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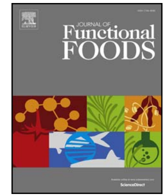

Hypoglycemic, anti-glycation and antioxidant *in vitro* properties of two Vaccinium species from Macaronesia: A relation to their phenolic composition

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ABSTRACT

Vaccinium cylindraceaum (Azores blueberry) and *V. padifolium* (Madeira blueberry) are two Portuguese endemic species, which phytochemical composition and beneficial properties lack scientific validation. In this work, their potential *in vitro* inhibitory effects against key enzymes linked to type 2 diabetes and obesity and glycation of proteins were evaluated. Anthocyanins were predominant in fruits, while hydroxycinnamic acids (in particular 5-*O*-caffeoylquinic acid) and flavonoids were the main compounds in leaves. Vaccinium extracts showed effective inhibitions of glucosidases and glycation of proteins and mild activity towards α-amylase and pancreatic lipase (by comparison with reference compounds). Phenolic composition was strongly correlated with reported bioactivities. In fact, 5-*O*-caffeoylquinic can be considered one of the main hypoglycemic and anti-glycation agents of analysed extracts. Consumption of both Vaccinium species should be encouraged since berries could offer a dietary option in the prevention and control of diabetes and obesity, while leaves are good candidates for development of nutraceuticals.

1. Introduction

Type II diabetes (T2D) is a major health concern worldwide (Sancho & Pastore, 2012; Wang, Camp, & Ehlenfeldt, 2012), characterized by chronic high blood glucose levels (hyperglycaemia). Inhibition of carbohydrate hydrolyzing enzymes is considered one of the effective measures for regulating T2D by reducing glucose availability in the digestion tract (Podsędek, Majewska, Redzynia, Sosnowska, & Koziołkiewicz, 2014; Wang et al., 2012). Currently, this is achieved by the use of approved oral antidiabetic agents (acarbose, miglitol and voglibose) for the management of hyperglycaemia (Boath, Grussu, Stewart, & McDougall, 2012; Sancho & Pastore, 2012). However, these drugs can cause side/adverse effects such as abdominal pain, flatulence, diarrhea and liver toxicity (Salehi, Asghari, Esmaeili, Dehghan, & Ghazi, 2013). As a result, the use of natural products as complementary strategies to existing medications for the management of T2D is growing worldwide (Nickavar & Amin, 2011; Podsędek et al., 2014).

Many studies (Boath, Grussu, et al., 2012; Kang, Racicot, Pilkenton, Kwon, & Apostolidis, 2015; McDougall, Kulkarni, & Stewart, 2008; Nickavar & Amin, 2011; Podsędek et al., 2014; Wang et al., 2012) reported on the impact of berries polyphenols in the management of T2D and obesity, through inhibition of digestive enzymes. Additionally, the intrinsic antioxidant activity of phenolic compounds can prevent the formation of advanced glycation end products (AGEs), responsible for many diabetes complications (cardiovascular disease, retinopathy, cataract development, atherosclerosis, neuropathy, and nephropathy) (Beaulieu et al., 2010; Ferrier et al., 2012; Harris et al., 2014; Liu et al., 2011; Wang, Yagiz, Buran, Nunes, & Gu, 2011). Therefore, polyphenols can offer a complementary dietary approach in prevention and control of diabetic complications.

The Macaronesia region (Azores and Madeira archipelagos, Canary and Cape Verde Islands and a coastal strip of Northwest Africa) (Fig. 1) is home to several endemic berry-producing species, with local/domestic use, but knowledge about their health properties is scarce. *Vaccinium cylindraceum* Sm. and *V. padifolium* Sm. (family Ericaceae), endemic to Azores and Madeira archipelagos (Portugal), respectively, are commonly known as *uveira* or *uva-da-serra* (blueberries) (Cabrita & Andersen, 1999; Lima, Baptista, & Albuquerque, 2009). These wild plants grow as semi-evergreen shrubs or small trees from 3 to 6 m tall. Leaves are oblong to elliptic, usually reddish when new. In the late summer, they grow ovoid-shape berries of blue-black color, when ripe, that are consumed fresh and/or used to make jams and liquors (Lima et al., 2009; Press & Short, 1994). *V. cylindraceum* is relatively common in rural areas (above 300 m of altitude) in all the Azores islands (except

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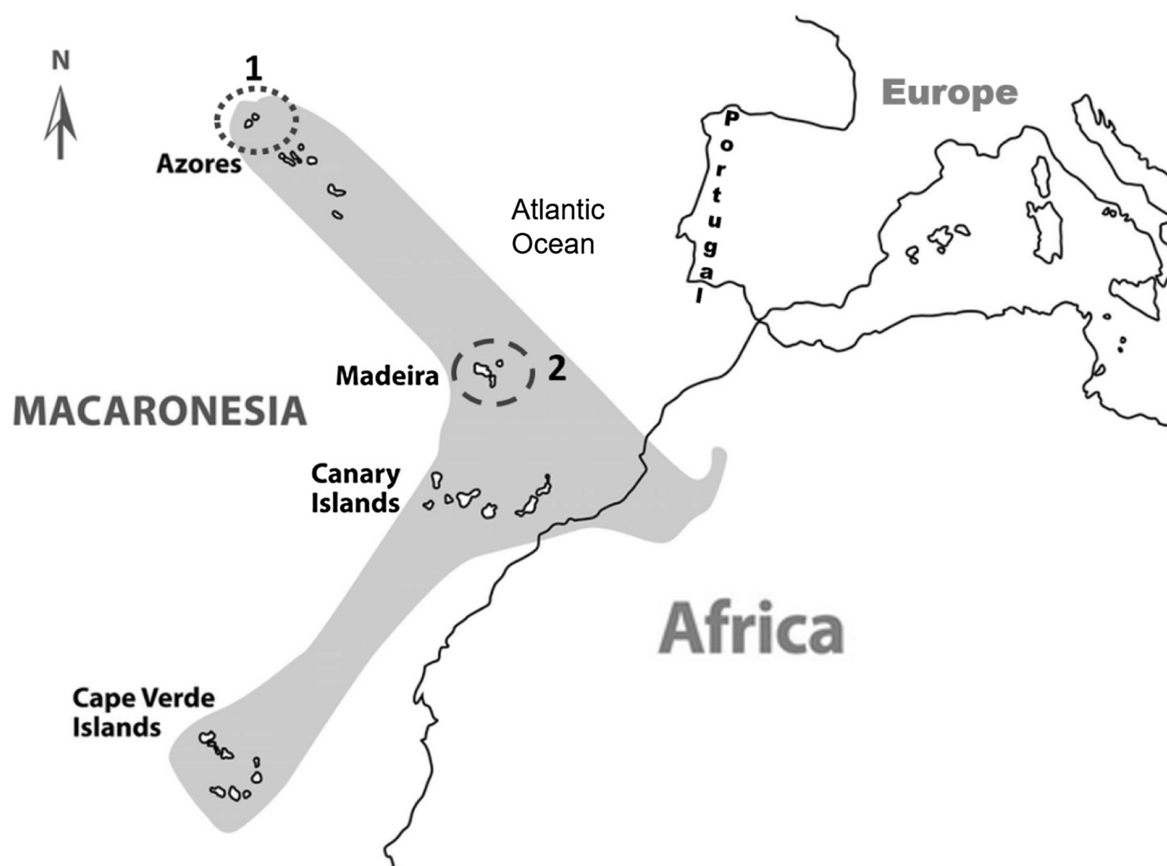

Fig. 1. Macaronesia region, composed of Azores (**1**) and Madeira (**2**) archipelagos (Portugal), Canary Islands, Cape Verde Islands and a coastal strip of Northwest Africa.

Graciosa) (Lima et al., 2009), while *V. padifolium* is present only at high altitudes (> 1300 m) of Madeira Island (Press & Short, 1994). Their occurrence is restricted to their natural habitats, although the presence of specimens in Botanical Gardens (but not extensive cultivation), has been reported. Previous investigations (Cabrita & Andersen, 1999; Cabrita, Frøystein, & Andersen, 2000; Lima et al., 2009) had focused only on the berries' anthocyanins content, and lacked detailed phenolic characterization of fruit and leaves.

