M, Leonti M, Nebel S, Peschel W (2005) Local food-nutraceuticals: an
389 example of a multidisciplinary research project on local knowledge. J Physiol
390 Pharmacol 56:5-22.

391 3. Poljuha P, Šola I, Bilić J, Dudaš S, Bilušić T, Markić J, Rusak G (2015) Phenolic
392 composition, antioxidant capacity, energy content and gastrointestinal stability of
393 Croatian wild edible plants. Eur Food Res Technol 241:573–585

394 4. Marengo A, Maxia A, Sanna C, Bertea CM, Bicchi C, Ballero M, Cagliero C,
395 Rubiolo P (2017) Characterization of four wild edible *Carduus* species from the

Mediterranean region via phytochemical and biomolecular analyses. Food Res Int 100:822–831

5. Pinela J, Carvalho AM, Ferreira ICFR (2017) Wild edible plants: Nutritional and toxicological characteristics, retrieval strategies and importance for today's society. Food Chem Toxicol 110:165–188

6. Renna M, Cocozza C, Gonnella M, Abdelrahman H, Santamaria P (2015) Elemental characterization of wild edible plants from countryside and urban areas. Food Chem 177:29–36.

7. Sánchez-Mata MC, Cabrera Loera RD, Morales P, Fernández-Ruiz V, Cámara M, Díez Marqués C, Pardo-de-Santayana M, Tardío J (2012) Wild vegetables of the Mediterranean area as valuable sources of bioactive compounds. Genet Resour Crop Evol 59:431–443

8. Romojaro A, Botella MA, Obón C, Pretel MT (2013) Nutritional and antioxidant properties of wild edible plants and their use as potential ingredients in the modern diet. Int J Food Sci Nutr 64:944–952

9. Leonti M (2012) The co-evolutionary perspective of the food-medicine continuum and wild gathered and cultivated vegetables. Genet Resour Crop Evol 59:1295–1302

10. Rivera D, Heinrich M, Obon C, Inocencio C, Nebel S, Verde A, Fajardo J (2006) Disseminating knowledge about ‘‘local foods plants’’ and ‘‘local plant foods’’. Forum Nutr 59:75–85

11. Zengin G, Mahomoodally MF, Aktumsek A, Ceylan R, Uysala S, Mocan A, Yilmaz MA, Picot-Allain CMN, Ćiriće A, Glamočlija J, Soković M (2018) Functional constituents of six wild edible *Silene* species: A focus on their phytochemical profiles and bioactive properties. Food Biosci 23:75–82

12. Pandey KB, Rizvi SI (2009) Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev 2:270–278

13. Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39, 44–84.

14. Niki E (2010) Assessment of antioxidant capacity *in vitro* and *in vivo*. Free Radic Biol Med 49:503–515

15. Prior RL, Cao GH (2000) Analysis of botanicals and dietary supplements for antioxidant capacity: a review. J AOAC Int 83:950–956

16. Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L (2004) Polyphenols: Food sources and bioavailability. Am J Clin Nutr 79:727–747

17. Manach C, Williamson G, Morand C, Scalbert A, Remesy C (2005) Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr 81:230S–242S

18. Bouayed J, Hoffman L, Bohn T (2011) Total phenolics, flavonoids, anthocyanins and antioxidant activity following simulated gastro-intestinal digestion and dialysis of apple varieties: Bioaccessibility and potential uptake. Food Chem 128:14–21

19. Toydemir G, Capanoglu E, Kamiloglu S, Boyacioglu D, de Vos RCH, Hall RD, Beekwilder J (2013) Changes in sour cherry (*Prunus cerasus* L.) antioxidants during nectar processing and *in vitro* gastrointestinal digestion. J Funct Foods 5:1402–1413

