Accepted Manuscript

Polyphenolic profile and antioxidant activities of Madeiran elderberry (Sambucus lanceolata) as affected by simulated in vitro digestion

Joana Pinto, Vítor Spínola, Eulogio J. Llorent-Martínez, María Luisa Fernández-de Córdova, Lucía Molina-García, Paula C. Castilho

PII: S0963-9969(17)30144-8
DOI: doi: 10.1016/j.foodres.2017.03.044
Reference: FRIN 6651

To appear in: *Food Research International*

Received date: 31 October 2016
Revised date: 22 March 2017
Accepted date: 23 March 2017

Please cite this article as: Joana Pinto, Vítor Spínola, Eulogio J. Llorent-Martínez, María Luisa Fernández-de Córdova, Lucía Molina-García, Paula C. Castilho , Polyphenolic profile and antioxidant activities of Madeiran elderberry (Sambucus lanceolata) as affected by simulated in vitro digestion. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Frin(2017), doi: 10.1016/j.foodres.2017.03.044

Polyphenolic profile and antioxidant activities of Madeiran elderberry (*Sambucus lanceolata*) as affected by simulated *in vitro* digestion

Joana Pinto[1†], Vítor Spínola[1†], Eulogio J. Llorent-Martínez[2], María Luisa Fernández-de Córdova[3], Lucía Molina-García[3], Paula C. Castilho[1*]

[1]*CQM - Centro de Química da Madeira, Universidade da Madeira, Campus da Penteada, 9020-105 Funchal, Portugal*

[2] *University of Castilla-La Mancha, Regional Institute for Applied Chemistry Research (IRICA), Ciudad Real 13071, Spain*

[3] *Department of Physical and Analytical Chemistry, University of Jaén, Campus Las Lagunillas S/N, E-23071 Jaén, Spain*

[†]These authors contributed equally to this work.

*Corresponding author

☏ +351 291 705 102

castilho@uma.pt

Abstract

The aims of this study were twofold: a) to provide a detailed report on the phenolic composition and antioxidant activity of fresh berries and leaves of *Sambucus lanceolata* (Madeiran elderberry); b) to study the effects caused by a simulated *in vitro* digestion on the composition and antioxidant activity of the berries and leaves.

Seventy-seven phytochemicals, mainly polyphenols, were identified in the methanol extracts of fresh berries and leaves, with the content of polyphenols higher in berries (27.2 mg g^{-1} dry extract, DE) than in leaves (25.9 mg g^{-1} DE). Anthocyanins were dominant in berries, while hydroxycinnamic acids (HCAs) and flavonols were abundant in leaves. Higher antioxidant activities were found in leaves than in berries, using several *in vitro* assays. After the simulated *in vitro* digestion, the levels of polyphenols were significantly reduced, in particular those of berries (81.8% decrease). Anthocyanins were the most affected compounds during the simulated digestion. However, despite the significant loss of phenolic compounds during digestion, methanol extracts of digested berries and leaves were still able to scavenge free-radicals. Hence, the consumption of leaves and/or berries of *S. lanceolata* may help prevent oxidative stress.

Keywords: *Sambucus lanceolata*; Whole fruit; Leaves; Polyphenols; *In vitro* digestion; Antioxidant activity.

Chemical compounds studied in this article

4-O-Caffeoylquinic acid (PubChem CID: 9798666); 5-Feruloylquinic acid (PubChem CID: 15901362); Caffeic acid (PubChem CID: 689043); Chlorogenic acid (PubChem CID: 1794427); Cyanidin (PubChem CID: 128861); Ellagic acid (PubChem CID:

5281855); p-Coumaric acid (PubChem CID: 637542); Quercetin (PubChem CID: 5280343); Roseoside (PubChem CID: 9930064).

1. Introduction

The genus *Sambucus* L. (Adoxaceae) includes approximately 30 species and are collectively known as elderberries, widely distributed around the world (Senica, Stampar, Veberic, & Mikulic-Petkovsek, 2016). The most economically important species include *S. cerulea* Raf. (blue elder), *S. javanica* Blume (Chinese elder), and *S. nigra* L. (common elderberry). Elderberries possess innumerous beneficial effects on human health, which are due to their many phytochemicals, such as flavonoids, phenolic acids, and vitamins (Mikulic-Petkovsek, Ivancic, Schmitzer, Veberic, & Stampar, 2016; Sidor & Gramza-Michałowska, 2015). Elderberry fruits have been used as food colorants in the preparation of concentrates, jams, juices, wines and dried powders, due to their high content in anthocyanins, which can function as both natural pigments and natural antioxidants (Duymuş, Göger, & Başer, 2014). Elderberry is not a toxic plant; however it has been reported that high consumption of leaves or unripe fruits can cause some adverse effects (nausea, vomiting and diarrhea) due to the presence of cyanogenic glycosides (Mikulic-Petkovsek et al., 2016; Senica et al., 2016). Therefore, elderberry is most widely consumed in its processed form than as fresh fruits.

Little is known about the stability of elderberries phytochemicals once they enter the gastrointestinal tract in the human body (Olejnik et al., 2016). Under gastrointestinal conditions, phenolic compounds are exposed to physiochemical changes (temperature, pH and digestive enzymes) that are known to affect their bioavailability (Chiang, Chen, Jeng, Lin, & Sung, 2014; Gullon, Pintado, Fernández-López, Pérez-Álvarez, & Viuda-Martos, 2015; Tavares et al., 2012). Therefore, it is important to verify the stability and

absorption of these compounds in the digestive tract in order to better understand and evaluate their potential biological properties (Liu et al., 2014; Marhuenda et al., 2016). *In vitro* digestion models are widely used and have been proven efficient to determine the stability of phytochemicals under gastrointestinal conditions (Chiang et al., 2014; Marhuenda et al., 2016). Even though *in vitro* systems are hindered by their inability to effectively reproduce the complexity of the gastrointestinal tract, these models have been increasingly used to study the changes in the dietary components throughout digestion (Guerra et al., 2012). Despite their limitations, the results obtained by *in vitro* models can be generally correlated with those from human studies and animal models (Gullon et al., 2015; Hur, Lim, Decker, & McClements, 2011). While most studies are performed in static models (with prefixed concentrations and volumes of digested materials, enzymes, salts, etc), there are also a limited number of dynamic systems that mimic the continuous changes of the physicochemical conditions, and aim to better simulate the passage of the digesta through the human digestive tract (Guerra et al., 2012). However, these models are much more expensive and time-consuming than those operated in static mode and still require validation and standardization (Alminger et al., 2014; Hur et al., 2011).