Various Vaccinium species (blueberries, lingonberries, bilberries, cranberries, etc.) are reputed to possess beneficial effects in the control of T2D, mainly achieved by *in vitro* inhibition of key digestive enzymes and AGEs formation and antioxidant activity (Beaulieu et al., 2010; Boath, Grussu, et al., 2012; Ferrier et al., 2012; Harris et al., 2014; Kang et al., 2015; Liu et al., 2011; Sarkar et al., 2017; Shi, Loftus, McAinch, & Su, 2017; Wu et al., 2017). In this view, the major aim of this study was to evaluate, for the first time, the anti-diabetic potential of *V. cylindraceum* and *V. padifolium* through inhibition of digestive enzymes linked to T2D and obesity (α,β-glucosidases, α-amylase and lipase) and glycation of bovine serum albumin (BSA) and antioxidant activities Additionally, the composition of methanolic extracts of berries and leaves of both species was determined by HPLC-DAD, improving the knowledge on these species phytochemical profiles. A comparison on the contents and diversity of phenolic compounds and bioactive effects between Macaronesia blueberries species was also established. These new data may contribute to stimulate the use of these plant resources as dietary sources of phytochemicals as well as phyto-medicines and/or health food supplements with anti-diabetic functions.

2. Experimental

2.1. Chemicals and reagents

All reagents and standards were of analytical reagent (AR) grade unless stated otherwise. Folin–Ciocalteu's phenol reagent (FCR), sodium chloride (> 99.0%), potassium chloride (99.5–100.5%), gallic acid (> 98%) and potassium acetate (> 99.5%) were obtained from Panreac (Barcelona, Spain). Ellagic acid (≥96%), 6-hydroxy-2578-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and methanol (99.9%) were obtained 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%), sodium carbonate (100%), *α*-glucosidase from *Saccharomyces cerevisiae* (type I), intestinal acetone powder from rat source of *α*-glucosidase, *β*-glucosidase from almonds, *α*-amylase from porcine pancreas (type VI-B), lipase (type II; from porcine pancreas), *p*-nitrophenyl-α-D-glucopyranoside (*α*-*p*NPG), *p*-nitrophenyl-*β*-D-glucopyranoside (*β*-*p*NPG), *p*-nitrophenyl butyrate (*p*NPB), bovine serum albumin (BSA, ≥98%), D-(−)-ribose (≥99%), aminoguanidine hydrochloride (AMG, ≥98%), conduritol B epoxide (≥95%), acarbose, orlistat and formic acid (98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5-*O*-caffeoylquininc acid (5-*O*-CQA, > 95%) and 1-deoxynojirimycin (1-DNJ; 95–99%) were obtained from Biopurify phytochemicals LTD (Chengdu, China). Nitroblue tetrazolium chloride (NBT, 90%) was obtained from Acros Organics, o-phosphoric acid (85%) from BDH AnalaR and (+)-catechin hydrated (> 99%) from Extrasynthese (Genay, France). Hydrochloric acid (37%), aluminium chloride (98%) and quercetin dihydrate (> 99%) from Riedel-de Haen (Hanover, Germany) and acetic acid

Table 1
Sample information, collection area, dates and voucher numbers. For a better visualization of collection sites, check Fig. 1.

Sample	Collection area	Date	Voucher
V. cylindraceum (Azores blueberry)	(1) Flores Island, Azores Archipelago (39°26′26″N, 31°13′9″W)	August 2014	MADJ 13281
V. padifolium (Madeira blueberry)	(2) Madeira Island, Madeira Archipelago (32°42′50.72″N, 16°55′55.07″W)	September 2014	MADJ 13283

glacial purchased from Fischer Scientific (Bishop Meadow, UK). Soluble starch (p.a.), D-(−)-fructose, potassium iodate (99.5%), sodium nitroprusside (99%), ethylenediaminetetraacetic acid (EDTA, > 99%) and sodium azide (> 99%) were obtained from Merck (Darmstadt, Germany). LC–MS grade acetonitrile (CH_3CN, 99%) (LabScan; Dublin, Ireland) ultrapure water (Milli-Q Waters purification system; 18 M Ω cm at 23 °C; Millipore; Milford, MA, USA) were used for the HPLC-MS analysis.

2.2. Sample preparation and extraction of phenolic compounds

Samples of *V. cylindraceum* and *V. padifolium* were collected in the wild in different archipelagos of Portugal (Azores and Madeira, respectively), between August and September of 2014 (Table 1). In the case of *V. cylindraceum,* plant material was send by air transportation to Madeira Island, the day after collection, in styrofoam boxes over dry ice and delivered to Centro de Química da Madeira (CQM), within 3 days after harvest. *V. padifolium* was collected, placed in adequate refrigerated transporters and freeze-dried in the same day as collection. Plants were authenticated by specialists of Madeira Botanical Garden, Herbarium (Madeira, Funchal) where vouchers have been deposited (Table 1).

For analysis, plant material was separated into leaves and berries (fully ripe), destemmed, and washed. In the case of *V. padifolium*, leaves were divided in two groups: young leaves (red colored tips) and mature leaves (fully green). This was not verified for *V. cylindraceum* species. Samples were lyophilized (Alpha 1–2 LD plus freeze dryer, CHRIST), ground to powder in a mechanic grinder, and stored at −20 °C. For phenolic extraction, 1 g of dry material was mixed with 25 mL of methanol in an ultra-sonic bath (Bandelin Sonorex, Germany) at 35 kHz and 200 W for 60 min (room temperature). For berries, an extraction solution composed of MeOH/H_2O (acidulated with 7% acetic acid) (80:20, v/v) 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. The extraction procedure was conducted in duplicate for all samples and the resulting extracts were stored at 4 °C until further analysis.

In the case of leaves, an additional step was used for removal of chlorophylls. After the first filtration step, a small amount of activated charcoal was added to the liquid extract and, after mixing for a few seconds, the solution was filtered. Then, it was concentrated to dryness and stored as aforementioned.

The total soluble solids (TSS) were determined in fresh pulp from berries using a digital Atago RX-1000 refractometer. The TSS content varied between 11.7 and 12.1 °Brix, which are within the range reported previously for other Vaccinium berries (6–15.5 °Brix) (Garzón, Narváez, Riedl, & Schwartz, 2010; Jaakola, Määttä-Riihinen, Kärenlampi, & Hohtola, 2004).

2.3. Chromatography and Mass spectrometry conditions

HPLC analysis was performed on a Dionex ultimate 3000 series instrument (Thermo Scientific Inc., California, USA) equipped with a binary pump, an autosampler, a column compartment (kept at 30 °C) and a diode array detector (DAD) coupled with a Bruker Esquire model 6000 ion trap mass spectrometer (Bremen, Germany). The experimental conditions were the same as previously reported (Spínola, Llorent-Martínez, Gouveia, & Castilho, 2014), which details were as follows: Phenomenex Gemini C_{18} chromatographic column (5 µm, 250 × 3.0 mm i.d.); mobile phase: A: acidified water (0.1% formic acid, v/v), B: CH_3CN; flow rate: 0.4 mL min^{-1}; gradient elution 20% B (0 in), 25% B (10–20 min), 50% B (40 min), 100% B (42–47 min), 20% A (49–55 min); injection volume: 5 µL; column temperature: 30 °C.

Mass spectrometry conditions: dry and nebulizer gas (N_2): flow 10 mL min^{-1} and pressure 50 psi; nebulizer temperature and temperature: 365 °C and +4500 V, respectively; scanning range: *m/z* 100–1000. For analysis, dry extracts (DE) were re-dissolved in the initial HPLC mobile phase (H_2O:CH_3CN; 80:20), filtered through 0.45 µm PTFE membrane filters, and injected in the HPLC equipment.

2.4. Quantification of main polyphenols

For this quantitative analysis, one polyphenol was selected as the standard for each group, and was used to calculate relative individual concentrations by HPLC-DAD (Spínola et al., 2014). Caffeic and 5-*O*-caffeoylquinic acids were used for hydroxycinnamic and caffeoylquinic acids determination, respectively. Anthocyanins standard was cyanidin 3-*O*-glucoside. Quercetin, (+)-catechin, hesperidin, apigenin and ellagic acid were the standards used for the flavonols, flavanols, flavanones, flavones and ellagitannins, respectively. Stock standard solutions (1000 mg L^{-1}) were prepared in methanol and six different concentrations (5–100 mg L^{-1}) were prepared for the calibration curves, plotting peak area versus concentration ($R^2 \geq 0.990$ in all cases). Total individual phenolic content (TIPC) was defined as the sum of the relative amounts of compounds in extracts.