20. Gaspar MC, Fonseca DA, Antunes MJ, Frigerio C, Gomes NGM, Vieira M, Santos AE, Cruz MT, Cotrim MD, Campos MG (2018) Polyphenolic characterisation and bioactivity of an *Oxalis pes-caprae* L. leaf extract. Nat Prod Res 32:732–738

21. Güçlütürk I, Detsi A, Weiss EK, Ioannou E, Roussis V, Kefalas P (2012) Evaluation of anti-oxidant activity and identification of major polyphenolics of the invasive weed *Oxalis pes-caprae*. Phytochem Anal 23:642–646

22. Jarić S, Mačukanović-Jocić M, Djurdjević L, Mitrović M, Kostić O, Karadžić B, Pavlović P (2015) An ethnobotanical survey of traditionally used plants on Suva planina mountain (south-eastern Serbia). J Ethnopharmacol 175:93-108

23. Marmouzi I, El Karbane M, El Hamdani M, Kharbach M, Mrabti HN, Alami R, Dahraoui S, El Jemli M, Ouzzif Z, Cherrah Y, Derraji S, El Abbes Faouzi M (2017) Phytochemical and pharmacological variability in Golden Thistle functional parts: comparative study of roots, stems, leaves and flowers. Nat Prod Res 31:2669-2674

24. Mazzutti S, Ferreira SRS, Herrero M, Ibañez E (2017a) Intensified aqueous-based processes to obtain bioactive extracts from *Plantago major* and *Plantago lanceolata*. J Supercrit Fluids 119:64-71

25. Morales P, Ferreira ICFR, Carvalho AM, Sánchez-Mata MC, Cámara M, Fernández-Ruiz V, Pardo-de-Santayana M, Tardío J (2014) Mediterranean non-cultivated vegetables as dietary sources of compounds with antioxidant and biological activity. LWT - Food Sci Technol 55:389-396

26. Polo S, Tardío J, Vélez-del-Burgo A, Molina M, Pardo-de-Santayana M (2009) Knowledge, use and ecology of golden thistle (*Scolymus hispanicus* L.) in Central Spain. J Ethnobiol Ethnomed 5:42

27. Samuelsen AB (2000) The traditional uses, chemical constituents and biological activities of *Plantago major* L. A review. J Ethnopharmacol 71:1–21

28. Ryan L, Connell OO, Sullivan O, Aherne LS, Brien ON (2008) Micellarisation of carotenoids from raw and cooked vegetables. Plant Foods for Hum Nutr 63:127–133

29. Ainsworth EA, Gillespie KM (2007) Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nat Protoc 2:875–877

30. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26:1231–1237

31. Soler-Rivas C, Espín JC, Wichers HJ (2000) An easy and fast test to compare total free radical scavenger capacity of foodstuffs. Phytochem Anal 11:330–338

32. Gillespie KM, Chae JM, Ainsworth EA (2007) Rapid measurement of total antioxidant capacity in plants. Nat Protoc 2:867‑870

33. Pulido R, Bravo L, Saura-Calixto F (2000) Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. J Agric Food Chem 48:3396–340234. Hertog MGL, Hollman PCH, Katan MB, Kromhout D (1993) Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. Nutr Cancer 20:21–29

35. Jacobo-Velazquez DA, Cisneros-Zevallos L (2009) Correlations of antioxidant activity against phenolic content revisited: A new approach in data analysis for food and medicinal plants. J Food Sci 74:107–113

36. Granica S, Lohwasser U, Jöhrer K, Zidorn C (2015) Qualitative and quantitative analyses of secondary metabolites in aerial and subaerial of *Scorzonera hispanica* L. (black salsify). Food Chem 173:321–331

37. Karaköse H, Müller A, Kuhnert N (2015) Profiling and quantification of phenolics in *Stevia rebaudiana* leaves. J Agric Food Chem 63:9188−9198

490 38. Sareedenchai V, Zidorn C (2010) Flavonoids as chemosystematic markers in the
491 tribe Cichorieae of the Asteraceae. Biochem Syst Ecol 38:935–957

