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Evaluation of *Rubus grandifolius* L. (wild blackberries) activities targeting management of type-2 diabetes and obesity using *in vitro* models

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ABSTRACT

Rubus grandifolius Lowe (wild blackberries) is an endemic species from Madeira Archipelago (Portugal) used in folk medicine for alleviating diabetic complications. In this work, *R. grandifolius* methanolic extracts were analysed for *in vitro* inhibitory effect on digestive enzymes linked to type-2 diabetes, as well as aldose reductase activity and protein glycation. The phenolic composition, antioxidant and cytotoxic activities were also determined. Methanolic extracts exhibited strong inhibition of glucosidases (α- and β), but were less potent for α-amylase and pancreatic lipase when compared to current pharmaceutical drugs. The total phenolic content determined by HPLC-DAD varied between 92.96 - 97.47 and 118.01–137.41 mg g^{-1} of dry extract for berries and leaves, respectively. Fifty polyphenols were quantified, anthocyanins and ellagitannins being the main compounds. Cyanidin-3-glucoside was identified as one of the main hypoglycaemic and hypolipidemic agents in all extracts. *R. grandifolius* also prevented glycation of bovine-serum albumin (BSA) and showed strong radical scavenging activity against tested free radicals. At low concentration, the extracts were not cytotoxic against Caco-2 cells. Based on the results of this study, wild blackberry extracts demonstrated a potential beneficial effect on the control/management of type-2 diabetes mellitus, validating their use in folk medicine.

1. Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by chronic hyperglycemia (high blood sugar levels) that arises from the unbalanced glucose homeostasis (insulin resistance of target cells along with insufficient production of insulin) (Salehi et al., 2013; Sarkar et al., 2016). The classical classification includes type-1, type-2, other types, and gestational DM (Kharroubi and Darwish, 2015). Among them, type-2 Diabetes mellitus (T2DM) is one of the most common chronic diseases worldwide. In fact, around 366 million people suffered this disease in 2011, a figure expected to rise to 552 million by 2030 (Sami et al., 2017).

Dietary carbohydrates are mainly hydrolyzed into monosaccharides by the joint action of intestinal α-amylase and α-glucosidase (Grussu et al., 2011; Tan and Chang, 2017). This aspect must be considered particularly in patients with T2DM, who suffer from post-prandial hyperglycaemia, a risk factor for diabetic complications pathogenesis (Cheplick et al., 2007; Podsędek et al., 2014). An effective approach to lower post-prandial hyperglycaemia consists in the inhibition of digestive enzymes, which modulate carbohydrate digestion and absorption (Sarkar et al., 2016; Zhang et al., 2010). Several pharmacological-drugs (acarbose, miglitol and voglibose) are highly effective. However, many patients develop side effects (bloating, diarrhea, flautulance, indigestion) due to the fermentation of undigested starch (Cheplick et al., 2007; Salehi et al., 2013). Therefore, the search for natural alternatives for prevention and treatment of T2DM arises as a research opportunity. Among plant-based foods, berries are a rich source of dietary bioactive compounds, in particular polyphenols (Azofeifa et al., 2013; Cheplick et al., 2007; Zhang et al., 2010). Besides their high antioxidant properties and potential to neutralize undesired reactive species in the human body, these molecules have shown strong inhibitory activities towards digestive enzymes. These activities are an indicator of potential modulators of hyperglycaemia for management of T2DM (Edirisinghe and Burton-Freeman, 2016; Sarkar et al., 2016; Tan and Chang, 2017). In addition, elevated glycated hemoglobin A1c (HbA1c) level has also been considered as one of the leading risk factors for developing microvascular and macrovascular complications (retinopathy, neuropathy, nephropathy) (Kharroubi and Darwish, 2015; Sami et al., 2017). Phenolic compounds from berries have also shown anti-glycation properties and can potentially mitigate or prevent the appearance of diabetic complications (Harris et al., 2014; Liu et al., 2011; Yeh et al., 2017). As a result, this research aims to investigate alternative sources

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Table 1
Sample information, collection area, dates and voucher numbers of *R. grandifolius* samples under study.

Sample	Collection Area	Date	Voucher
Funchal (FX)	Pico do Arieiro, Funchal, Madeira Island (32° 42′ 50.72″N, 16° 55′ 55.07″W)	September 2014	MADJ12384
Machico (MX)	Santo António da Serra, Machico, Madeira Island (32° 43′ 13.72″N, 16° 51′ 2.43″W)	September 2014	MADJ12385

of natural bioactive compounds, in particular *Rubus grandifolius* Lowe, for the treatment of T2DM. The subgenus Rubus is composed by hundreds of species distributed worldwide (blackberries, raspberries, etc) (Oszmiański et al., 2015a). In Madeira Island (Portugal), endemic species *Rubus grandifolius* Lowe (Rosaceae) grows in the wild in moist and shady areas. These plants are characterized by hardy and arcuate stems with prickles, ovate-oblong leaves and numerous white flowers borne in a large pyramidal panicle. The berries, locally known as *amoras,* are fleshy, subspherical to cylindrical fruits that become black when mature. These are commonly consumed fresh or used to prepare jams, juices and liquors (Gouveia-Figueira and Castilho, 2015). Traditionally, herbal teas or alcoholic infusions made of leaves, shoots, and fruits are used to treat diabetes, as depurative, diuretic and to relieve sore throat (Rivera and Obón, 1995). A previous qualitative analysis of this species (Gouveia-Figueira and Castilho, 2015) has documented the presence of anthocyanins, flavonols (quercetin and kaempferol glycosides) and caffeic acid conjugates.

Considering the traditional anti-diabetic use of *R. grandifolius* and the potential of other *Rubus* species to inhibit digestive enzymes (Cheplick et al., 2007; Grussu et al., 2011; Sarkar et al., 2016; Zhang et al., 2010), this study was developed to (*i*) assess the inhibitory activity of this species (berries and leaves) towards key enzymes (α-, β-glucosidase, α-amylase, lipase and aldose reductase) relevant to the management of T2DM, and (*ii*) anti-glycation through *in vitro* models. The major phenolic compounds of extracts were quantified and the *in vitro* antioxidant and cytotoxic activities were determined as well.

2. Experimental

2.1. Chemicals and reagents

The eluents for HPLC–MS analysis were acetonitrile (CH_3CN, 99%) (LabScan; Dublin, Ireland) and ultrapure water (Milli-Q Waters purification system; 18 MΩ cm at 23 °C; Millipore; Milford, MA, USA). D-(−)-fructose, sodium nitroprusside (99%), soluble starch (p.a.), sodium azide (> 99%), potassium iodate (99.5%) and ethylenediaminetetraacetic acid (EDTA, > 99%) were acquired from Merck (Darmstadt, Germany). Gallic acid (> 98%), Folin–Ciocalteu's phenol reagent (FCR), potassium acetate (> 99.5%), potassium chloride (99.5–100.5%) and sodium chloride (> 99.0%) were bought from Panreac (Barcelona, Spain). ABTS (2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl), ellagic acid (≥96%), methanol (99.9%) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were acquired from Fluka (Lisbon, Portugal). Acarbose, aminoguanidine hydrochloride (AMG, ≥ 98%), conduritol B epoxide (≥95%), caffeic acid (≥98%), bovine serum albumin (BSA, ≥ 98%), α-amylase from porcine pancreas (type VI-B), α-glucosidase from *Saccharomyces cerevisiae* (type I), intestinal acetone powder from rat source of α-glucosidase, β-glucosidase from almonds, lipase (type II; from porcine pancreas), ammonium sulfate (≥99%), DL-glyceraldehyde (≥98%), β-mercaptoethanol (≥99%), formic acid (98%), phenazine methosulfate (≥90%), nicotinamide adenine dinucleotide reduced (NADH, ≥94%), N-(1-naphthyl)ethylene-diamine dihydrochloride (NEDA, ≥ 98%), sulfanilamide (≥99%), potassium persulfate (99%), rezasurin sodium salt, sodium carbonate (100%), *p*-nitrophenyl-α-D-glucopyranoside (α-*p*NPG), *p*-nitrophenyl-β-D-glucopyranoside (β-*p*NPG), *p*-nitrophenyl butyrate (*p*NPB), orlistat and D-(−)-ribose (≥99%) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Human caucasian colon adenocarcinoma (Caco-2) cells were acquired from the European Collection of Authenticatd Cell Cultures (ECACC). Minimum essential medium (MEM), fetal bovine serum (FBS), penicillin, streptomycin, amphotericin B, non-essential amino acids (NEAA), and L-glutamine were obtained from Life Technologies Gibco (Paisley, UK). Human aldose reductase (HAR) was purchased from Prozomix (Northumberland, UK) and β-nicotinamide adenine dinucleotide reduced tetrasodium salt hydrate (NADPH, ≥ 97%) from Calbiochem (MA, USA). 5-*O*-caffeoylquininc acid (5-*O*-CQA, > 95%), cyanidin-3-glucoside (C3G) chloride (> 98%) and 1-deoxynojirimycin (1-DNJ; 95–99%) were obtained from Biopurify phytochemicals LTD (Chengdu, China). o-Phosphoric acid (85%) was purchased from BDH AnalaR and nitroblue tetrazolium chloride (NBT, 90%) from Acros Organics (Geel, Belgium). Apigenin (> 99%), (+)-catechin hydrated (> 99%) and protocatechuic acid (≥99%) were acquired from Extrasynthese (Genay, France). Aluminium chloride (98%), hydrochloric acid (37%) and quercetin dihydrate (> 99%) were purchased from Riedel-de Haën (Hanover, Germany); acetic acid glacial from Fischer Scientific (Bishop Meadow, UK).