2.5. In vitro anti-diabetic assays

2.5.1. Yeast α-glucosidase inhibition assay

This assay was adapted from a previous report (Podsędek et al., 2014). In a 96-well plate, 50 µL of sample extract (sequential dilutions) was combined with 50 µL of enzyme solution (0.1 mg mL^{-1}) and 50 µL of 5 mmol L^{-1} *α-p*NPG solution. All solutions were prepared in 0.1 M phosphate buffer (pH 6.9). The mixture was incubated at 37 °C for 20 min in the dark. Finally, 100 µL of 0.1 M Na_2CO_3 solution was added and the absorbance was read at 405 nm (Victor^3 microtiter reader, Perkin-Elmer, Germany). Acarbose and 1-DNJ were used as positive controls and the IC_{50} values (mg mL^{-1} DE) were determined from the least-squares regression line of the logarithmic concentrations plotted against percentage inhibition.

2.5.2. Rat α-Glucosidase inhibition assay

For this assay, 0.5 g of intestinal acetone powder from rat was dissolved in 10 mL of 0.1 M phosphate buffer (pH 6.9) and sonicated for 10 min. After centrifugation (Sigma 3K30) at 1753*g* for 10 min at 4 °C, the resulting supernatant was diluted 5 times with above buffer and was used as the enzyme solution. The measurement of enzyme inhibition was performed as described above (Section 2.5.1).

2.5.3. β-Glucosidase inhibition assay

The assay for β-glucosidase was carried out as above (Section 2.5.1) using *β-p*NPG as substrate. Conduritol B epoxide and 1-DNJ were used as positive controls.

2.5.4. α-Amylase inhibition assay

The assay was performed as described before (Podsędek et al., 2014), with slight modifications (Spínola, Llorent-Martínez, Gouveia-Figueira, & Castilho, 2016): 20 µL of sample extract (serial dilutions) and 40 µL of 2 g L^{-1} starch solution were mixed with 20 µL of α-

amylase (0.1 mg mL^{-1}). All solutions were prepared in 0.1 M phosphate buffer (pH 6.9). After incubation (20 min; 37 °C), the reaction was stopped by the addition of 80 µL of 0.4 M HCl followed by 100 µL of 5 mmol L^{-1} I_2 (in 5 mmol L^{-1} KI) and the absorbance was read at 620 nm (Victor^3 microtiter reader, Perkin-Elmer). Acarbose was used as positive control.

2.5.5. Lipase inhibition assay

The method for measuring lipase activity was adapted (Kim et al., 2010): 40 µL of sample extract (serial dilutions) was mixed with 20 µL of substrate solution (10 mM of *p*-NPB in ethanol) and 40 µL of the enzyme (2.5 mg mL^{-1} in 0.1 M phosphate buffer, pH 8.0). After incubation (20 min; 37 °C) absorbance was read at 405 nm (Victor^3 microtiter reader, Perkin-Elmer). Orlistat was used as positive control.

2.5.6. BSA glycation assay

Formation of AGEs was measured in 96 black well-plates, with slight modifications from a published protocol (Séro et al., 2013). Each well contained 50 µL of BSA solution (10 mg mL^{-1}), 80 µL of 0.1 M phosphate buffer (containing sodium azide, 3 mM, pH 7.4), 50 µL of ribose or fructose solution (0.5 M) and 20 µL of sample extracts (serial dilutions). Plates were incubated for 24 h at 37 °C and were analysed at an excitation wavelength of 355 nm and emission wavelength of 460 nm (Victor^3 microtiter reader, Perkin–Elmer). AMG and quercetin were used as positive controls and the anti-glycation activities were expressed as IC_{50} value (mg mL^{-1} DE) as described before (Section 2.5.1).

2.6. Total phenolic and flavonoid contents and in vitro antioxidant assays

2.6.1. Total phenolic content (TPC)

TPC determinations followed a previous protocol (Zheng & Wang, 2001), with some modifications (Spínola et al., 2016): 50 µL of sample extract (5 mg mL^{-1} in MeoH) were mixed with 1.25 mL of FCR (diluted 1:10 with water) and 1 mL of 7.5% Na_2CO_3 solution. After 30 min in the dark at room temperature, the absorbance was measured at 765 nm in a Perkin Elmer UV–Vis Lambda 2 spectrophotometer (Oberlingen, Germany). The amounts of total phenolics were expressed as mg of gallic acid equivalents (GAE) g^{-1} DE, based on the gallic acid calibration curve.

2.6.2. Total flavonoid content (TFC)

TFC was measured based on previous procedure (Akkol, Göger, Koşar, & Başer, 2008) with some adjustments (Spínola et al., 2016): 0.5 mL of sample solution (2.5 mg mL^{-1} in MeOH) was mixed with 1.5 mL of methanol, 2.8 mL of distilled water, 0.1 mL of CH_3COOK (1 mol L^{-1}), and 0.1 mL of $AlCl_3 \cdot 6H_2O$ (10% in MeOH). The absorbance was measured at 415 nm (UV–Vis Lambda 2, Perkin Elmer) after 30 min of reaction (room temperature, in the dark). The final results were expressed as mg of rutin equivalent (RUE) g^{-1} DE, based on the rutin calibration curve.

2.6.3. ABTS radical scavenging activity

Determination of antioxidant activity was adapted from previous works (Dudonné, Vitrac, Coutière, Woillez, & Mérillon, 2009): 40 µL of sample solution (5 mg mL^{-1} in MeOH) was added to 1.96 mL of $ABTS^{\cdot +}$ solution (diluted in PBS pH 7.4 until the absorbance is 0.700 ± 0.021 at 734 nm). The reduction of absorbance at 734 nm (UV–Vis Lambda 2, Perkin Elmer) was measured during 6 min, and the results were expressed as µmol of Trolox equivalent (TE) g^{-1} DE, based on the Trolox calibration curve.

2.6.4. DPPH radical scavenging activity

The DPPH assay followed a previously reported method (Dudonné et al., 2009), with slight adjustments (Spínola et al., 2016): 100 µL of sample solution (5 mg mL^{-1} in MeOH) was added to 3.5 mL of DPPH radical solution (0.06 mmol L^{-1} in MeOH). Absorbance was measured at 516 nm (UV–Vis Lambda 2, Perkin Elmer), after 30 min of reaction in the dark (room temperature) and results were expressed as µmol TE g^{-1} of DE.

2.6.5. Nitric oxide (NO) scavenging activity

Fifty microliters of 20 mM sodium nitroprusside was mixed with 50 µL of sample extract (5 mg mL^{-1}) for 60 min, at room temperature (under light) based on a previous work (Sousa, Valentão, Ferreres, Seabra, & Andrade, 2008). All solutions were prepared in 0.1 M phosphate buffer (pH 7.4). After incubation, 50 µL of Griess reagent (1% sulfanilamide and 0.1% NEDA in 2% phosphoric acid) was added. Then, the absorbance was read at 550 nm (Victor^3 microtiter reader; Perkin–Elmer) and the results were expressed as µmol TE g^{-1} DE.

2.6.6. Superoxide radical (SO) scavenging activity

Twenty-five microliters of sample extract (5 mg mL^{-1}) was mixed with 200 µL 0.1 mmol L^{-1} EDTA, 62 µmol L^{-1} NBT and 98 µmol L^{-1} NADH solution (Ewing & Janero, 1995). The reaction was initiated with the addition of 25 µL of 33 µmol L^{-1} PMS (containing 0.1 mM EDTA) to each well. All solutions were prepared in 0.1 M phosphate buffer (pH 7.4). The absorbance was read at 550 nm (Victor^3 microtiter reader; Perkin-Elmer) and the results were expressed as µmol TE g^{-1} DE.

2.7. Statistical analysis

All samples were assayed in triplicate and results are given as the means ± standard deviations. Data was analysed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using SPSS for Windows, IBM SPSS Statistics 20 (SPSS, Inc., USA). A value of $p < .05$ was considered statistically significant. Pearson correlation coefficients (r) were determined to corroborate relationships between selected parameters. Principal Component Analysis (PCA) was applied to the concentrations of polyphenols determined in different morphological parts of *Vaccinium* species.