Among the wild edible berries of Madeira Archipelago (Portugal), *Sambucus lanceolata* R. Br. in Buch (Madeira elderberry) is one of the species with less market penetration, despite its large domestic use. *Sabugueiro* is a small endemic tree or shrub, up to 7 m tall, with small yellowish round edible fruits that get dark-purple when ripe (Press & Short, 1994). Flowers are used in folk medicine as diuretic and emollient, while leaves are applied in poultices on bruises, wounds and sores (Rivera & Obón, 1995). Additionally, berries infusion is consumed to relieve colds, diarrhea and menstrual pains.

The aim of this study was to examine the effects of a simulated *in vitro* digestion on the phenolic composition and antioxidant activity of berries and leaves of *S. lanceolata*. The results here presented may provide the first insight into the behavior of the phenolics of this plant during the digestion process, and may yield useful information about its potential bioactive properties to facilitate its commercial interest.

2. Experimental

2.1. Chemicals and reagents

All reagents and standards were of analytical reagent grade unless stated otherwise. Folin–Ciocalteu's phenol reagent (FCR), calcium chloride (99 - 105 %), potassium chloride (99.5 – 100.5%), and potassium acetate (> 99.5%) were purchased from Panreac (Barcelona, Spain). Ellagic acid (≥ 96%), (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), luteolin (≥ 98%) and methanol (99.9%) were acquired from Fluka (Lisbon, Portugal). Caffeic acid (≥98%), N-(1-naphthyl)ethylene-diamine dihydrochloride (NEDA, ≥ 98%), phenazine methosulfate (PMS, ≥ 90%), sulfanilamide (≥ 99%), *β*-nicotinamide adenine dinucleotide reduced (NADH, ≥94%), potassium persulfate (99%), hydrochloric acid (37%), formic acid (98%), potassium dihydrogen phosphate (99.5%), disodium hydrogen phosphate (99%), ammonium chloride (99.8%), sodium carbonate, mucin (type II; from porcine stomach), *α*-amylase (porcine pancreas, type VI-B), pepsin (porcine gastric mucosa), pancreatin (porcine pancreas), lipase (type II; from porcine pancreas) and porcine bile extract (contains glycine and taurine conjugates of hyodeoxycholic acid and other bile salts) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cyanidin-3-glucoside (C3G) chloride (> 98%) and 5-*O*-caffeoylquininc acid (> 95%) were obtained from Biopurify Phytochemicals LTD

(Chengdu, China). Nitroblue tetrazolium chloride (NBT, 90%) was acquired from Acros Organics, *o*-phosphoric acid (85%) from BDH AnalaR, and apigenin (≥ 99%) was purchased from Extrasynthese (Genay, France). Quercetin dihydrate (> 99%) and magnesium chloride hexahydrated (99%) were purchased from Riedel-de Haen. Acetic acid glacial was purchased from Fischer Scientific (Bishop Meadow, UK), and urea (99%) was acquired from Harnstoff. Sodium nitroprusside (99%) and ethylenediaminetetraacetic acid (EDTA, > 99%) were acquired from Merck (Darmstadt, Germany). LC–MS grade acetonitrile (CH_3CN, 99%) (LabScan; Dublin, Ireland) and ultrapure water (Milli-Q Waters purification system; 18 M Ω cm at 23 °C; Millipore; Milford, MA, USA) were used for the HPLC-MS analyses.

2.2. Extraction of phenolic compounds from berries and leaves

Samples of *S. lanceolata* were collected in the wild at Santana (Madeira Island) in October 2014. Voucher specimens were stored at Madeira Botanical Garden Herbarium (Funchal, Madeira) (voucher: MADJ13284). For analysis, plant material was separated into leaves and fruits (fully ripe), destemmed, washed, lyophilized (Alpha 1-2 LD plus freeze dryer, CHRIST), ground to powder using a mechanical grinder, and stored at -20 ºC. Phenolic extraction was performed by a previous procedure (Spínola, Llorent-Martínez, Gouveia, & Castilho, 2014): 1 g of dry material was extracted with 25 mL of methanol (in a 100 mL erlenmeyer wrapped in foil) in an ultra-sonic bath (Bandelin Sonorex, Germany) at 35 kHz and 200 W for 60 min (room temperature). For berry fruits, an extraction solution composed of MeOH/H_2O (80:20, v/v) acidulated with 7% acetic acid was used. After sonication, solutions were filtered through Whatman No.1 filter papers, concentrated to dryness under reduced pressure in a rotary evaporator (Buchi Rotavapor R-114; USA) at 40 ºC (under reduced light), and the resulting extracts were stored in 5 mL capped flasks at 4 ºC until further analysis.

In the case of leaves, an additional step was required to remove chlorophylls from the leaves' extract. After the first filtration step, a small amount of activated charcoal was added to the methanol extract and, after mixing for a few seconds, the solution was filtered. Then, it was concentrated to dryness and stored as previously mentioned.

2.3. Simulation of in vitro digestion

A static model that simulated gastrointestinal digestion was employed (Flores, Singh, Kerr, Pegg, & Kong, 2014). The detailed composition of digestive juices (salivary, gastric, intestine and bile) is given in Table 1. Lyophilized berry fruits and leaves (approximately 2 g) were added, separately, to 50 mL Falcon tubes and incubated at 37 ºC in a water bath with agitation (150 rpm), protected from light. The samples were digested sequentially as follows: mouth – addition of 4 mL salivary juice and mixing for 5 min; stomach – addition of 10 mL gastric juice and mixing for 2 h; and intestines – addition of 10 mL duodenal and 4 mL bile juices and mixing for 2 h. After simulation, samples were frozen at -20 ºC, lyophilized and submitted to extraction as described above (section 2.2).

TABLE 1

2.4. HPLC analysis

The HPLC-ESI-MS^n analysis was performed on a Dionex ultimate 3000 series instrument (Thermo Scientific Inc.) equipped with a binary pump, an autosampler, a column compartment (kept at 30 ºC) and coupled with a Bruker Esquire model 6000 ion trap mass spectrometer (Bremen, Germany). Separation was carried out on a Phenomenex Gemini C_{18} column (5 µm, 250 x 3.0 mm i.d.) using the same conditions reported in (Spínola et al., 2014). Dry extracts (DE) from fruits and leaves were re-dissolved in the initial HPLC mobile phase (5 mg mL^{-1}), filtered through 0.45 µm PTFE

membrane filters, and injected (5 μL) in the HPLC system. Esquire control and Data Analysis software were used for data acquisition and processing, respectively.