2.2. Sample preparation and extraction of phenolic compounds

R. grandifolius samples were collected in the wild at two different locations (Table 1) of Madeira Island (Portugal) in September 2014. Specimens were identified by Madeira Botanical Garden and deposited at Madeira Botanical Garden Herbarium (Funchal, Madeira).

Before analysis, plant material was separated into berries (fully ripe) and leaves, destemmed, washed, lyophilized (Alpha 1–2 LD plus freeze dryer, CHRIST), ground to powder using a mechanic grinder (60 mesh size), and stored at −20 °C in sealed plastic bags. Extraction of polyphenols followed a previous detailed protocol (Gouveia-Figueira and Castilho, 2015). In this case, an extraction solution composed of MeOH:H_2O (acidulated with 7% acetic acid) (80:20, v/v) was used for berries. Duplicate extractions were made for each sample and the obtained dry extracts (DE) were stored at 4 °C.

Before freeze-drying, the total soluble solids (TSS) was determined for berries using a digital Atago RX-1000 refractometer. The measured TSS varied between 8.3 and 10.3 ºBrix, similar to previous report for *R. fruticosus* (7.8–9.8 ºBrix) (Zia-Ul-Haq et al., 2014).

2.3. Chromatographic analysis of methanolic extracts

Analysis was performed on a Dionex ultimate 3000 series HPLC instrument (Thermo Scientific Inc., CA, USA) equipped with a binary pump, an autosampler, a column compartment (kept at 30 °C) a diode array detector (DAD) coupled to a Bruker Esquire model 6000 ion trap mass spectrometer (Bremen, Germany). The same method and conditions, previously applied for the analysis of *R. grandifolius* (Gouveia-Figueira and Castilho, 2015), were followed in this work.

2.4. Quantification of polyphenols

The quantification of main polyphenols in the analysed extracts was performed using calibration curves established with appropriate standards for each phenolic class (R^2 ≥ 0.990 in all cases). Caffeic and 5-*O*-caffeylquinic acid (5-*O*-CQA) were used for the relative quantification of hydroxycinnamic and caffeoylquinic acids, respectively, Apigenin, (+)-catechin, cyanidin-3-glucoside (C3G), ellagic acid, and quercetin

were the standards used for the flavones, flavanols, anthocyanins, ellagitannins, and flavonols, respectively. Results were expressed as mg of phenolic compound g^{-1} of dry extract (DE). Total individual phenolic contents (TIPC) was defined as the sum of the relative concentrations of polyphenols determined in extracts.

2.5. Total phenolic and flavonoid contents

The total phenolic content (TPC) was determined using a previous reported method (Spínola et al., 2018b). Fifty μL of extract solution, 1.25 mL of FCR (diluted 1.10) and 1 mL of 7.5% Na_2CO_3 solution were mixed. After 30 min of incubation at room temperature, absorbance was at 765 nm (Lambda-2 UV-vis spectrophotometer, Perkin-Elmer) and the total phenolic contents present in extracts were expressed as mg of gallic acid equivalents (GAE) g^{-1} DE.

Total flavonoids content (TFC) was measured according to a previous procedure (Spínola et al., 2018b). Briefly, 0.5 mL of extract solution, 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 $AlCl_3.6H_2O$ were mixed. After 30 min, absorbance at 415 nm was recorded (Lambda-2 UV-vis spectrophotometer) and the results expressed as mg of rutin equivalent (RUE) g^{-1} DE.

2.6. Cell culture and cytotoxicity assay

Caco-2 cells were cultured in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) antibiotic mixture (10,000 units/mL of penicillin, 10,000 μg/mL of streptomycin, and 25 μg/mL of amphotericin B), 1% (v/v) nonessential aminoacids (NEAA) and 1% (v/v) L-glutamine (200 mM) in Petri dishes. The cells were grown at 37 °C in an incubator with 5% CO2. Growth medium was changed 3 times per week.

The cytotoxic effect of *R. grandifolius* extracts was evaluated in Caco-2 cells, as a model of intestinal epithelial cells, by the resazurin reduction assay (Xiao et al., 2016). Caco-2 cells were seeded on 96-well flat bottom plates at a concentration of 5×10^4 cells/well. Each well contained 100 μL of growth medium. Following a 24 h incubation (37 °C, 5% CO_2), the growth medium was removed. Then, different concentrations of extract solutions (dissolved in PBS) were added to 200 μL of growth medium. The cells were incubated at 37 °C and 5% CO_2 for 24 h. After this period, the growth medium was removed and 150 μL of 10% v/v resazurin (0.1 mg mL^{-1}) in cell culture was added to each well. After 3 h of incubation at 37 °C and 5% CO_2, resorufin's fluorescence was measured (λex = 530 nm, λem = 590 nm; Victor^3 1420 multilabel plate counter, Perkin-Elmer). All tests were performed in duplicate (n = 8). Cytotoxicity was expressed as the IC_{50} value (mg mL^{-1} DE). This value corresponds to the concentration of extracts able to reduce the fluorescence of treated cells by 50% with reference to the control (cells treated only with culture medium).

2.7. In vitro anti-diabetic and anti-obesity assays

2.7.1. Yeast α-glucosidase inhibition assay

This assay was performed using the procedures detailed in a previous work (Spínola et al., 2018b). Briefly, 50 μL of sample extract (sequential dilutions) was combined with 50 μL of enzyme solution and 50 μL of 5 mmol L^{-1} α-*p*NPG solution. The mixture was incubated at 37 °C for 20 min in the dark. Then, 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.

2.7.2. Rat α-glucosidase inhibition assay

For this assay, 0.5 g of intestinal acetone powder from rat was dissolved in 10 mL of 0.1M phosphate buffer (pH 6.9) and sonicated for 10 min. After centrifugation (Sigma 3K30) at 1753g 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).

2.7.3. β-Glucosidase inhibition assay

For this assay, the previous procedure (section 2.5.1) was applied using β-pNPG as substrate. Conduritol B epoxide and 1-DNJ were used as positive controls.

2.7.4. α-Amylase inhibition assay

This assay was performed as described before (Spínola et al., 2018b): 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.

2.7.5. Pancreatic lipase inhibition assay

This assay was performed using the same protocol as in Spínola et al., 2018b: 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.

2.7.6. Aldose reductase inhibition assay

This assay was conducted following the same procedures detailed in Spínola et al., 2018a. In a 96 well-plate (UV-transparent), 25 μL of extract solution (serial dilutions) were mixed with 25 μL of 10 mM DL-glyceraldehyde and 25 μL of enzyme solution (1 mg mL^{-1}). All solutions were prepared in 0.1 M phosphate buffer (pH 6.2) containing 0.2 mM ammonium sulfate and 5 mM β-mercaptoethanol. The reaction was initiated with the addition of 50 μL of 0.5 mM NADPH solution and incubation at 37 °C for 20 min. The decrease in the absorption of NADPH was measured at 340 nm (Victor3 1420 microtiter reader, Perkin-Elmer) over 0 and 20 min of reaction. Quercetin was used as positive control. The obtained inhibitory activities were expressed as the IC_{50} value (mg mL^{-1} DE).