3. Results and discussion

Identification of phytochemicals in analysed Vaccinium species was achieved based on spectrum data, authentic standards (when available) and literature (Tables S1 and S2 – Supplementary Material).

In case of *V. cylindraceum*, 81 phytochemicals were tentatively identified, with 75 in berries and 80 in leaves extracts (Tables S1 and S2 – Supplementary Material): 64 polyphenols and 17 non-phenolic compounds (oligosaccharides, organic acids, terpenoids, coumarins). Anthocyanins, ellagic acid and tricin derivatives were only detected in berries.

Similar phenolic profiles were found in berries and leaves of *V. padifolium*. In total, 100 phytochemicals were identified, with 76 in berries and 78 in leaves (young and mature) extracts (Tables S1 and S2 – Supplementary Material): 91 polyphenols and 9 other compounds (oligosaccharides and organic acids). In this case, anthocyanins were detected berries and young leaves, while ellagitannins were present only in berries.

3.1. Quantification of polyphenols

In the present study, the amounts of 66 polyphenols were determined *via* HPLC-DAD using the corresponding relative standards (Table 2). Only the main compounds were quantified, since the low amounts of other minor components did not allow an accurate quantification.

Quantitative results indicated that anthocyanins were the most abundant compounds in berries (78–81% of TIPC); other relevant families were hydroxycinnamic acids (HCAs) (9–13%), ellagic acid derivatives (2.39–5.50%), flavonols (3.20–3.81%) and flavanols

Table 2
Quantification of main polyphenolic compounds present in berries and leaves of targeted Vaccinium species (mg g^{-1} DE).

No.	Assigned identification	Berries		Leaves		
		VC	VP	VC	VP-YL	VP-ML
Anthocyanins						
3	Delphinidin-*O*-(pentosyl)hexoside	0.82 ± 0.03^{a}	1.88 ± 0.02^{b}			
5	Delphinidin-*O*-hexoside	7.88 ± 0.03^{a}	13.77 ± 0.20^{b}			
10	Cyanidin-*O*-glucoside				6.65 ± 0.20	
17	Delphinidin-*O*-hexoside	26.81 ± 0.96^{a}	29.40 ± 0.22^{b}			
22	Petunidin-*O*-hexoside	15.25 ± 0.11^{b}	10.02 ± 0.40^{a}			
25	Cyanidin-*O*-pentoside				3.58 ± 0.16	
31	Delphinidin-*O*-rhamnoside	2.14 ± 0.09				
35	Malvidin-*O*-hexoside		1.36 ± 0.03			
38	Peonidin-*O*-hexoside	2.04 ± 0.04^{a}	3.62 ± 0.11^{b}			
49	Malvidin-*O*-hexoside	10.57 ± 0.07^{b}	9.49 ± 0.28^{a}			
53	Malvidin-*O*-pentoside	2.13 ± 0.09^{b}	1.08 ± 0.04^{a}			
64	Malvidin-*O*-rhamnoside	0.63 ± 0.01^{b}	0.13 ± 0.01^{a}			
Total		68.27 ± 1.15^{b}	70.73 ± 1.01^{c}		10.23 ± 0.32^{a}	
Hydroxycinnamic acids						
10	Caffeoylisocitrate	0.35 ± 0.01				
18	Caffeoylquinic acid derivative				0.33 ± 0.01^{b}	0.29 ± 0.01^{a}
34	3-*O*-Caffeoylquinic acid dimer				0.65 ± 0.02^{b}	0.22 ± 0.01^{a}
55	Caffeoylquinic acid derivative				0.39 ± 0.01^{a}	0.38 ± 0.01^{a}
57	Caffeoylquinic acid derivative				0.48 ± 0.01^{a}	0.27 ± 0.01^{b}
58	Caffeic acid-*O*-hexoside	0.56 ± 0.04^{c}	0.40 ± 0.02^{a}	0.49 ± 0.02^{b}	0.60 ± 0.02^{d}	0.70 ± 0.02^{e}
68	5-*O*-Caffeoylquinic acid	5.57 ± 0.05^{a}	8.88 ± 0.10^{b}	33.11 ± 1.30^{c}	66.05 ± 0.52^{d}	87.27 ± 1.11^{e}
78	Caffeoylquinic acid	0.15 ± 0.02^{a}	0.22 ± 0.03^{b}	1.03 ± 0.04^{c}	2.64 ± 0.05^{e}	2.00 ± 0.01^{d}
80	Caffeoylshikimic acid	0.26 ± 0.01				
87	5-*p*-Coumaroylquinic acid		0.28 ± 0.003^{a}	0.55 ± 0.01^{b}	1.62 ± 0.04^{c}	1.58 ± 0.02^{c}
93	Caffeoylquinic acid derivative				0.33 ± 0.01^{b}	0.24 ± 0.04^{a}
95	5-*p*-Coumaroylquinic acid			0.78 ± 0.02		
100	Caffeoylquinic acid derivative				0.77 ± 0.02^{b}	0.25 ± 0.01^{a}
104	Methyl-5-*O*-caffeoylquinic acid	0.27 ± 0.04^{c}	0.24 ± 0.01b^{c}	0.19 ± 0.02^{a}	0.78 ± 0.02^{d}	0.96 ± 0.02^{e}
106	Coumaroyl iridoid	0.31 ± 0.01^{a}	0.34 ± 0.01^{a}	0.49 ± 0.01^{b}	1.06 ± 0.02^{d}	0.75 ± 0.01^{c}
107	Coumaric acid derivative				0.86 ± 0.01^{a}	1.62 ± 0.02^{b}
111	Coumaroyl iridoid	0.52 ± 0.02^{a}		0.55 ± 0.01^{a}		
126	Coumaric acid derivative			0.11 ± 0.01		
129	4-Benzoyl-9-*p*-coumaroyl-DOA		0.23 ± 0.01^{a}		1.05 ± 0.02^{b}	1.44 ± 0.03^{c}
135	Coumaric acid derivative		0.93 ± 0.02^{a}		13.02 ± 0.24^{b}	13.48 ± 0.06^{c}
142	Coumaric acid derivative		0.13 ± 0.01^{a}		1.18 ± 0.05^{b}	1.40 ± 0.01^{c}
159	Coumaric acid derivative				0.04 ± 0.01^{a}	0.08 ± 0.01^{b}
Total		7.99 ± 0.11^{a}	11.64 ± 0.14^{b}	38.10 ± 1.15^{c}	91.85 ± 0.86^{d}	112.91 ± 1.08^{e}
Hydroxybenzoic acids						
40	Protocatechuic acid-*O*-hexoside	0.80 ± 0.02^{b}		0.60 ± 0.01^{a}		
56	Protocatechuic acid derivative			1.15 ± 0.02		
Total		0.80 ± 0.02^{a}		1.74 ± 0.03^{b}		
Flavonols						
102	Myricetin-*O*-hexoside	0.19 ± 0.03^{a}	0.29 ± 0.01^{b}			
109	Rutin				0.71 ± 0.04^{a}	0.81 ± 0.06^{b}
115	Cinchonain Ib			0.24 ± 0.01		
117	Quercetin-*O*-hexoside			4.93 ± 0.11^{a}	17.98 ± 0.63^{c}	10.68 ± 0.01^{b}
118	Myricetin-*O*-deoxyhexoside	0.41 ± 0.01^{a}	0.58 ± 0.01^{b}			
119	Quercetin-*O*-hexoside	0.23 ± 0.01^{a}	0.88 ± 0.02^{b}	9.06 ± 0.30^{e}	7.26 ± 0.20^{d}	6.51 ± 0.16^{c}
125	Quercetin-*O*-pentoside				0.65 ± 0.02^{a}	0.63 ± 0.02^{a}
128	Cinchonain-Ib			1.38 ± 0.03		
131	Cinchonain-Ib			1.24 ± 0.05^{c}	0.54 ± 0.02^{b}	0.46 ± 0.01^{a}
133	Quercetin-*O*-(acetyl)hexoside					0.15 ± 0.01
134	Kaempferol-*O*-hexoside			11.65 ± 0.24		
139	6-Hydroxy-7,4-dimethoxy-quercetin-*O*-hexoside	0.14 ± 0.01				
140	Isorhamentin-*O*-hexoside	0.14 ± 0.01				
144	Quercetin-*O*-(acetyl)hexoside	0.11 ± 0.01^{a}		0.16 ± 0.02^{b}		
148	Kaempferol-*O*-(acetyl)hexoside				2.96 ± 0.05^{b}	2.57 ± 0.02^{a}
153	Cinchonain-Ib			4.40 ± 0.04^{c}	0.65 ± 0.01^{b}	0.60 ± 0.02^{a}
156	6-Hydroxy-7,4-dimethoxy-quercetin-*O*-(acetyl)hexoside		0.20 ± 0.01			
158	Kaempferol-*O*-(acetyl)hexoside			0.21 ± 0.01^{a}	0.26 ± 0.01^{b}	0.46 ± 0.02^{c}
160	6-Hydroxy-7,4-dimethoxy-quercetin derivative	0.39 ± 0.01^{b}	0.38 ± 0.01^{b}	0.34 ± 0.01^{a}		
Total		3.21 ± 0.04^{b}	2.90 ± 0.05^{a}	34.75 ± 0.74^{e}	31.39 ± 0.78^{d}	23.14 ± 0.27^{c}
Flavanols						
50	Procyanidin dimer (B type)			3.28 ± 0.08		
60	Procyanidin dimer (B type)			1.92 ± 0.03		
75	Procyanidin dimer (B type)	1.22 ± 0.03^{a}		11.31 ± 0.38^{b}		
79	Catechin	0.83 ± 0.04^{a}		8.14 ± 0.21^{d}	2.89 ± 0.07^{c}	1.66 ± 0.06^{b}

