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

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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 $mg \cdot g^{-1}$ dry extract, DE) than in leaves (25.9 $mg \cdot g^{-1}$ 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.

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

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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 °C. 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 °C (under reduced light), and the resulting extracts were stored in 5 mL capped flasks at 4 °C 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 °C 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 °C, lyophilized and submitted to extraction as described above (Section 2.2).

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 °C) 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 × 3.0 mm i.d.) using the same conditions reported in (Spínola et al., 2014). Dry extracts (DE) from fruits and

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
$NaHCO_3$	1.06 g	–	3.39 g	5.79 g
$CaCl_2 \cdot H_2O$	–	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	0.15 mL
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).