2.7.7. BSA glycation inhibition assay

Inhibition of AGEs formation was measured in 96 black well-plates, using the same protocol as previous (Spínola et al., 2018b). In brief, 50 μL of BSA solution (10 mg mL^{-1}), 80 μL of 0.1M phosphate buffer (containing sodium azide, 3 mM, pH 7.4), 50 μLof ribose or fructose solution (0.5 M) and 20 μL of sample extracts (serial dilutions). After incubation (24 h at 37 °C), plates were analysed at an excitation wavelength of 355 nm and emission wavelength of 460 nm (Victor3 microtiter reader, Perkin–Elmer). AMG and quercetin were used as positive controls and the anti-glycation activities were expressed as IC50 value (mg mL^{-1} DE) as described before (section 2.5.1).

2.8. In vitro antioxidant activity

2.8.1. ABTS radical cation ($ABTS^{\bullet+}$) scavenging activity

Determination of antioxidant activity by $ABTS^{\bullet+}$ was performed as detailed on a previous work (Spínola et al., 2018b). Extract solution (40 μL) was added to $ABTS^{\bullet+}$ solution (1.96 mL). Prior to beginning the assay, ABTS solution was diluted with PBS to an absorbance of 0.700 ± 0.02 at 734 nm. The sample absorbance was read at 734 nm during 6 min (at room temperature) (Lambda-2 UV-vis spectrophotometer). The scavenging activity was expressed as mmol Trolox equivalent (TE) g^{-1} DE, based on a Trolox calibration curve.

2.8.2. DPPH radical (DPPH$^{\bullet}$) scavenging activity

The scavenging activity against DPPH$^{\bullet}$ was measured according to a previous protocol (Spínola et al., 2018b). Sample solution (100 μL) was mixed with 0.06 mM DPPH$^{\bullet}$ solution (3.5 mL) and left to react during 30 min (protected from light). The sample absorbance was read at 517 nm and antioxidant activities were expressed as mmol TE g^{-1} DE.

2.8.3. Nitric oxide (NO$^{\bullet}$) scavenging activity

This assay was conducted as previous (Spínola et al., 2018b). In a 96-well plate, 20 mM sodium nitroprusside (50 μL) was mixed with the same volume of sample solution. This mixture reacted for 60 min, at room temperature, under light. Then, Greiss reagent (1% sulfanilamide and 0.1% NEDA in 2% phosphoric acid), was added to each well (50 μL). Sample absorbance was read at 550 nm (Victor^3 microtiter reader; Perkin–Elmer, Germany) and anti-radical activities were expressed as mmol TE g^{-1} DE.

2.8.4. Superoxide radical ($O_2^{\bullet-}$) scavenging activity

This assay was performed using the procedures detailed in a previous work (Spínola et al., 2018b). Briefly, sample solution (25 μL) was mixed with of 0.1 mM EDTA, 62 μM NBT and 98 μM NADH solution (200 μL). Then, 33 μM PMS (containing 0.1 mM EDTA) solution was added to each well (25 μL). After incubation for 5 min at room temperature, the absorbance was measured at 550 nm (Victor^3 microtiter reader) and scavenging activities were expressed as mmol TE g^{-1} DE.

2.9. Statistical analysis

Statistical analysis was performed using SPSS Statistics software v.20 (IBM SPSS Statistics for Windows, IBM Corp., USA). Data of all analysis, in triplicate, are expressed as mean ± standard deviation. A one-way analysis of variance (ANOVA) was performed to determine whether there are any statistically significant differences among parameters experimentally determined, followed by Tukey's HSD post-hoc test. The fulfilment the ANOVA requirements, specifically the normality of distribution and the homogeneity of variance, was tested by means of the Shapiro-Wilk's and the Levene's tests, respectively. A 5% significance level was considered for all tests. *p*-Values inferior to 0.05 were considered statistically significant. Principal component analysis (PCA) was applied to the quantified polyphenols from *R. grandifolius* extracts collected at different locations.

3. Results and discussion

Phytochemicals present in *R. grandifolius* collected at two different locations of Madeira Island were tentatively identified based on HPLC-ESI-MS^n spectrum information, authentic standards and literature data. A total of 108 compounds, including anthocyanins, other flavonoids, phenolic acids, terpenoids, and organic acids were characterized (Tables S1 and S2 – Supplementary Material), corroborative with previous qualitative analysis (Gouveia-Figueira and Castilho, 2015).

3.1. Quantification of individual phenolic compounds

Fifty main phenolic compounds were quantified by HPLC-DAD in *R. grandifolius* extracts (Table 2). Due to the low amounts of minor compounds, only the most abundant polyphenols were determined in this analysis.

TIPC of *R. grandifolius* extracts ranged between 92.96 and 137.41 mg g^{-1}, being higher in leaves (Table 2). Significant variations ($p < 0.05$) in the amounts of phenolic compounds were observed among different samples. In general, FX samples showed the highest concentrations of polyphenols ($p < 0.05$).

Anthocyanins were the most abundant polyphenols in berries (≈54%), followed by ellagitannins (37–39%), flavonols (8–9%) and HCAs (0.7–0.9%). Except for ellagitannins content, significant variations ($p < 0.05$) were observed in berries among different phenolic classes. Similarly to other blackberries species (Azofeifa et al., 2013; Oszmiański et al., 2015a; Zia-Ul-Haq et al., 2014), C3G was a major compound in berries (41–45%). Casuarinin (20.97–22.30%) (**53**) and ellagic acid-*O*-glucuronide (11.65–15.39%) (**48**) were also relevant compounds in berries (Table 2). Similar amounts of phenolic compounds were reported for other wild blackberries (Oszmiański et al., 2015a). *R. grandifolius* showed the highest TIPC among the berries species studied by our work group (*Myrica faya*, *Sambucus lanceolata*, *Vaccinium cylindraceum and V. padifolium*) (27.72–90.72 mg g^{-1} DE) (Pinto et al., 2017; Spínola et al., 2018a, 2018b); only the anthocyanin content of *Vaccinium* species was superior (63.68–70.77 mg g^{-1} DE) (Spínola et al., 2018b).

Leaves were composed mainly by ellagitannins (44–49%), which agrees with published data (Oszmiański et al., 2015b). However, a previous sample of *R. grandifolius* was poor in ellagitannins (Gouveia-Figueira and Castilho, 2015). The discrepancies could be due to differences in the period of collection and drying techniques. It is known that maturity stage and post-harvest management affect the phenolic composition of *Rubus* species (Skrovankova et al., 2015). HCAs (28–38%), flavonols (12–16%), flavanols (2–10%) and flavones (0.28–0.32%) were also present in leaves.

Casuarinin (**53**) was dominant in leaves (33–43%) (Table 2), however, significant compositional differences ($p < 0.05$) were found between leaves from different areas, as shown in TIPC, HCAS and flavanols contents. Quercetin-*O*-glucuronide (**81**) (9.56%), 3-*O*-CQA (**18**) (7.24%) and ellagic acid-*O*-hexoside (**74**) (5.7%) were major in FX; catechin (**39**) (8.91%), caffeic acid derivative (**49**) (6.57%) and 3-*O*-CQA (**18**) (6.21%) were relevant in MX counterparts. Flavan-3-ols contents seemed significantly affected by the collection area: catechin concentration was almost 4-fold higher in MX leaves. An explanation for the observed variations might be due to geographical differences of collection areas. Samples collected in Funchal grow at approximately 1400 m high, while Machico at about 500. Environmental factors like light/solar radiation, temperature, or rainfall, among others, have an impact on the phenolic composition of *Rubus* berries (Skrovankova et al., 2015).

TIPC of leaves was found within the range of other wild blackberries (83.02–334.24 mg g^{-1} DE) (Oszmiański et al., 2015b). Ellagitannins and flavonoids contents were lower than those in the aforementioned study (51.59–255.01 and 8.68–61.27 mg g^{-1} DE, respectively), while HCAs content was in the upper limit (8.62–43.14 mg g^{-1} DE). Leaves of *S. lanceolata* (Pinto et al., 2017), *M. faya* (Spínola et al., 2018a) *and V. cylindraceum* (Spínola et al., 2018b) showed lower TIPC (25.90 – 104.89 mg g^{-1} DE), while *V. padifolium* (128.85 – 134.42 mg g^{-1} DE) (Spínola et al., 2018b) were comparable with FX samples (Table 2).

TPC and TFC of leaves extracts, measured by colorimetric assays (Table 2), were comparable to the amounts found in dewberry (*R. caesius*) (280.26 mg GAE g^{-1} DE and 44.77 mg QE g^{-1} DE, respectively) (Velickovic et al., 2015), while lower contents were documented for blackberry crude extracts (87.25 mg GAE g^{-1} and 48.97 mg RUE g^{-1} DE) (Tan and Chang, 2017).