(*continued on next page*)

Table 2 (*continued*)

No.	Assigned identification	Berries		Leaves		
		VC	VP	VC	VP-YL	VP-ML
84	Procyanidin trimer (B type)	0.57 ± 0.02[a]		4.08 ± 0.04[c]	1.99 ± 0.06[b]	1.99 ± 0.03[b]
124	Procyanidin dimer (A type)		0.45 ± 0.01			
Total		2.62 ± 0.07[b]	0.45 ± 0.01[a]	28.73 ± 0.61[e]	4.88 ± 0.105[d]	3.65 ± 0.07[c]
Flavanones						
145	Phlorizin			1.02 ± 0.04		
Total				1.02 ± 0.04		
Ellagic acid derivatives						
24	Ellagic acid derivative		2.012 ± 0.09			
26	Tergallagic acid	1.15 ± 0.06				
39	Methyl-ellagic acid derivative		2.85 ± 0.13			
47	Methyl-ellagic acid-*O*-pentoside	0.86 ± 0.01[b]	0.10 ± 0.01[a]			
Total		2.01 ± 0.06[a]	4.96 ± 0.18[b]			
TIPC		84.13 ± 1.44[a]	90.68 ± 1.74[b]	103.37 ± 2.55[c]	134.42 ± 2.06[d]	129.70 ± 1.41[d]
TPC[1]		76.63 ± 1.30[a]	88.58 ± 4.01[b]	186.98 ± 2.64[c]	341.25 ± 2.27[e]	297.70 ± 9.07[d]
TFC[2]		11.49 ± 0.08[a]	14.67 ± 0.19[b]	35.41 ± 0.20[c]	127.91 ± 2.16[e]	94.09 ± 0.64[d]

[1] Determined by the Folin-Ciocalteu method (mg GAE g^{-1} DE).

[2] Determined by the aluminium chloride method (mg RUE g^{-1} DE). VC: *V. cylindraceum*; VP: *V. padifolium*; YL: young leaves; ML: mature leaves); DOA: 2,7-anhydro-3-deoxy-2-octulopyranosonic acid. Means in the same line not sharing the same letter are significantly different at $p < .05$ probability level.

(0.50–3.11%). Delphinidin-*O*-hexoside (**17**) was main compound in berries (31.80–32.42%), followed by petunidin-*O*-hexoside (**22**) (18.09%) and malvidin-*O*-hexoside (**49**) (12.54%) in *V. cylindraceum*; a different delphinidin-*O*-hexoside (**5**) (15.18%), petunidin-*O*-hexoside (**22**) (11.05%) and malvidin-*O*-hexoside (**49**) (10.46%) were quantified in *V. padifolium*. In leaves, HCAs were dominant (36.85–87.05%), followed by flavonols (17.84–33.62%) and flavanols (2.80–27.79%). Anthocyanins (7.61%) were only present in *V. padifolium* (young leaves), while flavanones and hydroxybenzoic acids were quantifiable in *V. cylindraceum* (0.99% and 1.68%, respectively). Leaves were rich sources of 5-*O*-caffeoylquinic acid (5-*O*-CQA) (**68**) (37.98–67.73%) (Table 2). Kaempferol-*O*-hexoside (**134**) (11.27%), B-type procyanidin dimer (**75**) (10.94%), quercetin-*O*-hexoside (**119**) (8.76%) and catechin (**79**) (7.81%) were also relevant in *V. cylindraceum*; quercetin-*O*-hexoside (**117**) (8.23–13.38%), coumaric acid derivative (135) (9.69–10.39%) and quercetin-*O*-hexoside (**119**) (5.02–5.40%) in *V. padifolium*. Cyanidin-3-glucoside (C3G) content was also significant in *V. padifolium* young leaves (4.95%).

Inter-species variations were found in this study, which are due to genetic factors (species/cultivar) and to edaphoclimatic conditions (soil, sun exposure, rainfall, etc.) of different growing locations (Sarkar et al., 2017; Shi et al., 2017). In general, *V. padifolium* extracts showed richer phenolic contents than *V. cylindraceum* ($p < .05$). Exceptions were observed in flavonols and flavanols contents. In the latter group, amounts were 6 to 8-times higher than in *V. padifolium* (Table 2). HCAs content was superior in *V. padifolium*, mostly due to the higher 5-*O*-CQA amounts (almost 3 times-fold higher).

Previously (Cabrita & Andersen, 1999; Cabrita et al., 2000; Lima et al., 2009), malvidin-3-*O*-glucoside, delphinidin-3-*O*-galactoside and petunidin-3-*O*-glucoside were described, as main anthocyanins of analysed *Vaccinium* species. This was not the case in the present study, which could be due to differences in sample preparation methodologies and collection year. By contrast, cyanidin glycosides were the main polyphenols found in *V. vitis-idea* and *V. meridionale* Swartz berries (Beaulieu et al., 2010; Garzón et al., 2010). Quercetin and kaempferol glycosides are reported as the most abundant flavonoids in Vaccinium berries (Chen, Xin, Yuan, Su, & Liu, 2014; Jaakola et al., 2004; Martineau et al., 2006; Vrhovsek, Masuero, Palmieri, & Mattivi, 2012), which agrees with obtained data (Table 2). Comparable flavonols values were reported in *V. myrtillus* leaves (Jaakola et al., 2004; Martineau et al., 2006).

Higher content of phenolic compounds in the leaves were also noted for other Vaccinium species (Bujor, Le Bourvellec, Volf, Popa, & Dufour, 2016; Jaakola et al., 2004; Oszmiański, Wojdyło, Lachowicz, Gorzelany, & Matłok, 2016; Teleszko & Wojdyło, 2015). 5-*O*-CQA was also main compound in *V. myrtillus* and *V. angustifolium* leaves (Bujor et al., 2016; Martineau et al., 2006), with relative higher contents in the latter species (104 mg g^{-1} DE). Similarly to *V. padifolium*, coumaroyl derivatives were very common in *V. macrocarpon* leaves (Oszmiański et al., 2016). Another interesting issue is the content of anthocyanins in *V. padifolium* (young leaves), which was less diverse (only cyanidin glycosides) and in considerably lower concentrations ($p < .05$) than in fruits. The designation of “young leaves” refers to those recently developed on the extremities of twigs. Cyanidin glycosides were also reported in *V. myrtillus* (Jaakola et al., 2004) and *V. macrocarpon* (Oszmiański et al., 2016) leaves, although, higher concentrations were found in the latter species (22.38–28.26 mg g^{-1} DE). The presence of anthocyanins in *V. padifolium* (young leaves) could be attributed to the higher sun exposure, which activates polyphenols production (Jaakola et al., 2004). C3G and quercetin glycosides are known to play simultaneously a predominant role in defense against high solar radiation in Vaccinium leaves (Jaakola et al., 2004). Intra-species differences ($p < .05$) were also observed in *V. padifolium* leaves. Anthocyanins were present only in young leaves not in mature ones). Both HCAs and anthocyanins can act as antioxidants/radiation protectors. It seems that the biosynthesis of anthocyanins is faster and happens first to protect leaves from radiation (Jaakola et al., 2004). HCAs take longer to be produced and when sufficient amounts are reached, anthocyanin production stops.