2.5. Quantification of main polyphenols

For this quantitative analysis, one polyphenol was selected as the standard for each group, and was used to calculate individual concentrations by HPLC-DAD (Díaz-García, Obón, Castellar, Collado, & Alacid, 2013). Caffeic and 5-*O*-caffeoylquinic acids were used for hydroxycinnamic and caffeoylquinic acids, respectively. Anthocyanins standard was cyanidin 3-glucoside (C3G); quercetin, (+)-apigenin and ellagic acid were the standards used for flavonols, flavones and ellagitannins, respectively. Stock standard solutions (1000 mg L^{-1}) were prepared in methanol, and calibration curves were prepared by diluting the stock solutions with the initial mobile phase. Six concentrations (5−100 mg L^{-1}) were used for the calibration, plotting peak area versus concentration. The calibration curves obtained had $R^2 \geq 0.967$ in all cases. The selected detection wavelengths were 520 nm for anthocyanins, and 280 nm for the other polyphenols. Total individual phenolic content (TIPC) was defined as the sum of the quantified phenolic compounds in *S. lanceolata* extracts.

2.6. *Total phenolic and flavonoid contents*

For the following analysis, dry extracts of berry fruits and leaves were re-dissolved in methanol (5 mg mL^{-1}). TPC determinations were carried out following a previous protocol (Zheng & Wang, 2001), with some modifications (Spínola et al., 2014). The amounts of total phenolics were expressed as mg of gallic acid equivalents (GAE) g^{-1} DE. TFC was determined based on a previous procedure (Akkol, Göger, Koşar, & Başer, 2008) with some adaptations (Spínola et al., 2014). The final results were expressed as mg of rutin equivalent (RUE) g^{-1} DE.

2.7. *In vitro antioxidant activity assays*

Determination of antioxidant activity by ABTS radical cation assay was based on a previous work (Dudonné, Vitrac, Coutière, Woillez, & Mérillon, 2009), with slight adjustments (Spínola et al., 2014): The DPPH assay was performed as (Dudonné et al., 2009) with some modifications (Spínola et al., 2014). Nitric oxide (NO) scavenging activity (Sousa, Valentão, Ferreres, Seabra, & Andrade, 2008) and superoxide radical ($O_2^{\cdot-}$) assays (Ewing & Janero, 1995) were performed as in the cited works. The results of antioxidant assays were expressed as μmol of Trolox equivalent (TE) g^{-1} DE, based on the Trolox calibration curves.

2.8. Statistical Analysis.

All samples were assayed in triplicate and results are given as the means ± standard deviations. Data analysis was carried out by means of a one-way ANOVA with Tukey's post-hoc test using SPSS for Windows, IBM SPSS Statistics 20 (SPSS, Inc., USA). A value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

This study represents the first report on the phytochemical profile of *S. lanceolata* morphological parts by HPLC-ESI-MS^n. The base peak chromatograms (BPC) of the analyzed extracts are shown in Fig. 1 & 2. Compounds were numbered by their order of elution and this numeration was kept identical for all samples.

3.1. HPLC-ESI-MS^n screening

Identification of phytochemicals was assigned based on data available in scientific literature and, in some cases, authentic standards were also used (Table S1 & S2, Supplementary Material). Additionally, some derivatives of phytochemicals were putatively assigned, on the basis of analogous fragmentations.

Relatively similar phenolic profiles were found in berries and leaves. In total, 77 phytochemicals were tentatively identified, with 54 in berries and 35 in leaves (Tables

S1 & S2, Supplementary Material): 60 polyphenols and 17 non-phenolic compounds (oligosaccharides, organic acids, terpenoids, lignans and fatty acids). Some classes of compounds were found only in fruits (anthocyanins, flavones, tannins and ellagic acid derivatives). As opposed to previous studies on *S. nigra* (Senica et al., 2016; Sidor & Gramza-Michałowska, 2015), no cyanogenic glycosides were detected in the analyzed methanol extracts. Moreover, some phytochemicals (**8**, **9**, **11**, **13, 27, 29, 72, 81** and **82**) have not been documented in *Sambucus* species so far.

3.2. *Quantification of polyphenols*

In the present study, 31 polyphenols were tentatively quantified by HPLC-DAD (Table 2) as described in section 2.5. Only the most abundant compounds have been considered, since the overlapping of some compounds or their low levels did not permit a proper integration of the peaks.

Berries showed a higher TIPC than leaves ($p < 0.05$). This difference is due to anthocyanins, present only in berries. The same trend was observed for *S. nigra* morphological parts (Dawidowicz, Wianowska, & Baraniak, 2006). However, superior contents of hydroxycinnamic acids (HCAs) and flavonols ($p < 0.05$) were determined in the analyzed leaves (Table 2).

TABLE 2

Quantitative results indicated that anthocyanins were the most abundant compounds in berries (≈ 91 % of TIPC), followed by HCAs (≈ 5 %), flavonols (≈ 2 %), ellagic acid derivatives (≈ 1.5 % each) and flavones (≈ 0.5 %). For leaves, a different profile was observed: HCAs (≈ 60 %) > flavonols (≈ 40 %).

Cyanidin-*O*-sambubioside (**A2**) was dominant in berries, while 3-*O*-caffeoylquinic acid (**25**), quercetin-*O*-rutinoside (**49**) and quercetin-*O*-(rhamnosyl)rutinoside (**38**) were representative in leaves.

Previous works on other Sambucus species have shown alike polyphenolic compositions. For example, cyanidin derivatives are dominant in elderberries, in particular cyanidin-*O*-sambubioside (Dawidowicz et al., 2006; Olejnik et al., 2016; Sidor & Gramza-Michałowska, 2015). Others report C3G as the main anthocyanin in elderberries (Duymuş et al., 2014; Mandrone et al., 2014), which was not confirmed in the present work. Comparable C3G (0.4 – 0.7 mg g^{-1} DE) (Duymuş et al., 2014) but lower anthocyanins total contents (8.8 – 13.4 mg g^{-1} DE) were documented in previous studies on *S. nigra* (Duymuş et al., 2014; Mandrone et al., 2014). Besides anthocyanins, quercetin-*O*-rutinoside, kaempferol and isorhamnetin glycosides are also documented as the main flavonols in elderberries (Mandrone et al., 2014; Sidor & Gramza-Michałowska, 2015). TIPC of *S. lanceolata* was slightly higher than those found on *S. nigra* (0.7 – 27.4 mg g^{-1} DE) (Dawidowicz et al., 2006; Mandrone et al., 2014).