3.2. Principal component analysis

PCA statistical tool was applied to the amounts of polyphenols from *R. grandifolius* extracts. The PCA score scatter plot of the two first principal components (which explains 100% of the total variability) is shown in Fig. 1A. PC1 that explained 99% of the total variability, shows morphological discrimination based on phenolic profile: berries are projected in PC1 positive and leaves are below the positive PC1 axis. Taking in account the loading plots (Fig. 1B), C3G (**12**) was the compound that most contribute for this differentiation. On the other hand, PC2 (that explained 1% of the total variability) separates samples based on collection area: Machico samples are below PC2 axis while Funchal counterparts are positioned in PC2 positive. According to Fig. 2B, the

Table 2
Contents of main polyphenols (mg g^{-1} DE) present in *R. grandifolius* methanolic extracts collected at two different locations. For compound identification please check Tables S1 – S2 (Supplementary Material). Data represent the mean ± standard deviation ($n = 3$).

Nº	Assigned identification	Berries		Leaves	
Anthocyanins		FX	MX	FX	MX
2	Cyanidin-O-hexoside	5.19 ± 0.14^{b}	3.46 ± 0.05^{a}		
12	Cyanidin-3-*O*-glucoside	39.49 ± 0.44^{a}	40.94 ± 1.20^{a}		
13	Cyanidin-*O*-pentoside	2.86 ± 0.03^{b}	1.67 ± 0.06^{a}		
30	Cyanidin-*O*-dioxaloylglucoside	4.76 ± 0.08^{b}	3.74 ± 0.13^{a}		
Total		**52.31 ± 0.72**^{b}	**48.81 ± 0.89**^{a}		
Hydroxycinnamic acids					
18	3-*O*-Caffeoylquinic acid		0.54 ± 0.02^{a}	9.96 ± 0.24^{c}	6.75 ± 0.32^{b}
19	Caffeic acid-*O*-hexoside			5.84 ± 0.13^{b}	3.09 ± 0.11^{a}
22	Caffeic acid-*O*-hexoside			6.41 ± 0.21^{b}	2.59 ± 0.08^{a}
24	Caffeic acid derivative			1.85 ± 0.05^{b}	1.27 ± 0.02^{a}
27	Salvianolic acid			1.55 ± 0.03^{a}	1.72 ± 0.03^{b}
29	5-*O*-Caffeoylquinic acid dimer	0.69 ± 0.02^{c}	0.35 ± 0.01^{a}	1.20 ± 0.02^{d}	0.58 ± 0.02^{b}
34	Ferulic acid-*O*-hexoside	0.04 ± 0.01			
40	Ferulic acid derivative			7.29 ± 0.12^{b}	3.97 ± 0.14^{a}
44	Caffeic acid-*O*-hexoside derivative			0.84 ± 0.02^{a}	1.09 ± 0.02^{b}
49	Caffeic acid derivative			6.42 ± 0.14^{a}	7.14 ± 0.12^{b}
54	Caffeic acid derivative			0.24 ± 0.01	
56	Caffeic acid-*O*-(galloyl)hexoside			1.99 ± 0.03^{b}	0.08 ± 0.01^{a}
65	Caffeic acid derivative			1.18 ± 0.03	
77	Caffeic acid derivative			0.25 ± 0.01^{b}	0.13 ± 0.01^{a}
93	Umbelliferone			0.46 ± 0.01^{b}	0.38 ± 0.02^{a}
96	Caffeic acid-*O*-dihexoside			4.76 ± 0.07^{b}	1.56 ± 0.06^{a}
98	Umbelliferone			0.72 ± 0.01^{b}	0.35 ± 0.01^{a}
104	Caffeic acid-*O*-(Co)hexoside			0.20 ± 0.01	
109	Umbelliferone			0.18 ± 0.01	
121	Coumaric acid				0.08 ± 0.01
Total		**0.72 ± 0.02**^{a}	**0.89 ± 0.02**^{b}	**51.69 ± 1.15**^{d}	**30.79 ± 0.95**^{c}
Flavonols					
55	Quercetin-*O*-(pentosyl)hexoside	0.65 ± 0.02^{b}	0.58 ± 0.01^{a}		
62	Isorhamnetin-*O*-glucuronide	0.47 ± 0.01^{a}	0.48 ± 0.01^{a}		
64	Rutin	0.44 ± 0.01^{a}	0.65 ± 0.01^{b}		2.14 ± 0.02^{c}
71	Quercetin-*O*-hexoside	1.11 ± 0.02^{a}	2.02 ± 0.04^{b}	3.70 ± 0.09^{d}	2.14 ± 0.02^{c}
81	Quercetin-*O*-glucuronide	1.74 ± 0.02^{b}	1.08 ± 0.02^{a}	13.13 ± 0.05^{d}	10.22 ± 0.20^{c}
82	3-Hydroxy-3-MG-quercetin-*O*-hexoside	1.21 ± 0.02^{b}	1.00 ± 0.02^{a}		
85	Quercetin-*O*-pentoside	1.37 ± 0.02^{b}	0.60 ± 0.01^{a}	4.82 ± 0.08^{b}	2.84 ± 0.04^{a}
90	Quercetin-*O*-(acetyl)hexoside	0.32 ± 0.01^{a}	0.30 ± 0.01^{a}	2.08 ± 0.06^{b}	1.99 ± 0.06^{b}
91	Kaempferol-*O*-hexoside			1.75 ± 0.04^{a}	1.80 ± 0.02^{a}
92	Isorhamnetin derivative	0.31 ± 0.01^{a}	0.38 ± 0.01^{a}		
114	Kaempferol-*O*-(coumaroyl)hexoside	0.34 ± 0.01^{a}	0.48 ± 0.02^{b}	2.07 ± 0.04^{d}	0.71 ± 0.03^{c}
115	Quercetin	0.27 ± 0.01			
Total		**8.23 ± 0.16**^{b}	**7.81 ± 0.14**^{a}	**27.54 ± 0.49**^{d}	**21.98 ± 0.39**^{c}
Flavanols					
32	Proanthocyanidin dimer (B type)				1.23 ± 0.02
39	Catechin			2.67 ± 0.07^{a}	9.68 ± 0.15^{b}
Total				**2.67 ± 0.07**^{a}	**10.91 ± 0.17**^{b}
Flavones					
99	Apigenin-*O*-glucorunide			0.39 ± 0.01^{a}	0.35 ± 0.01^{a}
Total				**0.39 ± 0.01**^{a}	**0.35 ± 0.01**^{a}
Ellagic acid derivatives/Ellagitannins					
9	HHDP-*O*-hexoside				1.83 ± 0.04
15	Pedunculagin I			0.38 ± 0.01^{a}	0.36 ± 0.01^{a}
21	Pedunculagin I		1.99 ± 0.06^{c}	0.51 ± 0.01^{a}	1.01 ± 0.03^{b}
37	Trigalloyl-*O*-hexoside			1.42 ± 0.04	
42	Ellagic acid-*O*-glucuronide	1.02 ± 0.02			
48	Ellagic acid-*O*-glucuronide	11.35 ± 0.18^{a}	14.31 ± 0.31^{b}		
53	Casuarinin	21.74 ± 0.72^{b}	19.49 ± 0.34^{a}	44.83 ± 0.77^{c}	47.00 ± 0.67^{d}
58	Ellagic acid-*O*-pentoside	0.44 ± 0.01^{a}	0.40 ± 0.02^{a}	0.73 ± 0.03^{b}	
61	Ellagic acid derivative				0.27 ± 0.01
73	Ellagic acid-*O*-hexoside	0.20 ± 0.01^{a}	0.25 ± 0.01^{a}	7.86 ± 0.16^{c}	1.01 ± 0.04^{b}
113	Ellagic acid derivative	1.47 ± 0.02			
Total		**36.21 ± 0.95**^{a}	**36.45 ± 0.73**^{a}	**59.93 ± 1.10**^{c}	**53.98 ± 0.80**^{b}

(*continued on next page*)

Table 2 (*continued*)

Nº	Assigned identification	Berries		Leaves	
Anthocyanins		FX	MX	FX	MX
TIPC		**97.47 ± 1.85**[b]	**92.96 ± 1.94**[a]	**137.41 ± 2.22**[d]	**118.01 ± 1.89**[c]
TPC[1]		103.42 ± 1.63[b]	87.20 ± 3.44[a]	270.23 ± 1.45[c]	263.51 ± 8.06[c]
TFC[2]		14.88 ± 0.27[a]	14.25 ± 0.20[a]	71.81 ± 1.80[c]	40.11 ± 1.19[b]

[1]determined by the Folin-Ciocalteau method (mg GAE g^{-1} DE); [2]determined by the aluminium chloride method (mg RUE g^{-1} DE). FX: Funchal; MX: Machico. HHDP: Hexahydroxydiphenoyl; MG: methlyglutaroyl. Bold values represent the sum of each type of components. Means in the same line not sharing the same letter are significantly different at $p < 0.05$ probability level.