TIPC of analysed Vaccinium species varied from 84.13 to 134.42 mg g^{-1} DE (Table 2). Previously (Teleszko & Wojdyło, 2015), lower TIPC were documented for berries and leaves extracts of *V. macrocarpon* and *V. myrtillus* (16.24–18.83 and 68.84–110.95 mg g^{-1} DE, respectively). By contrast, superior amounts were determined in *V. macrocarpon* extracts (91.11–137.44 and 215.50–388.94 mg g^{-1} DE for berries and leaves, respectively) (Oszmiański et al., 2016). TIPC of analysed leaves were within the range reported for *V. angustifolium* and *V. myrtillus* (Bujor et al., 2016; Martineau et al., 2006) (97.4–185.18 mg g^{-1} DE), while berries had lower total phenolic and anthocyanins contents (4.59–41.1 and 22.3–34.5 mg g^{-1} DE, respectively).

TPC and TFC, measured by colorimetric assays (Table 2), showed

Table 3
In vitro inhibitory activities (IC_{50}, mg mL^{-1}) of *Vaccinium* extracts towards digestive enzymes and glycation of BSA.

	α-Glucosidase		β-Glucosidase	α-Amylase	Lipase	BSA Glycation	
	Yeast	Rat				Ribose	Fructose
Berries							
VC	1.10 ± 0.04[e]	4.36 ± 0.14[g]	9.88 ± 0.40[f]	2.78 ± 0.04[f]	6.50 ± 0.34[e]	2.35 ± 0.15[e]	1.53 ± 0.11[d]
VP	1.03 ± 0.03[e]	4.13 ± 0.16[g]	9.36 ± 0.09[f]	2.63 ± 0.09[f]	5.97 ± 0.38[e]	2.09 ± 0.14[e]	1.35 ± 0.06[d]
Leaves							
VC	0.85 ± 0.03[d]	1.64 ± 0.06[f]	4.59 ± 0.40[d]	1.05 ± 0.03[b]	4.20 ± 0.27[d]	1.21 ± 0.02[d]	0.60 ± 0.01[c]
VP-YL	0.58 ± 0.01[b]	0.82 ± 0.04[d]	2.33 ± 0.19[c]	1.75 ± 0.06[d]	2.18 ± 0.09[c]	0.75 ± 0.04[b]	0.41 ± 0.01[b]
VP-ML	0.76 ± 0.02[c]	0.86 ± 0.02[e]	2.60 ± 0.09[c]	2.03 ± 0.04[e]	3.56 ± 0.31[d]	0.88 ± 0.03[c]	0.49 ± 0.01[b]
5-*O*-CQA	0.36 ± 0.02[a]	0.27 ± 0.02[c]	0.39 ± 0.01[a]	1.20 ± 0.04[c]	0.31 ± 0.01[a]	0.14 ± 0.01[a]	0.20 ± 0.01[a]
Acarbose	2.06 ± 0.04[f]	0.12 ± 0.01[b]	–	0.02 ± 0.001[a]	–	–	–
1-DNJ	0.65 ± 0.02[b]	0.01 ± 0.01[a]	0.45 ± 0.02[b]	–	–	–	–
Conduritol B epoxide	–	–	8.94 ± 0.19[e]	–	–	–	–
Orlistat	–	–	–	–	0.47 ± 0.02[b]	–	–
Aminoguanidine	–	–	–	–	–	9.56 ± 0.36[e]	2.29 ± 0.13[e]
Quercetin	–	–	–	–	–	0.11 ± 0.01[a]	0.24 ± 0.02[a]

VP: *Vaccinium padifolium*; VC: *Vaccinium cylindraceum*; YL: Young Leaves; ML: Mature Leaves. Means in the same column not sharing the same letter are significantly different at $p < .05$ probability level.

the same power trend between morphological parts and species ($p < .05$). TPC was within the range reported in literature for other Vaccinium berries (12.4–630 mg GAE g^{-1} DE) but lower TFC (114.21 mg RUE g^{-1} DE) were documented (Bujor et al., 2016; Ferrier et al., 2012; Harris et al., 2014; Salehi et al., 2013; Venskutonis et al., 2016; Wang et al., 2011).

3.2. In vitro digestive enzyme inhibition assays

Consumption of blueberries and its by-products has been suggested to have potential health benefits in ameliorating the development of T2D (Shi et al., 2017). In the present work, the inhibitory capacity of berries and leaves extracts of both *Vaccinium* species under study, was investigated on digestive enzymes responsible for the carbohydrates and lipids metabolism (α-,β-glucosidases, α-amylase and lipase) (Table 3).

From different morphological parts, extracts showed different inhibitory activities against targeted enzymes. For the yeast α-glucosidases assay, leaves showed the best activities ($p < .05$). Higher inhibitory activities were observed for the leaves of *V. padifolium* (namely young leaves). All samples showed stronger inhibitory efficacy ($p < .05$) than commercial drug acarbose (Table 3). The same trend was previously observed for other Vaccinium berries (Salehi et al., 2013; Wu et al., 2017). Young leaves of *V. padifolium* showed comparable activities to 1-DNJ, a known glucosidases inhibitor extracted from mulberry leaves (*Morus* spp. L.). Remarkable differences were observed between results of yeast and rat glucosidase assays. In the latter case, higher IC_{50} values were determined (Table 3). A correlation of 0.840 at $p < .05$ was obtained from results of both models. Additionally, acarbose and 1-DNJ were more effective towards mammalian enzyme (about 17 and 65 times higher, respectively) and displayed the higher inhibitory activities among tested samples. The discrepancy of results could be attributed to structural differences in the catalytic site of enzymes (Shai, Magano, Lebelo, & Mogale, 2011). Data obtained from the mammalian enzyme seem to be closer to the human digestion system, thus, it is a preferable model to search for new hypoglycemic agents.

For the β-glucosidase assay, berries extracts were less effective than the standards conduritol B epoxide (a known β-glucosidase inhibitor) and 1-DNJ ($p < .05$).

Our experiments have shown a mild inhibition towards α-amylase activity (Table 3), since all samples presented higher IC_{50} values than acarbose ($p < .05$). Leaves extracts were the most active (except for *V. padifolium* mature leaves). In this case, higher inhibitory activities were observed for leaves of *V. cylindraceum*. Berries of *V. arctostaphylos* showed comparable IC_{50} values (1.91 mg mL^{-1}) towards α-amylase (Nickavar & Amin, 2010) while leaves were more effective (0.53 mg mL^{-1}) (Nickavar & Amin, 2011) than analysed extracts (Table 3).

Leaves were more active than berries ($p < .05$) against lipase, albeit showing lower activities than commercial drug orlistat (0.47 mg mL^{-1}). Similarly, other Vaccinium berries have been documented as active digestive enzymes inhibitors (Boath, Grussu, et al., 2012; McDougall et al., 2008; Podsędek et al., 2014; Wang et al., 2012).

High correlations between TIPC and inhibition of glucosidases and lipase ($r \geq -0.937$) were observed. Similar results were stated in literature (Flores, Singh, Kerr, Pegg, & Kong, 2013; Salehi et al., 2013; Wang et al., 2012). However, lack of correlation was obtained for α-amylase ($r \geq -0.498$). These data are consistent with that of previous works (Flores et al., 2013; Podsędek et al., 2014), suggesting that the type of polyphenols is more important for the inhibitory effects than total phenolic amounts (Kang, Racicot, Pilkenton, & Apostolidis, 2016; Podsędek et al., 2014). Moreover, synergistic and/or antagonistic effects can occur between phytochemicals present in crude extracts (Boath, Grussu, et al., 2012). In the present work, HCAs and anthocyanins seem to be the main glucosidases inhibitors ($r \geq -0.818$) Flavonols and flavan-3-ols were key contributors for α-amylase inhibition ($r \geq -0.860$), while HCAs and flavanols ($r \geq -865$) were important for lipase inhibition.