492 39. Gonçalves S, Romano A (2016) The medicinal potential of plants from the genus
493 *Plantago* (Plantaginaceae). Ind Crops Prod 83:213–226

494 40. Gonçalves S, Grevenstuk T, Martins N, Romano A (2015) Antioxidant activity and
495 verbascoside content in extracts from two uninvestigated endemic *Plantago* spp. Ind
496 Crops Prod 65:198–202

497 41. IUPAC Commission on the Nomenclature of Organic Chemistry (CNOC) and
498 IUPACIUB Commission in Biochemical Nomenclature (CBN) (1976).
499 Nomenclature of cyclitols. Recommendations. Biochem J 153:23-31

500 42. Bajko E, Kalinowska M, Borowski P, Siergiejczyk L, Lewandowski W (2016) 5-*O*-
501 Caffeoylquinic acid: A spectroscopic study and biological screening for
502 antimicrobial activity. LWT - Food Sci Technol 65:471-479

503 43. Mazzutti S, Riehl CAS, Ibañez E, Ferreira SRS (2017b) Green-based methods to
504 obtain bioactive extracts from *Plantago major* and *Plantago lanceolata*. J Supercrit
505 Fluids 119:211-220

506 44. Attri S, Singh N, Singh TR, Goel G (2017) Effect of *in vitro* gastric and pancreatic
507 digestion on antioxidant potential of fruit juices. Food Biosci 17:1–6

508 45. Jara-Palacios JM, Gonçalves S, Hernanz D, Heredia JF, Romano A (2018) Effects
509 of *in vitro* gastrointestinal digestion on phenolic compounds and antioxidant activity
510 of different white winemaking byproducts extracts. Food Res Int 109:433–439.

511 46. Martínez-Las Heras R, Pinazo A, Heredia A, Andrés A (2017) Evaluation studies of
512 persimmon plant (*Diospyros kaki*) for physiological benefits and bioaccessibility of

513 antioxidants by *in vitro* simulated gastrointestinal digestion. Food Chem 214:478-
514 485

515 47. Siracusa L, Kulišić-Bilušić T, Politeo O, Krause I, Dejanović B, Ruberto G (2011)
516 Phenolic composition and antioxidant activity of aqueous infusions from *Capparis*
517 *spinosa* L. and *Crithmum maritimum* L. before and after submission to a two-step *in*
518 *vitro* digestion model. J Agric Food Chem 59:12453–12459

519 48. Jayawardena N, Watawana MI, Waisundara VY (2015) Evaluation of the total
520 antioxidant capacity, polyphenol contents and starch hydrolase inhibitory activity of
521 ten edible plants in an *in vitro* model of digestion. Plant Foods Hum Nutr 70:71–76

522 49. Chen G-L, Chen S-G, Xie Y-Q, Chen F, Zhao Y-Y, Luo C-X, Gao Y-Q (2015)Total
523 phenolic, flavonoid and antioxidant activity of 23 edible flowers subjected to *in vitro*
524 digestion. J Func Food 17:243–259

525 50. Pavan V, Sancho RAS, Pastores GM (2014) The effect of *in vitro* digestion on the
526 antioxidant activity of fruit extracts (*Carica papaya*, *Artocarpus heterophillus* and
527 *Annona marcgravii*). LWT-Food Sci Technol 59:1247-1251

528 51. Celep E, Charehsaz M, Akyüz S, Acar ET, Yesilada E (2015) Effect of *in vitro*
529 gastrointestinal digestion on the bioavailability of phenolic components and the
530 antioxidant potentials of some Turkish fruit wines. Food Res Int 78:209–215

531 52 Wootton-Beard, PC, Moran A, Ryan L (2011) Stability of the total antioxidant
532 capacity and total polyphenol content of 23 commercially available vegetable juices
533 before and after *in vitro* digestion measured by FRAP, DPPH, ABTS and Folin–
534 Ciocalteu methods. Food Res Int 44:217–224.