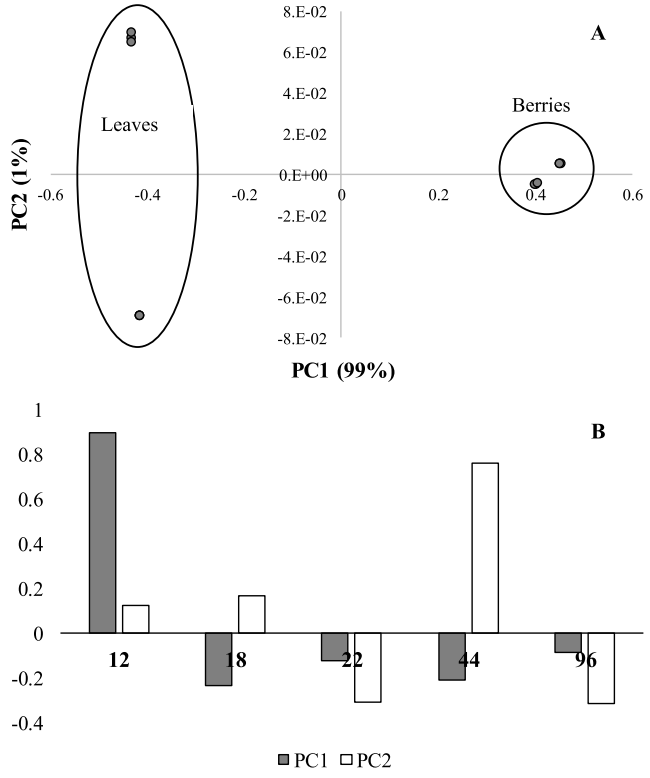

Fig. 1. (A) PC1 × PC2 of scores scatter plot between *R. grandifolius* morphological parts; (B) PC1 × PC2 of loading plot of the main source of variability between *R. grandifolius* morphological parts.

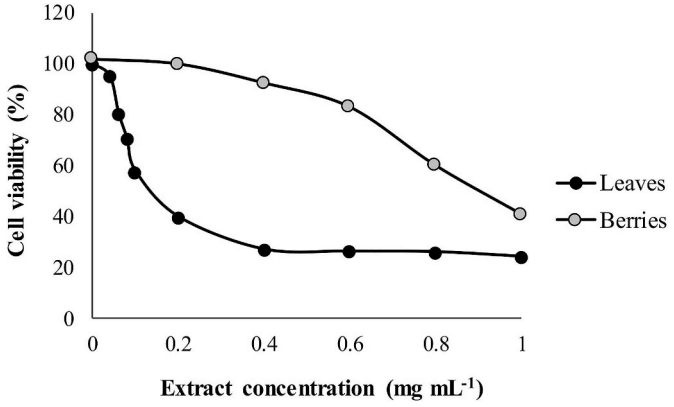

Fig. 2. *In vitro* cytotoxic activities of *R. grandifolius* methanolic extracts towards Caco-2 cells after 24 h of exposure. Cell viability was assessed by the resazurin reduction assay (IC_{50}: 0.99 and 0.17 mg mL^{-1} for berries and leaves, respectively). Data were obtained from two independent experiments with eight replicates ($n = 8$).

polyphenols responsible for the obtained results are caffeic acid derivatives (**18**, **22**, **49** and **96**).

3.3. Cytotoxicity activity

The cytotoxic effect of *R. grandifolius* (berries and leaves) was evaluated against human cancer cell line Caco-2, by the resazurin reduction assay. The exposure of cells to extracts for 24 h caused a decrease of cell viability in a dose-dependent manner in relation to the control (cells cultured only in the presence of cell culture medium) (Fig. 2).

Leaves were found to be more cytotoxic than berries, suggesting that the inhibition of cell growth was related to the phenolic composition (Table 2).

Berries of *M. faya* and *Vaccinium* spp., analysed under same conditions, were more cytotoxic than *R. grandifolius* (Table S5 – Supplementary Material). Higher cytotoxic activities were also observed for different *Rubus* berries against Caco-2 cell lines (IC_{50}: 0.02–0.12 mg mL^{-1} DE) (McDougall et al., 2008b; Nowak et al., 2017). By contrast, *R. idaeus* extract was not cytotoxic to Caco-2 cells at the tested concentrations (1, 0.1, and 0.01 mg mL^{-1} DE) (Kreander et al., 2006). The discrepancies among these studies could be related to differences in phenolic composition but also the time of exposure (48–72 h) and cell density. Anyway, present data point out that the extracts of *R. grandifolius* (especially those from the berries) can be used at low concentrations without compromising cell viability.

3.4. In vitro inhibition of digestive enzymes

Berries polyphenols are known to inhibit digestive enzymes involved in the glucose and lipids digestion (Edirisinghe and Burton-Freeman, 2016; McDougall et al., 2008a). Retarding or inhibiting the digestion of these nutrients has therapeutic implications for controlling postprandial hyperglycemia and hyperlipidaemia (Boath et al., 2012b; Tan and Chang, 2017). The main aim of this study was to evaluate the ability of *R. grandifolius* extracts to inhibit glucosidases (α-, β-), α-amylase and lipase.

All the extracts strongly inhibited yeast α-glucosidase (Table 3), showing lower IC_{50} values than commercial drug acarbose ($p < 0.05$). Leaves were the most active samples ($p < 0.05$), while berries showed similar activities to 1-DNJ, an effective natural inhibitor isolated from mulberry roots. Although the yeast α-glucosidase assay has been commonly applied for the search of new hypoglycemic compounds (Salehi et al., 2013; Sarkar et al., 2016; Tan and Chang, 2017; Zhang et al., 2010), rat α-glucosidase is a better model to validate results due to higher similarities to the human counterpart (Shai et al., 2011). In this work, the analysed extracts were less potent towards the mammalian enzyme, showing higher IC_{50} values (up to 9-fold). This trend has been reported previously as it is related to the dissimilarities of the aminoacid sequences of the catalytic region of enzymes (Shai et al., 2011). Nevertheless, a positive correlation ($r \leq 0.929$) was observed among results from the two models. Contrary to the extracts, acarbose and 1-

Table 3
In vitro inhibitory activities of *R. grandifolius* extracts ($IC_{50;}$ mg mL^{-1}) towards key enzymes linked to type-II diabetes and obesity and glycation of BSA. Data represent the mean ± standard deviation ($n = 3$).

	α-Glucosidase		β-glucosidase	α-Amylase	Lipase	Aldose reductase	BSA-glycation	
	Yeast	Rat					Ribose	Fructose
Berries								
FX	0.61 ± 0.02^{d}	3.26 ± 0.17^{f}	8.27 ± 0.15^{e}	0.94 ± 0.04^{d}	4.45 ± 0.13^{g}	0.52 ± 0.02^{d}	1.77 ± 0.05^{e}	0.88 ± 0.02^{e}
MX	0.68 ± 0.02^{e}	3.78 ± 0.23^{g}	8.79 ± 0.22^{f}	0.97 ± 0.02^{d}	3.97 ± 0.25^{f}	0.64 ± 0.03^{e}	1.87 ± 0.04^{f}	0.94 ± 0.04^{e}
Leaves								
FX	0.11 ± 0.01^{a}	0.97 ± 0.03^{e}	3.24 ± 0.08^{c}	0.83 ± 0.03^{c}	2.97 ± 0.11^{c}	0.35 ± 0.01^{c}	0.96 ± 0.05^{d}	0.55 ± 0.01^{c}
MX	0.15 ± 0.01^{b}	0.89 ± 0.05^{d}	3.87 ± 0.12^{d}	0.72 ± 0.05^{b}	3.33 ± 0.09^{d}	0.26 ± 0.01^{b}	1.07 ± 0.04^{d}	0.68 ± 0.02^{d}
Acarbose	2.06 ± 0.04^{g}	0.12 ± 0.01^{b}	N.I.	0.02 ± 0.01^{a}	–	–	–	–
1-DNJ	0.65 ± 0.02^{de}	0.01 ± 0.01^{a}	0.45 ± 0.02^{a}	–	–	–	–	–
Conduritol B epoxide	–		8.94 ± 0.19^{f}	–	–	–	–	–
Orlistat	–		–	–	0.47 ± 0.02^{b}	–	–	–
Aminoguanidine	–		–	–	–	–	9.56 ± 0.36^{g}	2.29 ± 0.13^{e}
C3G	0.38 ± 0.02^{c}	0.23 ± 0.01^{c}	0.86 ± 0.03^{b}	0.97 ± 0.03^{d}	0.30 ± 0.01^{a}	0.38 ± 0.01^{c}	0.24 ± 0.01^{b}	0.18 ± 0.01^{a}
Quercetin	–		–	–	–	0.10 ± 0.01^{a}	0.11 ± 0.01^{a}	0.24 ± 0.02^{b}

FX: Funchal; MX: Machico. N.I.: no inhibition. Means in the same column not sharing the same letter are significantly different at $p < 0.05$ probability level.