Previous studies have shown that α-glucosidase is susceptible to inhibition by a range of berries polyphenols, in particular cyanidin glycosides and caffeoylquinic acids (CQAs) (Boath, Grussu, et al., 2012; Kang et al., 2015; Shi et al., 2017). In fact, the high contents of 5-*O*-CQA may explain the more potent inhibitory activity of blueberries than other berry species (McDougall et al., 2008). Considering that 5-*O*-CQA was the most abundant in leaves of targeted Vaccinium species, its inhibitory potential was further analysed and it was found to be a powerful inhibitor of targeted enzymes (Table 3), with lower IC_{50} values than positive controls in yeast a-glucosidase, β-glucosidase and lipase assays ($p < .05$). In case of rat α-glucosidase, its effectiveness was only inferior to 1-DNJ and acarbose. For α-amylase, its potency was comparable to berries extracts (Table 3). It seems that 5-*O*-CQA is a more effective inhibitor of glucosidases than of other digestive enzymes. In fact, extracts with the highest contents in 5-*O*-CQA were the most active (*V. padifolium* > *V. cylindraceum*). Previously (Boath, Stewart, & McDougall, 2012; Spínola & Castilho, 2017; Worsztynowicz, Napierała, Białas, Grajek, & Olkowicz, 2014), 5-*O*-CQA has been reported as a strong inhibitor of digestive enzymes. Inhibition of α-glucosidase by

this molecule appears to be non-competitive, suggesting binding to, or interaction with, other sites than the recognized active site of the enzyme (McDougall et al., 2008). The present data (Table 3) demonstrate that 5-*O*-CQA can be considered as one of the main active hypoglycemic agents present leaves extracts. Nevertheless, the action of flavonoids cannot be ruled out and may also contribute for the obtained results.

Tannins (ellagitannins and proanthocyanidins) are active α-amylase and lipase inhibitors, but not effective against α-glucosidase (Boath, Grussu, et al., 2012; Kang et al., 2015; McDougall et al., 2008). However, their effectiveness may be potentiated by anthocyanins (Podsędek et al., 2014). For example, quercetin and malvidin-3-*O*-glucoside, isolated from leaves and berries of *V. arctostaphylos,* have been reported as active α-amylase components (Nickavar & Amin, 2010; Nickavar & Amin, 2011). Therefore, it seems that different berry components could interact to additively or synergistically inhibit amylase and glucosidases (McDougall et al., 2008). The higher effect of *V. cylindraceum* on α-amylase is indicative of its high flavanol content (Table 3). In fact, if we remove young leaves from *V. padifolium* we obtain a higher correlation ($r = -0.72$).

Previously (Boath, Grussu, et al., 2012; Sancho & Pastore, 2012), a synergistic effect between acarbose, cyanidin glycosides and proanthocyanidins from berry extracts was observed. Therefore, this combination may lead to a reduction of the dose of acarbose in the management of hyperglycaemia, consequently reducing side effects of acarbose consumption (flatulence and diarrhea). In addition, identifying extracts with high α-glucosidase but mild α-amylase inhibitory potential is recommended to prevent fermentation of undigested starch in colon (Boath, Grussu, et al., 2012).

3.3. *In vitro anti-glycation assays*

Inhibition of AGEs formation, which is induced by hyperglycaemia, represents another potential therapeutic approach for some diabetic complications (nephropathy, retinopathy, among others) (Beaulieu et al., 2010; Chinchansure, Korwar, Kulkarni, & Joshi, 2015; Harris et al., 2014). Tested extracts showed potent *in vitro* inhibitory potential to glycation of BSA ($IC_{50} \leq 2.35\,mg\,mL^{-1}$ DE for both models) (Table 3). Leaves showed the highest anti-glycation activities (*V. padifolium* > *V. cylindraceum*) ($p < .05$). Higher glycation rates were found in BSA-ribose assay ($p < .05$). It is known that glycation ability occurs in the following increasing order: D-glucose < D-fructose < D-ribose. This is explained by the almost planar structure of the aldofuranose ring of ribose which causes its instability and high rate of anomerization; the unstable ring is thus susceptible to reactions with nucleophiles, such as amino groups, giving rise to its high efficiency in protein glycation (Sompong, Meeprom, Cheng, & Adisakwattana, 2013).

All samples were more effective than AMG (an experimental anti-diabetic drug) in both models ($p < .05$), in agreement with previous data (Liu et al., 2011). However, an opposite trend was found in another study (Wang et al., 2011). Quercetin standard proved to be the best inhibitor ($p < .05$). Other authors found similar results (Beaulieu et al., 2010; Harris et al., 2014). The analysed extracts potency was inferior to those reported for other Vaccinium species (Beaulieu et al., 2010; Ferrier et al., 2012; Harris et al., 2014; Liu et al., 2011). These discrepancies can arise from different analytical methodologies (type and concentration of sugar, time of reaction, etc.) or sample composition. Nevertheless, these data demonstrate other beneficial effects of targeted extracts in the control of diabetes, besides lowering hyperglycemia.

A strong correlation was observed for inhibition of AGEs formation and TIPC ($r \geq -0.906$), HCAs and flavonols as main contributors ($r \geq -0.860$). Cyanidin-3-glycosides and quercetin-glycosides were the main active antiglycation agents in *V. vitis-idaea* berry extract (Beaulieu et al., 2010), while proanthocyanidins appeared to be the potent inhibitors from *V. macrocarpon* (Liu et al., 2011). Although anthocyanins are effective AGEs inhibitors, it seems that anthocyanin-procyanidin mixture or total phenolic composition is more pertinent to anti-glycation activity (Harris et al., 2014; Wang et al., 2011). 5-*O*-CQA, main compound in leaves extracts, is known as a potent inhibitor of AGEs formation (Chinchansure et al., 2015; Spínola & Castilho, 2017). The obtained results (Table 3) are corroborative to this fact: 5-*O*-CQA has a better superior IC_{50} value to AMG and comparable to quercetin ($p < .05$). In fact, *V. padifolium* leaves showed the strongest inhibitions compared with other berry species studied by our group (*Myrica faya* and *Rubus grandifolius*) with lower CQAs content (unpublished data). Polyphenols are thought to act by several mechanisms of action in the prevention of AGEs formation (Chinchansure et al., 2015), and in the case of 5-*O*-CQA its potential effect results probably from its antioxidant activity or chelating of transitional metal ions (Gugliucci, Bastos, Schulze, & Souza, 2009). Therefore, 5-*O*-CQA can be considered as one of the main anti-glycation agents on analysed Vaccinium species.

3.4. *In vitro antioxidant assays*

The increased free-radical production (like NO and O_2^-) by oxidative stress is associated with the initiation and progression of diabetes and related complications (Sancho & Pastore, 2012; Wang et al., 2012). Therefore, exogenous scavengers can be useful in the prevention and/or reduction of oxidative stress involved in this metabolic disorder (Sarkar et al., 2017). For that reason, the antioxidant effects of targeted Vaccinium extracts towards and synthetic ($ABTS^{\cdot+}$ and DPPH) and biological (NO and O_2^-) radicals were measured (Fig. 2).

The antioxidant activities of *Vaccinium* spp. extracts varied between 0.27 and 7.91 mmol TE g^{-1} DE (Fig. 2). Leaves were the most active samples ($p < .05$), regardless of the assay, which is in agreement with the higher phenolic composition (Table 2). A similar trend was observed previously (Bujor et al., 2016; Oszmiański et al., 2016; Teleszko & Wojdyło, 2015). *V. padifolium* extracts (berries and leaves) showed the strongest antioxidant activities ($p < .05$). According to literature (Sarkar et al., 2017; Shi et al., 2017; Venskutonis et al., 2016), differences in blueberries species/cultivars antioxidant activities are mainly related to genetic factors, edaphoclimatic and cultivation conditions. Other Vaccinium species have shown high antioxidant activities (Bujor et al., 2016; Chen et al., 2014; Garzón et al., 2010; Podsędek et al., 2014; Wang et al., 2012; Wu et al., 2017). By comparison, *V. myrtillus* and *V. macrocarpon* extracts presented lower scavenging activity towards ABTS and DPPH radicals (0.14–0.40 mmol TE g^{-1} DE) (Bujor et al., 2016; Teleszko & Wojdyło, 2015) than analysed species (Fig. 2). On another study, *V. macrocarpon* presented higher antioxidant effects on $ABTS^{\cdot+}$ assay (2.29 and 9.81 mmol TE g^{-1} DE) (Oszmiański et al., 2016). A simultaneous scavenging activity of targeted species towards NO and O_2^- was demonstrated (Fig. 2), which might also prevent the formation of other more reactive species like peroxynitrite and hydroxyl radical (Sousa et al., 2008). *Sambucus lanceolata* extracts, analysed under the same conditions (Pinto et al., 2017), presented lower antioxidant activities (0.02–2.87 mmol TE g^{-1} DE) than analysed Vaccinium species. This may be related to the inferior TIPC of both berries and leaves ($\leq$27.22 mg g^{-1} DE).