535 53. Henning SM, Zhang Y, Rontoyanni VG, Huang J, Lee RP, Trang A, Heber D
536 (2014) Variability in the antioxidant activity of dietary supplements from

537 pomegranate, milk thistle, green tea, grape seed, goji, and acai: Effects of *in vitro*
538 digestion. J Agric Food Chem 62:4313–4321

539 54. Wong Y, Tan C, Long K, Nyam K (2014) *In vitro* simulated digestion on the
540 biostability of *Hibiscus cannabinus* L. seed extract. Czech J Food Sci 32:177–181

541 55. Arenas EH, Trinidad TP (2017) Fate of polyphenols in pili (*Canarium ovatum*
542 Engl.) pomace after *in vitro* simulated digestion. Asian Pac J Tropical Biomed 7:53-
543 58

544 56. Guldiken B, Toydemir G, Nur Memis K, Okur S, Boyacioglu D, Capanoglu E
545 (2016) Home-processed red beetroot (*Beta vulgaris* L.) products: changes in
546 antioxidant properties and bioaccessibility. Int J Mol Sci 17:1-13

547 57. Jayawardena N, Watawana MI, Waisundara VI (2015) The total antioxidant
548 capacity, total phenolics content and starch hydrolase inhibitory activity of fruit
549 juices following pepsin (gastric) and pancreatin (duodenal) digestion. J Verbr
550 Lebensm 10:349–357

551 58. Alipieva K, Korkina L, Orhan IE, Georgiev MI (2014) Verbascoside - A review of
552 its occurrence, (bio)synthesis and pharmacological significance. Biotechnol Adv
553 32:1065–1076

554 59. Zhou A, Sadik AO (2008) Comparative analysis of quercetin oxidation by
555 electrochemical, enzymatic, autoxidation, and free radical generation techniques: a
556 mechanistic study. J Agric Food Chem 56:12081–91

557

558 **Figure captions**

559 **Fig. 1** Aspect of the plants studied in their natural habitat. A: B. *maritima*; B: *O. pes-*
560 *caprae*; C: *P. major* and D: *S. hispanicus*.

561 **Fig. 2** HPLC-DAD chromatograms of *B. maritima* (A), *O. pes-caprae* (B), *P. major* (C)
562 and *S. hispanicus* (D). Detection at 320 nm; identity of compounds as in Table 2.

563 **Fig. 3** Total phenolic content of extracts from the four plant species studied before
564 (undigested samples) and after *in vitro* digestion (gastric and intestinal digests). Values
565 are expressed as mean ± SE (n = 3). * denotes significantly different ($p < 0.05$) in
566 comparison with undigested extract.

567 **Fig. 4** Antioxidant activity of extracts from the four plant species studied before
568 (undigested samples) and after *in vitro* digestion (gastric and intestinal digests). Values
569 are expressed as mean ± SE (n = 3). * denotes significantly different ($p < 0.05$) in
570 comparison with undigested extract.

Table 1 Edible and medicinal uses of the studied species.