TPC and TFC, measured by colorimetric assays (Table 2), were performed inorder to facilitate comparison with other species and showed an opposite trend compared to TIPC (leaves > berries) ($p < 0.05$). Higher TPCs (49.2 – 90.6 mg GAE g^{-1} DE) were determined previously in *S. nigra* and *S. ebulus* (Duymuş et al., 2014; Topuzović, Stanković, Jakovljević, & Bojović, 2016). In another study (Namiesnik et al., 2014), gooseberry and cranberry showed lower TPC and TFC than in the analyzed extracts; lower TFC were also reported for blueberry. It should be noted that differences in sample preparation, extraction and analytical procedures could justify the discrepancies between obtained results and data from other studies. Additionally, these colorimetric methods are susceptible to other components present in extracts (sugars, proteins, among others) (Flores et al., 2014).

Human digestion is a complex process, and the study of this multistage process is difficult and complex. Hence, different attempts to model it have been reported

(Alminger et al., 2014). Static models are the most widespread digestive systems, although they do not reproduce the dynamic processes occurring during human digestion (Guerra et al., 2012). Nevertheless, static models present several advantages, such as their low-cost, simplicity and reproducibility, which make them a desirable alternative to studies on animals and humans, and suitable for preliminary studies (Hur et al., 2011).

Significant changes ($p < 0.05$) in the composition of the major phenolic compounds were observed in the *S. lanceolata* extracts following *in vitro* digestion (Table 2). Berries components were more affected than leaves, with 81.8% and 61.5% reduction of TIPC, respectively, upon simulated digestion. The most significant reduction was mainly with anthocyanins (87.2% reduction), with only cyanidin-*O*-sambubiosidedetected in the digested extract (Table 2). Moreover, degradation of HCAs (35.8 - 54.9%) and flavonoids (56.3 - 70.6%) was observed as result of the digestion process. An exception was observed with the 3-*O* and 5-*O*-caffeoylquinic acids in berries, whose amounts were increased ($p < 0.05$) by 13% and 57%, respectively. Similarly, an increase of 53% was reported for 5-*O*-caffeoylquinic acid in elderberries, after *in vitro* digestion (Zhou, Zhu, Yang, & Zhou, 2016). Gullon and co-workers (Gullon et al., 2015) found an increase by 113% of ellagic acid after simulated digestion of pomegranate peel. According to these authors, the increased concentration might be due to this compound being bound to proteins or fibre in the original matrix and, as a result of enzymatic digestion, it was released from these structures.

A previous study conducted on *S. nigra* berries (Olejnik et al., 2016) reported similar degradation rates upon *in vitro* digestion process: 88.4% for anthocyanins and 80.5% for TIPC. The losses of polyphenols contents following *in vitro* gastrointestinal

digestion of different berries have been documented in the literature for other berry species (Chiang et al., 2014; Gullon et al., 2015; Huang, Sun, Lou, Li, & Ye, 2014; Marhuenda et al., 2016; Tavares et al., 2012). However, none was conducted on *S. lanceolata* so far.

Phenolic compounds are known to be unstable under gastrointestinal digestion due to pH variations and interactions with dietary constituents (proteins, fibre or iron) and digestive enzymes (Chiang et al., 2014; Gullon et al., 2015). The passage through the intestinal tract lead to their hydrolysis and degradation and/or their conversion/breakdown into other unknown or undetected compounds under dramatic pH changes (Huang et al., 2014; Olejnik et al., 2016). Anthocyanins appear to be the most sensitive class and may largely disappear in the intestinal step (Alminger et al., 2014; Gullon et al., 2015). Anthocyanins are very reactive compounds and susceptible to multiple factors such as temperature, light, pH, and enzymes/oxygen action (Huang et al., 2014). Anthocyanins can be found in different chemical forms which depend on the pH of the solution. The flavylium cation is the predominant species, at pH 1, and contributes to red and purple colours. At pH 2 – 4, the quinoidal species (blue) are main components. At higher pH values (5 - 6) only two colourless species can be observed (carbinol pseudobase and chalcone). At alkaline environments (pH > 7), the anthocyanins are degraded depending on their substituent groups (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). During digestion, the chemical structure of anthocyanins would vary along the broad digestion, and pH-dependent balance between the aforementioned species would lead to a disproportion on the initial structures of anthocyanins (Marhuenda et al., 2016). During oral processing, the enzyme activity or agitation conditions could facilitate the breakage of large molecules, such as high molecular weight phenols, which initially may be

insoluble (Gullon et al., 2015). Gastric digestion promotes the hydrolysis of the polymerized polyphenols to monomers or aglycones caused by the acidic gastric juice and pepsin (Chiang et al., 2014). Anthocyanins are generally stable in the acidic conditions of the stomach, but the pH increase in the small intestine has been reported to especially affect anthocyanins, which form colorless chalcone pseudobases that may be further degraded upon opening at the C ring (Alminger et al., 2014; Bohn, 2014; Tavares et al., 2012); which might be the main reason for the decrease in anthocyanin content. Also, the anthocyanins stability is influenced by the ring B substituents and the presence of additional hydroxyl or methoxyl groups which decrease the aglycone stability in neutral media (Castañeda-Ovando et al., 2009; Liu et al., 2014). Glycation seems to increase the stability of anthocyanins in neutral pH conditions. This behavior is explained because the sugar molecules avoid the degradation of instable intermediaries into phenolic acid and aldehyde compounds (Castañeda-Ovando et al., 2009).

3.5. *In vitro* antioxidant assays

The antioxidant activity of *S. lanceolata* extracts was assessed by different assays (Fig. 3). Leaves extract was the most active, regardless of the assayed method ($p < 0.05$). This trend is opposite to previous HPLC quantification (Table 2), suggesting that in this case the type of phenolics seems more important for antioxidant activities than total phenolic amounts.

From a previous study (Mandrone et al., 2014), *S. nigra* extracts were more active towards $ABTS^{\bullet+}$ and DPPH radical than *S. lanceolata* (0.6 – 2.2 mmol TE g^{-1} DE). However, analyzed extracts showed higher antioxidant activities in relation to ABTS cation and DPPH radicals than gooseberry, cranberry and blueberry extracts (0.02 – 0.26 mmol TE g^{-1} DE) (Namiesnik et al., 2014), which are species with major

economic importance (Manganaris, Goulas, Vicente, & Terry, 2014).It seems that the aforementioned antioxidant activities are related to the phenolic content of berry fruits, since samples with the highest amounts showed, in general, the greatest antioxidant activities.

An overproduction of reactive species like O_2^-, hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), NO and peroxynitrite ($ONOO^-$), caused by oxidative stress, is associated with acute and chronic diseases (inflammation, cardiovascular, neurodegenerative, diabetes) (Denev et al., 2010; Olejnik et al., 2015; Sousa et al., 2008). Therefore, exogenous scavengers like polyphenols are expected to play a beneficial therapeutic role by elimination of these free-radicals (Denev et al., 2010; Sousa et al., 2008). A simultaneous scavenging activity of *S. lanceolata* towards NO and O_2^- was demonstrated (Fig. 3), which might also prevent the formation of other more reactive species like OH^- and $ONOO^-$ (Sousa et al., 2008). Hence, the overall antioxidant capacity might provide more relevant biological information compared to that obtained by other assays that use non-biological radicals (like $ABTS^{\bullet+}$ and DPPH radicals). *S. nigra* extracts were also effective NO scavengers (Denev et al., 2010), but since results are expressed in different units it is difficult to establish a comparison with present data.