DNJ displayed superior inhibitory activity on rat α-glucosidase, about 17 and 65 times higher, respectively (Table 3).

1-DNJ showed the strongest inhibitory activity towards β-glucosidase (Table 3) and different potencies were found among extracts: leaves were more active than berries and conduritol B epoxide (a known β-glucosidase inhibitor). Berries from MX showed similar activity to that of conduritol B epoxide.

The analysed extracts showed a modest inhibitory activity against α-amylase (Table 3), compared with acarbose. Significant differences ($p < 0.05$) were observed among leaves extracts (MX > FX), more active than berries.

In the pancreatic lipase assay, C3G showed the strongest inhibitory activity (Table 3), higher than orlistat (commercial drug). Again, leaves showed lower IC_{50} values than berries and statistical variations ($p < 0.05$) were found among samples from different areas.

The potential beneficial effects of polyphenols on controlling blood glucose levels might derive from their ability to bind to proteins and, consequently, modulate digestion/breakdown of glucose by inhibition of digestive enzymes (Edirisinghe and Burton-Freeman, 2016). Although inhibition of digestive enzymes is a therapeutic approach used for control and management of diabetes and obesity, strong inhibition α-amylase is not recommended. Extracts that exhibited a mild α-amylase and high glucosidases inhibitions are the most desirable in order to avoid excessive fermentation of undigested starch in the colon that lead to stomach distention, abdominal pain and flatulence (Boath et al., 2012b; Cheplick et al., 2007; Sarkar et al., 2016).

Different raspberry cultivars (*R. idaeus*) strongly inhibited α-glucosidase (IC_{50} = 16.8–34.3 μg/mL) but, contrary to the present work, none showed inhibitory activity on pancreatic α-amylase or lipase (Zhang et al., 2010). On another work (Salehi et al., 2013), *R. fruticosus* extracts were also potent α-glucosidase and α-amylase inhibitors (1.1 and 53.7 μg/mL, respectively). Blackberries studied by Tan and collaborators (Tan and Chang, 2017) were less active than *R. grandifolius* in α-amylase assay (1.56 mg mL^{-1}), but the contrary was observed for yeast α-glucosidase and lipase (0.04 and 0.27 mg mL^{-1}, respectively). On another study, blackberry (*Rubus* spp.) extracts showed inferior activities towards yeast α-glucosidase and α-amylase (≥26 mg mL^{-1}) (Sarkar et al., 2016).

A good correlation was observed for TIPC and inhibition of targeted enzymes ($r \geq -0.850$), except for amylase ($r \geq -0.498$). By contrast, no significant correlation between TIPC and carbohydrate digestive enzymes was found among other *Rubus* extracts (Sarkar et al., 2016; Zhang et al., 2010), suggesting that enzyme inhibition is more influenced by specific phenolic types rather than the total amounts (Cheplick et al., 2007). In this work, flavonols ($r \geq -0.897$), HCAs ($r \geq -0.864$), ellagitannins ($r \geq -0.824$) and anthocyanins ($r \geq -0.818$) were the main inhibitors of glucosidases. Amylase activity seemed to be more affected by flavonols ($r \geq -0.943$) and flavan-3-ols ($r \geq -0.863$) contents, while flavonols ($r \geq -0.865$) and ellagitannins ($r \geq -0.854$) were the main inhibitors of lipase.

α-Glucosidase activity is highly affected by anthocyanins and HCAs (Cheplick et al., 2007; McDougall et al., 2008a; Zhang et al., 2010). C3G was the main compound of berries extracts and has demonstrated potent inhibitory activities towards α-glucosidase (Boath et al., 2012b, 2012a; Sancho and Pastore, 2012). Therefore, its inhibitory activity was further evaluated in detail. C3G inhibited targeted enzymes (Table 3), showing stronger effects ($p < 0.05$) than positive controls in yeast α-glucosidase and lipase assays. Inhibition of α-glucosidase by anthocyanins appears to be competitive, suggesting binding to the enzyme active site, like in acarbose (McDougall et al., 2008a; Sancho and Pastore, 2012). Additionally, an interaction between hydroxyl groups of anthocyanins and polar groups present in the active site of the enzyme can also occur and modulate enzyme activity (Boath et al., 2012b). Previously (Akkarachiyasit et al., 2010; Boath et al., 2012a), combination of acarbose with cyanidin glycosides has shown a synergetic effect towards inhibition of α-glucosidase, which could contribute to a reduction of the acarbose dose in the management of hyperglycaemia and, consequently, diminish side effects associated with its consumption (flatulence, diarrhea, liver toxicity) (Sancho and Pastore, 2012).

Previously, yellow and red raspberries extracts showed alike inhibitory effects on α-amylase, suggesting that anthocyanins are not crucial for amylase inhibition (Grussu et al., 2011). The same was observed in the present work ($r \geq$ - 0.548), where C3G standard was less effective than the tested samples (Table 3). Tannins (ellagitannins and proanthocyanidins) are reported to be the main active compounds from berries for lipase inhibition (Cheplick et al., 2007; McDougall et al., 2008a), which agrees with the obtained data. From a parallel study, berries of *Myrica faya* (Spínola et al., 2018a), *V. cylindraceum* and *V. padifolium* (Spínola et al., 2018b) were less effective than *R. grandifolius*. Although the anthocyanin content of *Vaccinium* berries was higher (Spínola et al., 2018b), their lack of ellagitannins could justify this outcome, since the combination of ellagitannins and anthocyanins increase the inhibitory activity of blackberries (Grussu et al., 2011). Given its demonstrated activities, C3G can be considered as one of the main inhibitory hypoglycemic agents of *R. grandifolius*.

3.5. *In vitro* inhibition of human aldose reductase (HAR) and protein glycation assays

Hyperglycaemia-induced complications (retinopathy, neuropathy, nephropathy, etc) are linked with the increased activity of the polyol pathway and accumulation of AGEs (Grewal et al., 2016; Veeresham

et al., 2014). HAR is the first enzyme of the polyol pathway that reduces glucose to sorbitol, which is further oxidized to fructose by sorbitol dehydrogenase (Grewal et al., 2016; Suryanarayana et al., 2004). The overactivity of the polyol pathway contributes also to the formation dicarbonyls that promotes protein glycation (Grewal et al., 2016). In this sense, inhibition of HAR activity seems to offer the possibility to ameliorate long-term diabetic complications (Grewal et al., 2016; Suryanarayana et al., 2004; Veeresham et al., 2014). *R. grandifolius* inhibited the HAR activity (Table 3), although in lower extent than by quercetin standard (positive control) ($p < 0.05$). Leaf extracts showed lower IC_{50} values than pure C3G (Table 3). *R. grandifolius* extracts were more effective towards HAR inhibition than *M. faya* (Spínola et al., 2018b) and other berry-producing plants analysed by our research group (Table S3 – Supplementary Material). A good correlation was found between TIPC and the reported bioactivities ($r \geq -0.856$), ellagitannins being the main agents ($r \geq -0.913$). Similarly, ellagitannins were the main active compounds of Indian gooseberry (*Emblica officinallis*) against aldose reductase activity (Suryanarayana et al., 2004).