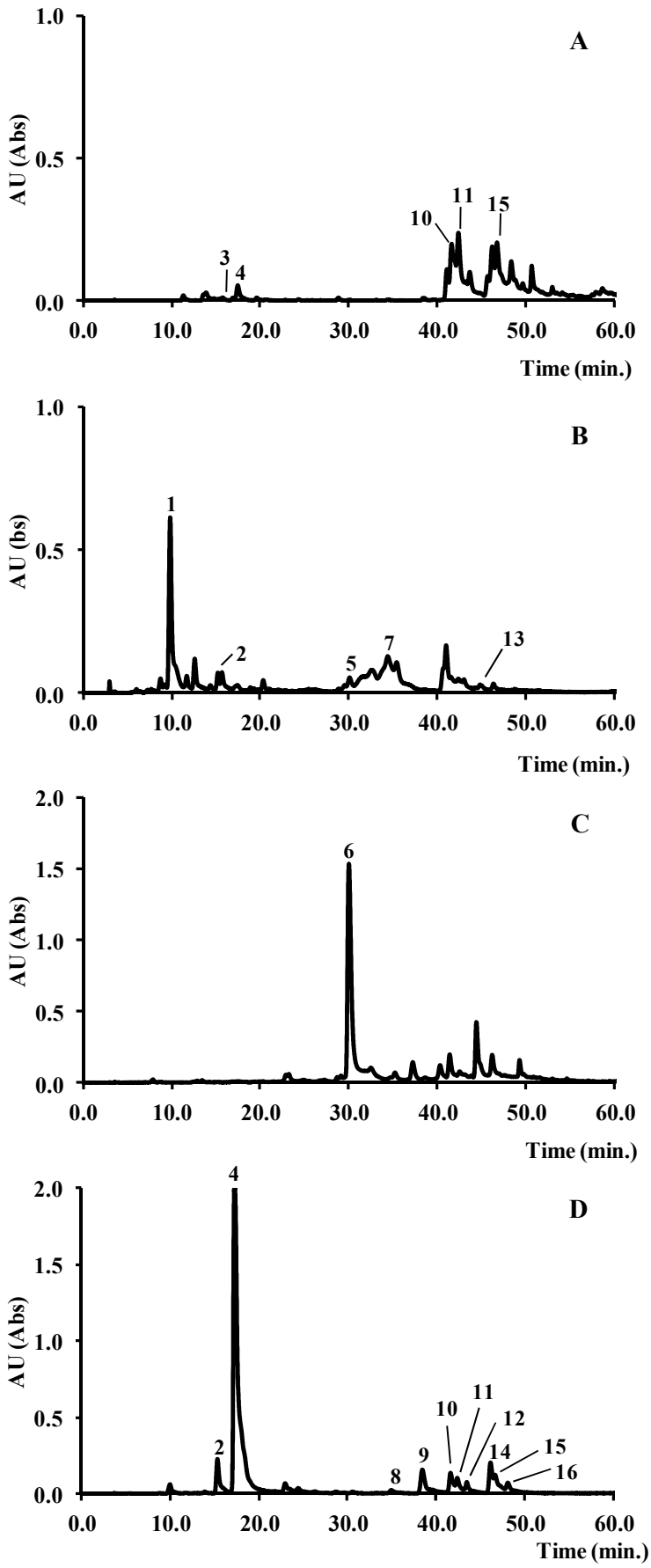
Plant species	Family	English common name	Edible part	Food use	Medicinal use
Beta maritima L.	Chenopodiaceae	Wild beet; Sea beet	Basal leaves	Stewed	Digestive disorders, burns and throat pains and anaemia
Plantago major L.	Plantaginaceae	Common plantain	Leaves and seeds	The leaves in salads and soups; the seeds in snacks, cakes and breads	Skin infections and other infectious diseases, digestive and respiratory disorders, to enhance the circulation and reproduction, for pain and fever relief, and to prevent cancer
Oxalis pes-caprae L.	Oxalidaceae	Bermuda buttercup; Cape sorrel	Leaves and peduncle of inflorescence	Salads	Not found
Scolymus hispanicus L.	Asteraceae	Golden thistle	Peeled basal leaves; roots and flowers	Peeled leaves boiled and fried in olive oil with garlic or raw in salads; roots employed as a coffee substitute and flowers as a colouring alternative to saffron	Diuretic, depurative, digestive, choleretic and lithiuretic

Table 2 Phenolic compounds identified in the extracts (mg/g of dry extract).[a]