Regarding the digestion process, the lower ($p < 0.05$) antioxidant activities of digested extracts (Fig. 3) was associated with significant losses of the polyphenolic composition. This is in agreement with previous works on *S. nigra* (Olejnik et al., 2015, 2016; Zhou, Zhu, Yang, & Zhou, 2016) and other berry fruits (Gullon et al., 2015; Huang et al., 2014; Liu et al., 2014; Marhuenda et al., 2016) that stated loss of antioxidant capacity of phenolic compounds after *in vitro* digestion. The decrease of antioxidant activity was more notorious for leaves (35 - 67%) than for berries (24 –

54%). This result may suggest that the antioxidant properties of analyzed extracts are mainly attributed to the particular structures and functional groups of their components, and not so much to the total amounts of these components. Since polyphenols are highly sensitive to the alkaline conditions in the small intestine (in particular anthocyanins) and are subjected to structural modifications (Gullon et al., 2015), their bioactivities are obviously changed due to such alterations. Nevertheless, digested samples were still active towards targeted free-radicals (Fig. 3). Although *in vitro* antioxidant assays do not provide an accurate estimation of the *in vivo* situation, they are highly useful for preliminary screening of the antioxidant potential of natural products (López-Alarcón & Denicola, 2013). Additional *in vivo* experiments are required to confirm the biological relevance of analyzed extracts; nevertheless, *S. lanceolata* showed promising antioxidant properties, even after simulated digestion.

FIGURE 3

Conclusions

In this work, a static *in vitro* digestion model has been simulated to study the phenolic composition and antioxidant activity of *S. lanceolata*. Different assays were carried out to determine the (individual and total) phenolic contents and the *in vitro* antioxidant activity of berries and leaves. All the assays were performed on fresh and digested samples, and the obtained data were compared. Although significant differences were observed in the phenolic profiles of fresh leaves and berries, their behavior towards the simulated digestion was similar: a significant loss of bioactive compounds. However, although the antioxidant activity was reduced, the digested extracts still presented significant antioxidant potential. It is obvious that the *in vitro* digestion model here used cannot mimic a complex *in vivo* digestion, so these results are only indicative of the effects caused by digestion on the analyzed berries and leaves.

As a follow-up investigation we aim to further evaluate the effect of released polyphenols from *S. lanceolata* and other berry-producing species on digestive enzymes. However, the remaining antioxidant activity observed after the simulated digestion may suggest the potential use of *S. lanceolata* in the food industry; hence, further experiments are required to confirm these preliminary results.

Acknowledgments

The authors wish to express their gratitude to Madeira Natural Park, and to Francisco Fernandes and José Carvalho from Madeira Botanical Garden for the identification of plant material. V. Spínola is grateful to Fundação para a Ciência e a Tecnologia (FCT, Portugal) for the Ph.D. grant SFRH/BD/84672/2012. This research was sponsored by FCT (Project PEst-OE/QUI/UI0674/2013, CQM, Portuguese Government funds) and the Portuguese National Mass Spectrometry Network (Contract RNEMREDE/1508/REM/2005). Funding through the project M1420-01-0145-FEDER-000005 - Centro de Química da Madeira - CQM+ (Madeira 14-20) is also acknowledged. E. J. Llorent-Martínez acknowledges the financial assistance from the UCLM Research Plan.

REFERENCES

Akkol, E. K., Göger, F., Koşar, M., & Başer, K. H. C. (2008). Phenolic composition and biological activities of *Salvia halophila* and *Salvia virgata* from Turkey. *Food Chemistry*, *108*(3), 942–949.

Alminger, M., Aura, A.-M., Bohn, T., Dufour, C., El, S. N., Gomes, A., Santos, C. N. (2014). *In vitro* models for studying secondary plant metabolite digestion and bioaccessibility. *Comprehensive Reviews in Food Science and Food Safety*, *13*(4), 413–436.

Bohn, T. (2014). Dietary factors affecting polyphenol bioavailability. *Nutrition Reviews*, *72*(7), 429–452.

Castañeda-Ovando, A., Pacheco-Hernández, M. de L., Páez-Hernández, M. E., Rodríguez, J. A., & Galán-Vidal, C. A. (2009). Chemical studies of anthocyanins: A review. *Food Chemistry*, *113*(4), 859–871.

Chiang, Y. C., Chen, C. L., Jeng, T. L., Lin, T. C., & Sung, J. M. (2014). Bioavailability of cranberry bean hydroalcoholic extract and its inhibitory effect against starch hydrolysis following *in vitro* gastrointestinal digestion. *Food Research International*, *64*, 939–945.

Dawidowicz, A. L., Wianowska, D., & Baraniak, B. (2006). The antioxidant properties of alcoholic extracts from *Sambucus nigra* L. (antioxidant properties of extracts). *LWT - Food Science and Technology*, *39*(3), 308–315.

Denev, P., Ciz, M., Ambrozova, G., Lojek, A., Yanakieva, I., & Kratchanova, M. (2010). Solid-phase extraction of berries' anthocyanins and evaluation of their antioxidative properties. *Food Chemistry*, *123*(4), 1055–1061.

Díaz-García, M. C., Obón, J. M., Castellar, M. R., Collado, J., & Alacid, M. (2013). Quantification by UHPLC of total individual polyphenols in fruit juices. *Food Chemistry*, *138*(2–3), 938–949.

Dudonné, S., Vitrac, X., Coutière, P., Woillez, M., & Mérillon, J.-M. (2009). Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *Journal Agricultural and Food Chemistry*, *57*, 1768–1774.

Duymuş, H. G., Göger, F., & Başer, K. H. C. (2014). *In vitro* antioxidant properties and anthocyanin compositions of elderberry extracts. *Food Chemistry*, *155*, 112–119.

Ewing, J. F., & Janero, D. R. (1995). Microplate superoxide dismutase assay employing

a nonenzymatic superoxide generator. *Analytical Biochemistry*, *232*(2), 243–8.

Flores, F. P., Singh, R. K., Kerr, W. L., Pegg, R. B., & Kong, F. (2014). Total phenolics content and antioxidant capacities of microencapsulated blueberry anthocyanins during *in vitro* digestion. *Food Chemistry*, *153*, 272–278.

Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., & Alric, M. (2012). Relevance and challenges in modeling human gastric and small intestinal digestion. *Trends in Biotechnology*, *30*(11), 591–600.

Gullon, B., Pintado, M. E., Fernández-López, J., Pérez-Álvarez, J. A., & Viuda-Martos, M. (2015). *In vitro* gastrointestinal digestion of pomegranate peel (*Punica granatum*) flour obtained from co-products: Changes in the antioxidant potential and bioactive compounds stability. *Journal of Functional Foods*, *19*, 617–628.

Huang, H., Sun, Y., Lou, S., Li, H., & Ye, X. (2014). *In vitro* digestion combined with cellular assay to determine the antioxidant activity in Chinese bayberry (*Myrica rubra* Sieb. et Zucc.) fruits: a comparison with traditional methods. *Food Chemistry*, *146*, 363–70.

Hur, S. J., Lim, B. O., Decker, E. A., & McClements, D. J. (2011). *In vitro* human digestion models for food applications. *Food Chemistry*, *125*(1), 1–12.

Liu, Y., Zhang, D., Wu, Y., Wang, D., Wei, Y., Wu, J., & Ji, B. (2014). Stability and absorption of anthocyanins from blueberries subjected to a simulated digestion process. *International Journal of Food Sciences and Nutrition*, *65*(4), 440–448.

López-Alarcón, C., & Denicola, A. (2013). Evaluating the antioxidant capacity of natural products: A review on chemical and cellular-based assays. *Analytica Chimica Acta*, *763*, 1–10.

Mandrone, M., Lorenzi, B., Maggio, A., La Mantia, T., Scordino, M., Bruno, M., & Poli, F. (2014). Polyphenols pattern and correlation with antioxidant activities of

berries extracts from four different populations of Sicilian *Sambucus nigra* L. *Natural Product Research*, *28*(16), 1246–53.

Manganaris, G. A., Goulas, V., Vicente, A. R., & Terry, L. A. (2014). Berry antioxidants: Small fruits providing large benefits. *Journal of the Science of Food and Agriculture*, *94*(5), 825–833.

Marhuenda, J., Alemán, M. D., Gironés-Vilaplana, A., Pérez, A., Caravaca, G., Figueroa, F., Zafrilla, P. (2016). Phenolic composition, antioxidant activity, and *in vitro* availability of four different berries. *Journal of Chemistry*, 1–7.

Mikulic-Petkovsek, M., Ivancic, A., Schmitzer, V., Veberic, R., & Stampar, F. (2016). Comparison of major taste compounds and antioxidative properties of fruits and flowers of different Sambucus species and interspecific hybrids. *Food Chemistry*, *200*, 134–140.

Namiesnik, J., Vearasilp, K., Leontowicz, H., Leontowicz, M., Ham, K. S., Kang, S. G., Gorinstein, S. (2014). Comparative assessment of two extraction procedures for determination of bioactive compounds in some berries used for daily food consumption. *International Journal of Food Science and Technology*, *49*(2), 337–346.

Olejnik, A., Kowalska, K., Olkowicz, M., Rychlik, J., Juzwa, W., Myszka, K., Białas, W. (2015). Anti-inflammatory effects of gastrointestinal digested *Sambucus nigra* L. fruit extract analysed in co-cultured intestinal epithelial cells and lipopolysaccharide-stimulated macrophages. *Journal of Functional Foods*, *19*, 649–660.

Olejnik, A., Olkowicz, M., Kowalska, K., Rychlik, J., Dembczyński, R., Myszka, K., Moyer, M. P. (2016). Gastrointestinal digested *Sambucus nigra* L. fruit extract protects *in vitro* cultured human colon cells against oxidative stress. *Food*

Chemistry, *197*, 648–657.

Press, J. R., & Short, M. J. (1994). *Flora of Madeira*. London: HMSO.

Rivera, D., & Obón, C. (1995). The ethnopharmacology of Madeira and Porto Santo Islands, a review. *Journal of Ethnopharmacology*, *46*(2), 73–93.

Senica, M., Stampar, F., Veberic, R., & Mikulic-Petkovsek, M. (2016). Processed elderberry (*Sambucus nigra* L.) products: A beneficial or harmful food alternative? *LWT - Food Science and Technology*, *72*, 182–188.

Sidor, A., & Gramza-Michałowska, A. (2015). Advanced research on the antioxidant and health benefit of elderberry (*Sambucus nigra*) in food - a review. *Journal of Functional Foods*, *18*, 941–958.

Sousa, C., Valentão, P., Ferreres, F., Seabra, R. M., & Andrade, P. B. (2008). Tronchuda Cabbage (*Brassica oleracea* L . var . costata DC): scavenger of reactive nitrogen species. *Journal Agricultural and Food Chemistry*, *56*, 4205–4211.

Spínola, V., Llorent-Martínez, E. J., Gouveia, S., & Castilho, P. C. (2014). *Myrica faya*: a new source of antioxidant phytochemicals. *Journal of Agricultural and Food Chemistry*, *62*(40), 9722–35.

Tavares, L., Figueira, I., Macedo, D., McDougall, G. J., Leitão, M. C., Vieira, H. L. A., Santos, C. N. (2012). Neuroprotective effect of blackberry (Rubus sp.) polyphenols is potentiated after simulated gastrointestinal digestion. *Food Chemistry*, *131*(4), 1443–1452.

Topuzović, M. D., Stanković, M. S., Jakovljević, D. Z., & Bojović, B. M. (2016). Plant part variability of *Sambucus ebulus* L. Secondary metabolites content and antioxidant activity. *Agro Food Industry Hi-Tech*, *27*(2), 60–63.

Zheng, W., & Wang, S. Y. (2001). Antioxidant activity and phenolic compounds in

selected herbs. *Journal of Agricultural and Food Chemistry*, *49*(11), 5165–70.

Zhou, N., Zhu, W., Yang, F., & Zhou, K. (2016). *In vitro* gastrointestinal digestion model to monitor the the antioxidant properties and bioavailability of phenolic antioxidants from elderberry. *Reactive Oxygen Species*, *2*(6), 421–431.

Figure captions

Fig. 1. HPLC-ESI/MS^n base peak chromatograms (BPC) of *S. lanceolata* extracts (negative mode).

Fig. 2. HPLC-ESI/MS^n base peak chromatogram (BPC) of *S. lanceolata* berries (positive mode).

Fig. 3. *In vitro* antioxidant activities of *S. lanceolata* extracts measured by different assays (B-ND: berries non-digested extract; B-D: berries digested extract; L-ND: leaves non-digested extract; L-D: leaves digested extract). Different letters refer to statistically significant differences at $p < 0.05$.