Prolonged post-prandial hyperglycemia can result in glycation of proteins (Maillard reaction) and, consequently, accumulation of advanced glycation end-products (AGEs) in human tissues (Xie and Chen, 2013; Yeh et al., 2017). Those compounds result from a non-enzymatic reaction between the free amino groups of proteins and carbonyl groups of reducing sugars and contribute to the initiation and development of retinopathy, neuropathy, and nephropathy in diabetic patients (Chinchansure et al., 2015). The inhibitory activities of phenolic compounds on the generation of AGEs have suggested that these compounds are effective anti-glycations agents (Harris et al., 2014; Liu et al., 2011; Wang et al., 2011). *R. grandifolius* showed a strong ability to prevent formation of AGEs ($IC_{50} \leq 1.87\ mg\ mL^{-1}$ DE for both models) (Table 3). Significant differences were found between samples of different areas/morphological parts ($p < 0.05$) (Table 3). Lower IC_{50} values were obtained in the BSA-fructose model (Table 3) than ribose analogue ($p < 0.05$). Glycation starts as the formation of a Schiff base (glycosamine) between a sugar in its open chain form and an amino acid from a protein; Schiff bases are easily oxidized, producing free radicals. The glycosamine undergoes Amadori rearrangement to form a ketamine, which, is oxidized by transition metal catalysts and a series of reactions occurs where dicarbonyl compounds form isomers with arginine and lysine residues of proteins (AGEs formation) (Chinchansure et al., 2015; Yeh et al., 2017). The reactivity of each individual sugar (ribose, fructose, glucose) depends largely on the stability of its open chain form and its keto group (Gugliucci, 2017). For that reason, the glycating ability of monosaccharides occur in the following increasing order: D-glucose < D-fructose < D-ribose. Inhibitors of AGE formation can act by binding to protein, quench free radicals in Schiff base oxidation, chelate metal ions, trap reactive carbonyls species and blocking the generation of Amadori products and subsequent dicarbonyls formation (Gugliucci et al., 2009; Yeh et al., 2017). The extracts showed higher inhibitory activities than the experimental anti-diabetic agent aminoguanidine (AMG) ($p < 0.05$). One justification for this result is that AMG is not effective in the early stage of protein glycation since it acts mainly as a carbonyl scavenger (Liu et al., 2011; Xie and Chen, 2013). In this work, quercetin and C3G standards exhibited the best anti-glycation activities (Table 3). Previously, quercetin has been reported as a powerful protein glycation inhibitor (Beaulieu et al., 2010; Chinchansure et al., 2015; Harris et al., 2014). Previous works (Harris et al., 2014; Liu et al., 2011) have documented potent anti-glycation activities for other berry extracts. In a parallel work (Spínola et al., 2018b), leaves of *V. padifolium* where of 5-*O*-CQA is the main component of the phenolic fraction were more active than *R. grandifolius*; this could be a consequent of the low CQAs content of *Rubus*. CQAs are very effective nucleophilic traps for reactive carbonyl intermediates, such as methylglyoxal, on the propagation phase of the Maillard reaction (Bains and Gugliucci, 2017; Gugliucci et al., 2009).

Inhibition of AGEs formation was correlated with TIPC ($r \geq -0.959$), suggesting that extracts with higher TIPC were more active. Flavonols ($r \geq -0.985$) and ellagitannins ($r \geq -0.964$) were the most effective anti-glycation agents. C3G showed a very high inhibitory potential (Table 3) and, along with quercetin, have been reported as main anti-glycation agents of *V. vitis-idaea* (lingonberry) (Beaulieu et al., 2010). Phenolic compounds bind to albumin and other proteins and may reduce glycation the first stage of AGEs formation. The inhibitory effects are also due to their intrinsic antioxidant activity, by scavenging the free radicals formed during the Schiff bases oxidation (Yeh et al., 2017). Quercetin, C3G and phenolic acids with a 1,3,5 hydroxylation pattern are able to interfere in all three phases of AGEs formation (Pal and Saha, 2014; Xie and Chen, 2013). Overall, it seems that, for the inhibition of AGEs formation, the total amounts of polyphenols is more relevant than individual components (Harris et al., 2014; Wang et al., 2011).

3.6. In vitro antioxidant activities

Daily intake of polyphenol-rich fruit and vegetables is highly associated with reduced incidence of chronic diseases like T2DM (Sarkar et al., 2016; Zhang et al., 2010). These health beneficial effects are mainly due to the antioxidant properties of phenolic compounds that scavenge reactive species and prevent the development of oxidative-stress related complications (Azofeifa et al., 2013; Tan and Chang, 2017). Therefore, *R. grandifolius* extracts were estimated as scavenging potential towards different radicals ($ABTS^{\bullet+}$, $DPPH^{\bullet}$, $NO^{\bullet}$ and $O_2^{-\bullet}$).

Significant differences ($p < 0.05$) were found among the analysed samples in the *in vitro* antioxidant activities. The values ranged between 0.31 and 7.54 mmol TE g^{-1} DE (Fig. 3, Table S4 – Supplementary

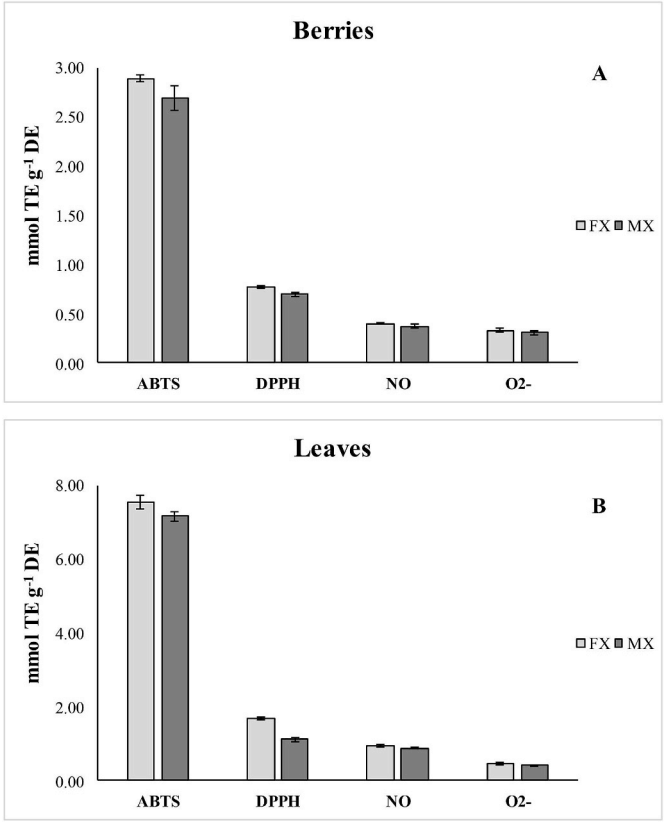

Fig. 3. Measurement of the antioxidant activities of *R. grandifolius* methanolic extracts by four *in vitro* assays. Data represent the mean ± standard deviation ($n = 3$). FX: Funchal; MX: Machico. For detailed values please check Table S4 (Supplementary Material).

Material). Overall, leaves were the most active samples ($p < 0.05$) regardless of the assay and FX samples showed the strongest activities (Fig. 2). Previously (Gouveia-Figueira and Castilho, 2015), the antioxidant activities of *R. grandifolius* were determined. However, different units make it difficult to establish a comparison with present data. By contrast, berries showed stronger anti-radical values than leaves. Differences in phenolic composition could justify this outcome. The analysed berries presented higher activities than other *Rubus* species (0.01–0.63 mmol TE g^{-1} DE); but lower than leaves and root extracts (20.51–212.69 mmol TE g^{-1} DE) (George et al., 2017; Oszmiański et al., 2015b). Alike the analysed species (Fig. 1), *R. adenotrichus* efficiently scavenged $NO^{\bullet}$ radicals (Azofeifa et al., 2013), suggesting a potential beneficial effect against oxidative stress. The measured antioxidant activities were strongly correlated with the phenolic amounts ($r \geq 0.929$), as previously reported for different blackberries (Bobinaitė et al., 2013; Oszmiański et al., 2015b, 2015a; Sarkar et al., 2016). Flavonols seemed the main contributors for the antioxidant activities ($r \geq 0.947$), followed by HCAs ($r \geq 0.943$) and ellagitannins ($r \geq 0.884$). The high antioxidant activities of *Rubus* species have been attributed mainly to their high content on ellagitannins and anthocyanins (Azofeifa et al., 2013; Oszmiański et al., 2015b, 2015a).