In the present study, relevant correlations between TIPC, TPC and TFC and measured antioxidant activities were observed ($r \geq 0.890$). Thus, polyphenols are significant contributors to measured activities: HCAs ($r \geq 0.865$) > anthocyanins ($r \geq 0.774$) > flavonoids ($r \geq 0.648$). Other works also noticed strong correlation between polyphenols and antioxidant activity of berry producing plants (Bujor et al., 2016; Chen et al., 2014; Podsędek et al., 2014; Sarkar et al., 2017; Teleszko & Wojdyło, 2015; Wang et al., 2012). Anthocyanins and 5-*O*-CQA seem the major contributors to the *in vitro* antioxidant properties of blueberries (Oszmiański et al., 2016; Shi et al., 2017; Venskutonis et al., 2016; Wang et al., 2012; Wu et al., 2017).

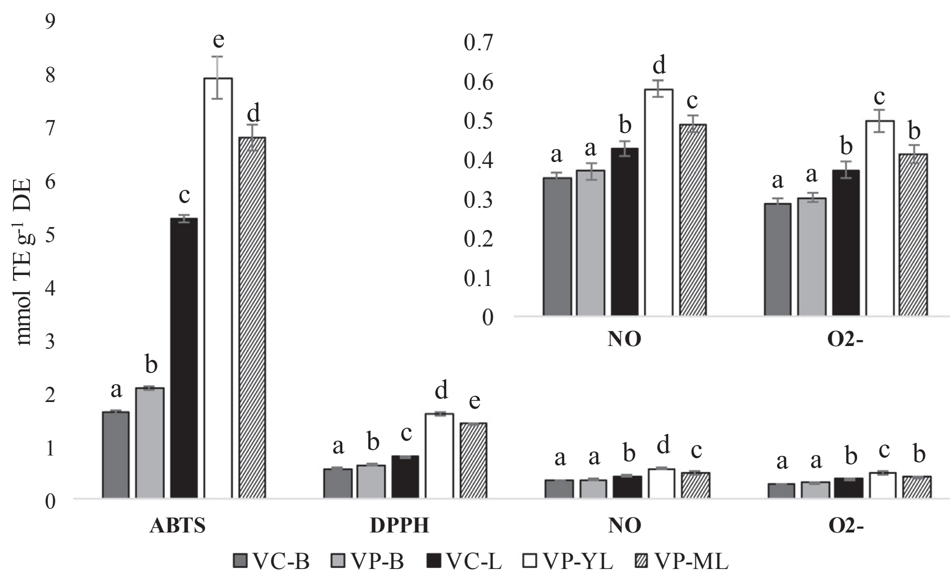

Fig. 2. Antioxidant activities of Vaccinium extracts measured by different *in vitro* assays. Data represent the mean ± standard deviation ($n = 3$). VC: *V. cylindraceum*; VP: *V. padifolium*; B: berries; L: leaves; YL: young leaves; ML: mature leaves.

3.5. Principal Component Analysis (PCA)

PCA statistical tool was applied to concentrations of 66 compounds (Table 2), in order to establish a relationship between morphological parts and Vaccinium species. PC1, that explained 86% of the total variability (Fig. 3A), shows Vaccinium samples discrimination based on morphological parts, where the berries are below PC1 axis and leaves are projected in PC1 positive. On the other hand, PC2 (that explained 12% of the total variability) separated leaves based on species: *V. cylindraceum* are above PC2 axis, while *V. padifolium* is positioned in PC2 negative (Fig. 3A). Taking into account the loading plot (Fig. 3B), the compounds that most contribute to these results were 5-*O*-caffeoylquinic acid (**68**), B-type procyanidin dimer (**75**) and kaempferol-*O*-hexoside (**134**). From this data, we can infer that Vaccinium berries have alike phenolic compositions, while previous compounds can be used as potential discriminatory geographical/taxonomical markers for leaves of Vaccinium species endemic to Azores and Madeira archipelagos.

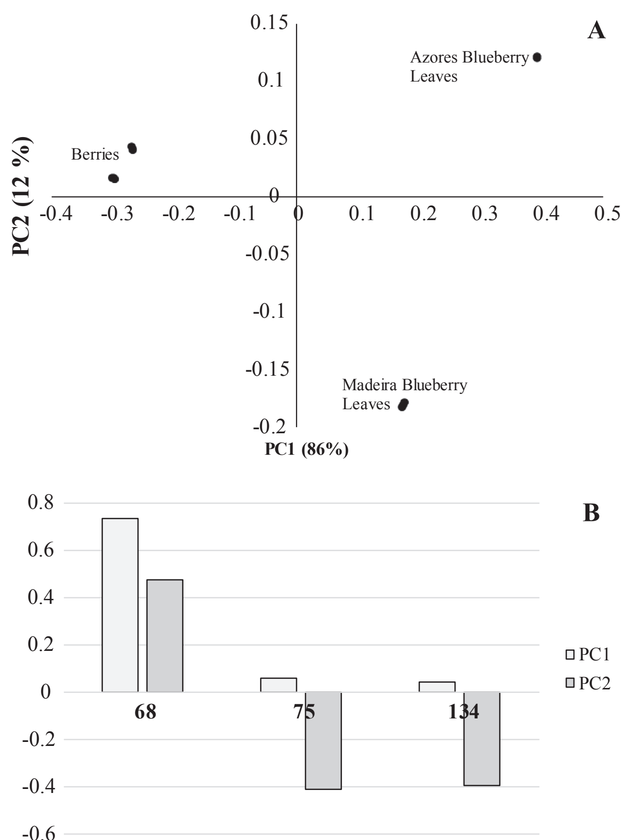

Fig. 3. (A) PC1 × PC2 of scores scatter plot between Vaccinium species morphological parts and collection areas; (B) PC1 × PC2 of loading plot of the main source of variability between Vaccinium species morphological parts and collection areas (Azores blueberry: *V. cylindraceum*; Madeira blueberry: *V. padifolium*).

4. Conclusions

This study reports the first full phytochemical analysis ever conducted on *V. cylindraceum* and *V. padifolium*, leading to the identification of 160 phenolic compounds. Quantitative analysis showed that anthocyanins were the main compounds of berries and HCAS in leaves, with special emphasis to 5-*O*-CQA. Berries from different species revealed a similar composition; variations were more significant for leaves (HCAs and flavanols contents). High inhibitory activities were observed for glucosidases and glycation of BSA, but extracts showed a mild inhibition for α-amylase and lipase. Specifically, 5-*O*-CQA can be considered to contribute most for the potential bioactivities of leaves. The phenolic content was also highly correlated to the antioxidant activity of analysed extracts.

Both Vaccinium species proved to be suitable for application in the management and control of hyperglycaemia and obesity, but the most promising results were obtained for *V. padifolium* (in particular young leaves). Genotype × environment interactions are most critical factors which determine *in vitro* anti-diabetic effects of blueberry extracts (Sarkar et al., 2017). Fruits are the main commercial products of berry plants and there has been an increased interest and consumption in recent years of these wild blueberry species. Considering their composition and potential health benefits, this study provided scientific data to reinforce their agricultural and commercial exploitation with some future economic impact in the local economy. Additionally, the obtained results may assist in developing high added value functional ingredients for food supplements, nutraceuticals and/or functional foods from berries leaves, which are a comparatively cheap plant source considered essentially as wastes or by-products resulting from pruning.

From a parallel study, targeted species have been submitted to *in vitro* gastrointestinal digestion simulation to evaluate the stability/availability of their polyphenol components that showed potential

beneficial properties (results to be published elsewhere). Additional toxicity and clinical studies are a follow-up investigation to determine the "real" impact of these berry species on T2D and obesity therapy.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.12.002.

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