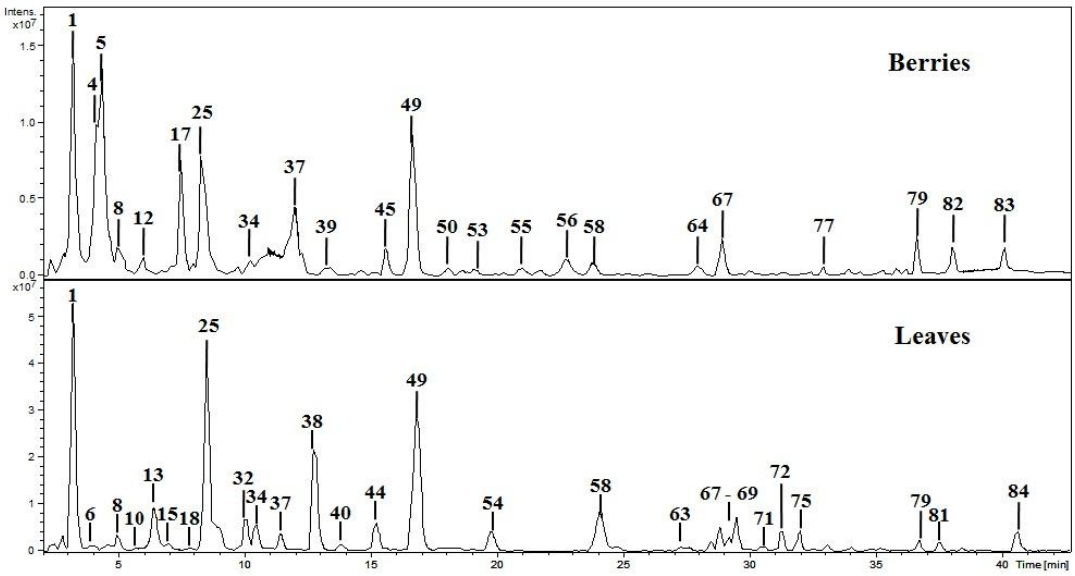

Figure 1

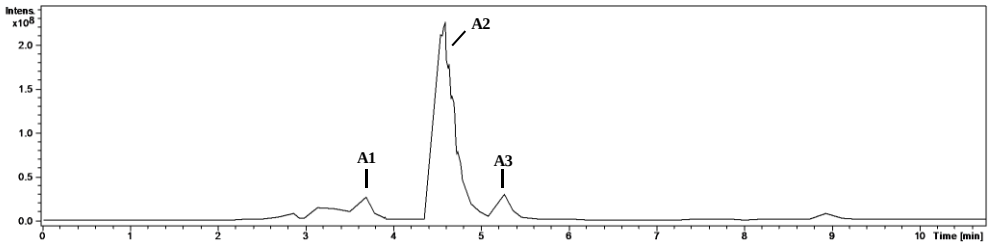

Figure 2

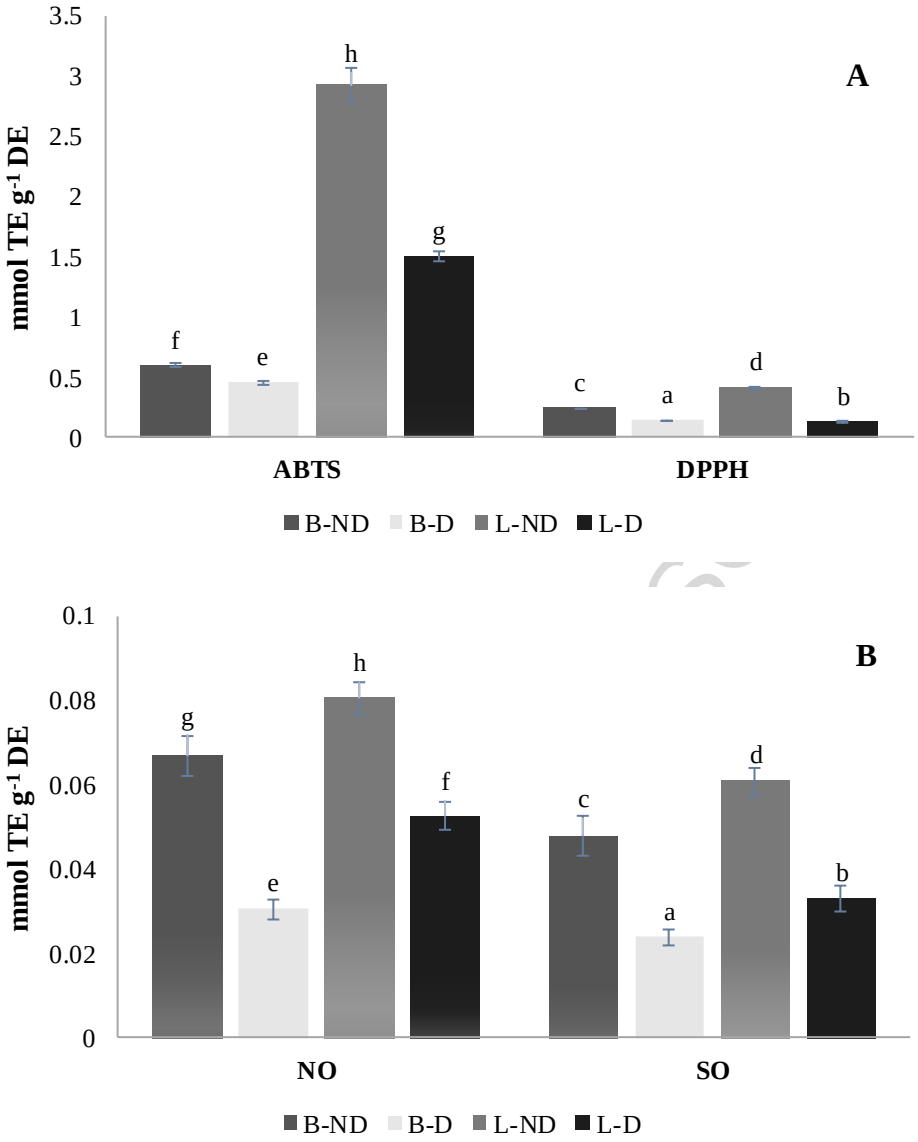

Figure 3

Table 1

Composition of simulated gastrointestinal juices.