Peak	Compound	RT (min)	*B. maritima*	*O. pes-caprae*	*P. major*	*S. hispanicus*
1	3-*O*-Caffeoylquinic acid	9.95	-	4.25 ± 0.34	-	-
2	4-*O*-Caffeoylquinic acid	15.43	-	0.75 ± 0.18	-	2.28 ± 0.13
3	2,4-Dihydroxybenzoic acid	16.51	1.53 ± 0.19	-	-	-
4	5-*O*-Caffeoylquinic acid	17.38	0.73 ± 0.02	-	-	33.30 ± 1.09
5	Luteolin-8-*C*-glucoside	30.49	-	0.74 ± 0.02	-	-
6	Verbascoside	31.87	-	-	32.37 ± 0.50	-
7	Luteolin-6-*C*-glucoside	34.88	-	2.92 ± 0.12	-	-
8	3,4-di-*O*-Caffeoylquinic acid	36.00	-	-	-	0.24 ± 0.02
9	3,5-di-*O*-Caffeoylquinic acid	38.58	-	-	-	2.05 ± 0.19
10	Quercetin 3-*O*-galactoside	41.51	3.62 ± 0.29	-	-	2.68 ± 0.19
11	Quercetin 3-*O*-rutinoside	42.84	5.47 ± 0.76	-	-	2.09 ± 0.22
12	4,5-di-*O*-Caffeoylquinic acid	43.51	-	-	-	0.80 ± 0.05
13	Apigenin-7-*O*-glucoside	44.5	-	0.21 ± 0.01	-	-
14	Kaempferol-3-*O*-glucoside	45.98	-	-	-	29.34 ± 3.22
15	Kaempferol-3-*O*-rutinoside	46.72	3.85 ± 0.54	-	-	2.27 ± 0.27
16	Isorhamnetin-3-*O*-rutinoside	47.56	-	-	-	0.28 ± 0.03
	Σ		15.20	8.87	32.38	75.33

[a] Values are expressed as mean ± SD (n = 3); -: not detected.