Stock solutions[1]	***Saliva***	***Gastric***	***Duodenal***	***Bile***
Distilled water	500 mL	500 mL	500 mL	500 mL
NaCl	58.50 mg	2.75 g	7.03 g	5.27 g
KCl	74.50 mg	0.82 g	0.57 g	0.38 g
$NaHCO_3$	1.06g	-	3.39 g	5.79g
$CaCl_2.H_2O$	-	0.40 g	-	-
NaH_2PO_4	-	0.266 g	-	-
KH_2PO_4	-	-	80.30 mg	-
NH_4Cl	-	0.306 g	-	-
$MgCl_2$	-	-	50.40 mg	-
Urea	0.20 g	0.09 g	0.10 g	0.26 g
Concentrated HCl	-	6.50 mL	0.15 mL	0.15mL
Adjuncts	0.50 g mucin	2.50 g pepsin	9.02 g pancreatin	12.01 g Bile salts
	1.06 g α-amylase	3.00 g mucin	1.50 g lipase	-
pH	6.8 ± 0.2	1.30 ± 0.02	8.1 ± 0.2	8.2 ± 0.2

[1]adapted from Flores *et al*. (2014)

Table 2
Quantification of main polyphenolic compounds present in berries and leaves of *S. lanceolata* (mg g^{-1} DE) before and after *in vitro* digestion.

Nº	[M-H]	Assigned identification	Berries		Leaves	
Anthocyanins			Non-digested	Digested	Non-digested	Digested
A1	581	Cyanidin-*O*-sambubioside	3.16 ± 0.04			
A2	581	Cyandin-*O*-sambubioside	20.13 ± 0.11^{b}	3.10 ± 0.02^{a}		
A3	449	Cyanidin-3-*O*-glucoside	0.85 ± 0.03			
Total			24.13 ± 0.13^{b}	3.10 ± 0.02^{a}		
Hydroxycinnamic acids						
4	353	Caffeoylisocitrate	0.15 ± 0.01^{b}	0.06 ± 0.01^{a}		
8	315	Hydroxytyrosol-*O*-hexoside	0.53 ± 0.02^{b}		1.03 ± 0.01^{c}	0.02 ± 0.01^{a}
10	707	Caffeoylquinic acid dimer			0.08 ± 0.01	
13	707	Dicaffeoylquinic acid-*O*-hexoside			0.43 ± 0.01^{b}	0.17 ±0.01^{a}
15	707	Caffeoylquinic acid dimer	0.04 ± 0.01^{a}		0.53 ± 0.01^{c}	0.14 ± 0.01^{b}
18	341	Caffeic acid-*O*-hexoside			0.31 ± 0.01	
25	353	3-*O*-Caffeoylquinic acid	0.68 ± 0.03^{a}	0.77 ±0.01^{b}	10.62 ± 0.17^{d}	4.91 ± 0.07^{c}
34	353	5-*O*-Caffeoylquinc acid	0.06 ± 0.01^{a}	0.14 ± 0.01^{b}	0.50 ± 0.01^{c}	0.15 ± 0.01^{b}
39	367	5-*O*-Feruloylquinic acid			0.06 ±0.01	
63	815	Coumaric acid derivative			0.28 ± 0.01^{b}	0.23 ± 0.01^{a}
65	523	Caffeic acid derivative			0.14 ± 0.01	
66	653	Coumaric acid derivative			0.20 ± 0.01^{b}	0.14 ± 0.01^{a}

67	699	Coumaric acid derivative			0.23 ± 0.01[b]	0.12 ± 0.01[a]
72	507	Coumaric acid derivative			0.16 ± 0.01[b]	0.08 ± 0.01[a]
75	507	Coumaric acid derivative			0.16 ± 0.01[b]	0.09 ± 0.01[a]
76	537	Ferulic acid derivative			0.06 ± 0.01[b]	0.02 ± 0.01[a]
Total			1.51 ± 0.05[b]	0.98 ± 0.01[a]	14.78 ± 0.11[d]	6.66 ± 0.07[c]
Flavonols						
38	755	Quercetin-*O*-(rhamnosyl)rutinoside	0.10 ± 0.01[a]		3.80 ± 0.04[c]	2.12 ± 0.11[b]
40	679	Myricetin derivative			0.46 ± 0.01[b]	0.25 ± 0.01[a]
43	595	Quercetin-*O*-(pentosyl)hexoside	0.11 ± 0.01[b]	0.07 ± 0.01[a]	0.74 ± 0.01[d]	0.41 ± 0.01[c]
45	595	Quercetin-*O*-(pentosyl)hexoside	0.13 ± 0.01			
49	609	Rutin	0.29 ± 0.01[b]	0.19 ± 0.01[a]	4.42 ± 0.01[c]	1.90 ± 0.01[b]
53	463	Quercetin-*O*-hexoside	0.21 ± 0.01[b]	0.12 ± 0.01[a]		
57	623	Isorhamnetin-*O*-rutinoside	0.08 ± 0.01[b]	0.01 ± 0.01[a]		
58	593	Kaempferol-*O*-rutinoside			1.52 ± 0.03[b]	0.36 ± 0.01[a]
77	615	Isorhamnetin-*O*-pentoside derivative			0.18 ± 0.01	
80	301	Quercetin	0.12 ± 0.01			
Total			0.89 ± 0.01[b]	0.39 ± 0.01[a]	11.12 ± 0.08[d]	3.33 ± 0.01[c]
Flavones						
17	597	Luteolin derivative	0.09 ± 0.01			
35	537	Tricin-*O*-hexoside	0.06 ± 0.01			

Total			0.15 ± 0.01			
Ellagic acid derivatives						
31	627	Ellagic acid-*O*-(pentosyl)hexoside derivative	0.16 ± 0.01			
78	645	Ellagic acid derivative	0.39 ± 0.01			
Total			0.55 ± 0.01			
TIPC			**27.22 ± 0.20^{d}**	**4.97 ± 0.02^{a}**	**25.90 ± 0.19^{c}**	**11.53 ± 0.07^{b}**
TPC[1]			**27.73 ± 1.00^{b}**	**19.35 ± 0.85^{a}**	**90.65 ± 3.11^{c}**	**27.92 ± 0.09^{b}**
TFC[2]			**7.35 ± 0.01^{b}**	**4.55 ± 0.16^{a}**	**40.66 ± 0.09^{d}**	**10.65 ± 0.27^{c}**

[1]determined by the Folin-Ciocalteau method; [2]determined by the aluminium chloride method.
Means in the same column not sharing the same letter are significantly different at $p < 0.05$ probability level.

Highlights:

- The polyphenolic profile of *Sambucus lancelolata* was established for the first time

- The contents of polyphenols were significantly reduced upon the digestion process

- Anthocyanins were the most affected (87.2% reduction) by alkaline conditions

- The extracts of digested berries and leaves still had antioxidant activity

- Consumption of *S. lanceolata* berries and leaves may be health beneficial