Figure 1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B
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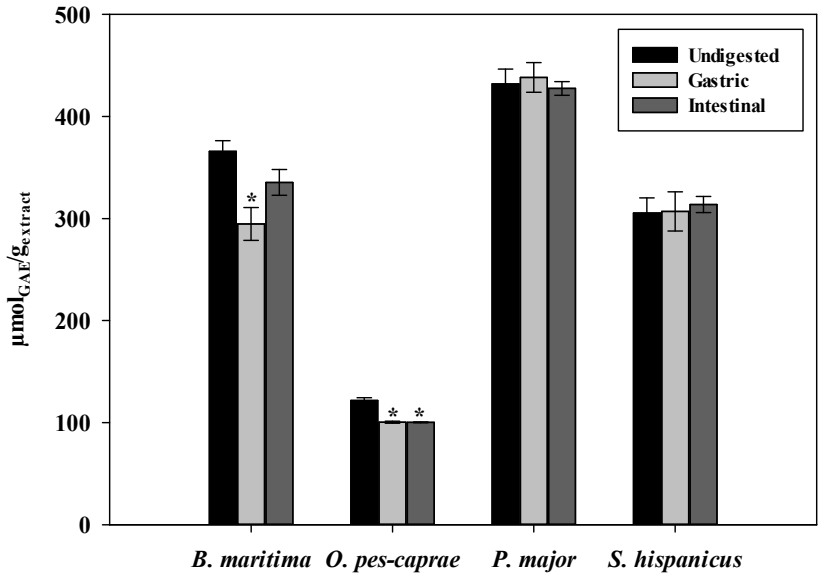
Figure 2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Undigested
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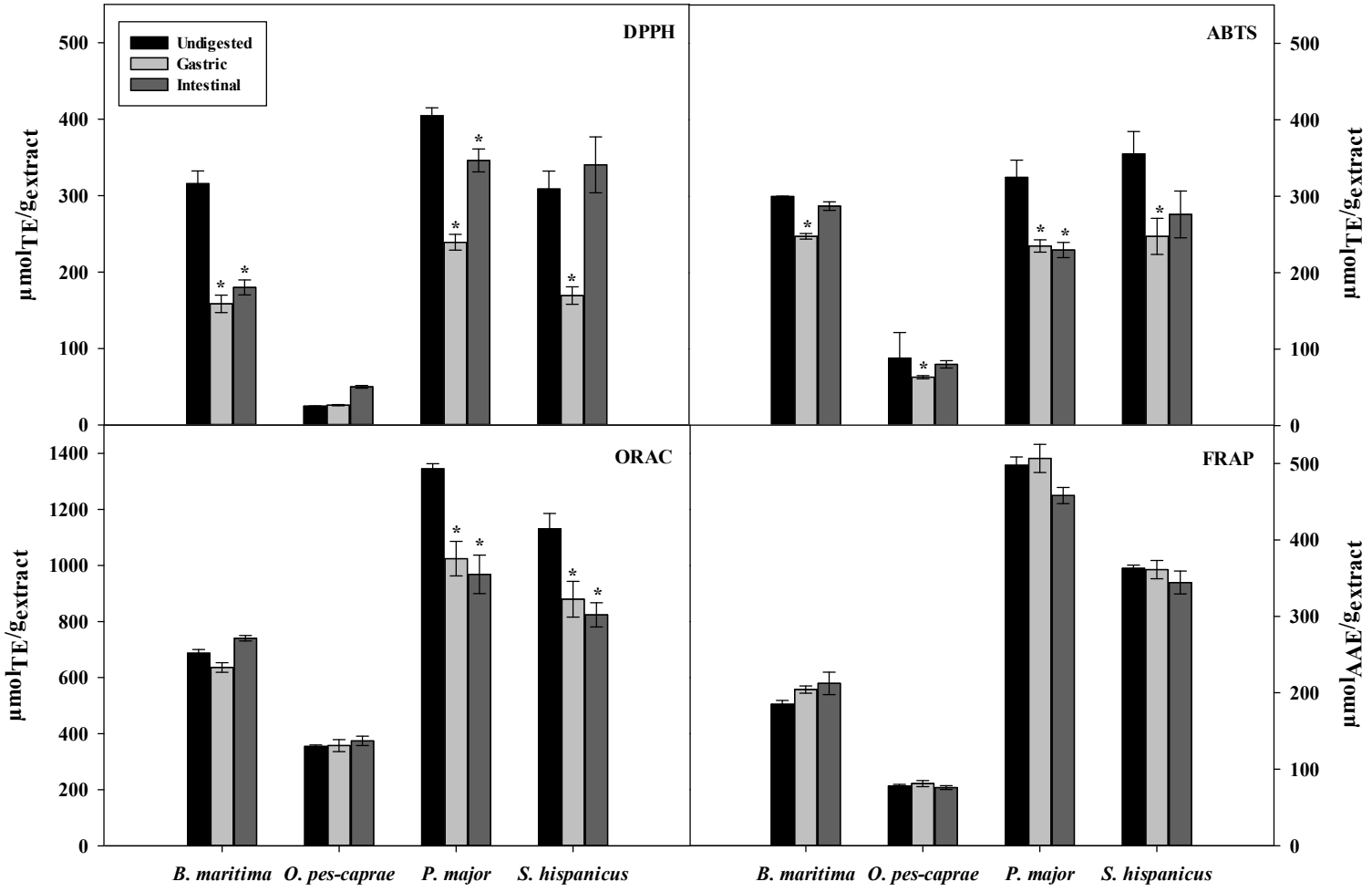
B. maritima
O. pes-caprae
P. major
S. hispanicus

Figure 3


Undigested
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B. maritima
O. pes-caprae
P. major
S. hispanicus
B. maritima
O. pes-caprae
P. major
S. hispanicus

Figure 4