4. Conclusions

In this study, *R. grandifolius* extracts displayed potent α- and β-glucosidases inhibitory activity and moderate effects towards α-amylase and lipase using *in vitro* models. This suggests that some specific phenolic structures might have different inhibitory effects on certain enzymes. Formation of AGES was also efficiently prevented by the components of *R. grandifolius* methanolic extracts. Based on the observed bioactivities, C3G could be considered as one of the main active agents of these samples. However, the role of other main compounds like flavonols and ellagitannins could not be ignored. This is the first report describing the cytotoxic activities of *R. grandifolius* although the precise mechanism underlying this effect on Caco-2 or other cell lines has still to be determined. In conclusion, this study provides scientific foundation to validate the anti-diabetic uses of *R. grandifolius*, through inhibition of key enzymes, anti-glycation and protection against oxidative stress. There is a clear potential for the utilization of *R. grandifolius* as whole fruit and/or source of functional ingredients for dietary management of early stages T2DM.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.11.006.

Transparency document

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References

Akkarachiyasit, S., Charoenlertkul, P., Yibchok-Anun, S., Adisakwattana, S., 2010. Inhibitory activities of cyanidin and its glycosides and synergistic effect with acarbose against intestinal α-glucosidase and pancreatic α-amylase. Int. J. Mol. Sci. 11, 3387–3396.

Azofeifa, G., Quesada, S., Boudard, F., Morena, M., Cristol, J.-P., Pérez, A.M., Vaillant, F., Michel, A., 2013. Antioxidant and anti-inflammatory *in vitro* activities of phenolic compounds from tropical highland blackberry (*Rubus adenotrichos*). J. Agric. Food Chem. 61, 5798–5804.

Bains, Y., Gugliucci, A., 2017. Ilex paraguariensis and its main component chlorogenic acid inhibit fructose formation of advanced glycation endproducts with amino acids at conditions compatible with those in the digestive system. Fitoterapia 117, 6–10.

Beaulieu, L.-P., Harris, C.S., Saleem, A., Cuerrier, A., Haddad, P.S., Martineau, L.C., Bennett, S.A.L., Arnason, J.T., 2010. Inhibitory effect of the Cree traditional medicine Wiishichimanaanh (*Vaccinium vitis-idaea*) on advanced glycation endproduct formation: identification of active principles. Phyther. Res. 24, 741–747.

Boath, A.S., Grussu, D., Stewart, D., McDougall, G.J., 2012a. Berry polyphenols inhibit digestive enzymes: a source of potential health benefits? Food Dig. 3, 1–7.

Boath, A.S., Stewart, D., McDougall, G.J., 2012b. Berry components inhibit α-glucosidase *in vitro*: synergies between acarbose and polyphenols from black currant and rowanberry. Food Chem. 135, 929–936.

Bobinaitė, R., Viškelis, P., Šarkinas, A., Venskutonis, P.R., 2013. Phytochemical composition, antioxidant and antimicrobial properties of raspberry fruit, pulp, and marc extracts. CyTA - J. Food 11, 334–342.

Cheplick, S., Kwon, Y.-I., Bhowmik, P., Shetty, K., 2007. Clonal variation in raspberry fruit phenolics and relevance for diabetes and hypertension management. J. Food Biochem. 31, 656–679.

Chinchansure, A.A., Korwar, A.M., Kulkarni, M.J., Joshi, S.P., 2015. Recent development of plant products with anti-glycation activity: a review. RSC Adv. 5, 31113–31138.

Edirisinghe, I., Burton-Freeman, B., 2016. Anti-diabetic actions of berry polyphenols - review on proposed mechanisms of action. J. Berry Res. 6, 237–250.

George, B.P., Abrahamse, H., Hemmaragala, N.M., 2017. Phenolics from *Rubus fairholmianus* induces cytotoxicity and apoptosis in human breast adenocarcinoma cells. Chem. Biol. Interact. 275, 178–188.

Gouveia-Figueira, S.C., Castilho, P.C., 2015. Phenolic screening by HPLC–DAD–ESI/MSn and antioxidant capacity of leaves, flowers and berries of *Rubus grandifolius* Lowe. Ind. Crop. Prod. 73, 28–40.

Grewal, A.S., Bhardwaj, S., Pandita, D., Lather, V., Sekhon, B.S., 2016. Updates on aldose reductase inhibitors for management of diabetic complications and non-diabetic diseases. Mini Rev. Med. Chem. 16, 120–162.

Grussu, D., Stewart, D., McDougall, G.J., 2011. Berry polyphenols inhibit α-amylase *in vitro*: identifying active components in rowanberry and raspberry. J. Agric. Food Chem. 59, 2324–2331.

Gugliucci, A., 2017. Formation of fructose-mediated advanced glycation end products and their roles in metabolic and inflammatory diseases. Adv. Nutr. 8, 54–62.

Gugliucci, A., Bastos, D.H.M., Schulze, J., Souza, M.F.F., 2009. Caffeic and chlorogenic acids in *Ilex paraguariensis* extracts are the main inhibitors of AGE generation by methylglyoxal in model proteins. Fitoterapia 80, 339–344.

Harris, C.S., Cuerrier, A., Lamont, E., Haddad, P.S., Arnason, J.T., Bennett, S.A.L., Johns, T., 2014. Investigating wild berries as a dietary approach to reducing the formation of advanced glycation endproducts: chemical correlates of *in vitro* antiglycation activity. Plant Foods Hum. Nutr. 69, 71–77.

Kharroubi, A.T., Darwish, H.M., 2015. Diabetes mellitus: the epidemic of the century. World J. Diabetes 6, 850–867.

Kreander, K., Galkin, A., Vuorela, S., Tammela, P., Laitinen, L., Heinonen, M., Vuorela, P., 2006. *In-vitro* mutagenic potential and effect on permeability of co-administered drugs across Caco-2 cell monolayers of *Rubus idaeus* and its fortified fractions. J. Pharm. Pharmacol. 58, 1545–1552.

Liu, H., Liu, H., Wang, W., Khoo, C., Taylor, J., Gu, L., 2011. Cranberry phytochemicals inhibit glycation of human hemoglobin and serum albumin by scavenging reactive carbonyls. Food Funct. 2, 475–482.

McDougall, G.J., Kulkarni, N.N., Stewart, D., 2008a. Current developments on the inhibitory effects of berry polyphenols on digestive enzymes. Biofactors 34, 73–80.

McDougall, G.J., Ross, H.A., Ikeji, M., Stewart, D., 2008b. Berry extracts exert different antiproliferative effects against cervical and colon cancer cells grown *in vitro*. J. Agric. Food Chem. 56, 3016–3023.

Nowak, A., Sójka, M., Klewicka, E., Lipińska, L., Klewicki, R., Kołodziejczyk, K., 2017. Ellagitannins from *Rubus idaeus* L. exert geno- and cytotoxic effects against Human colon adenocarcinoma cell line Caco-2. J. Agric. Food Chem. 65, 2947–2955.

Oszmiański, J., Nowicka, P., Teleszko, M., Wojdyło, A., Cebulak, T., Oklejewicz, K., 2015a. Analysis of phenolic compounds and antioxidant activity in wild blackberry fruits. Int. J. Mol. Sci. 16, 14540–14553.

Oszmiański, J., Wojdyło, A., Nowicka, P., Teleszko, M., Cebulak, T., Wolanin, M., 2015b. Determination of phenolic compounds and antioxidant activity in leaves from wild Rubus L. Species. Molecules 20, 4951–4966.

Pal, S., Saha, C., 2014. A review on structure-affinity relationship of dietary flavonoids with serum albumins. J. Biomol. Struct. Dyn. 32, 1132–1147.

Pinto, J., Spínola, V., Llorent-Martínez, E.J., Fernández-de Córdova, M.L., Molina-García, L., Castilho, P.C., 2017. Polyphenolic profile and antioxidant activities of Madeiran elderberry (*Sambucus lanceolata*) as affected by simulated *in vitro* digestion. Food Res. Int. 100, 404–410.

Podsędek, A., Majewska, I., Redzynia, M., Sosnowska, D., Koziołkiewicz, M., 2014. *In vitro* inhibitory effect on digestive enzymes and antioxidant potential of commonly consumed fruits. J. Agric. Food Chem. 62, 4610–4617.

Rivera, D., Obón, C., 1995. The ethnopharmacology of Madeira and porto santo islands, a review. J. Ethnopharmacol. 46, 73–93.
Salehi, P., Asghari, B., Esmaeili, M.A., Dehghan, H., Ghazi, I., 2013. α -Glucosidase and α -amylase inhibitory effect and antioxidant activity of ten plant extracts traditionally used in Iran for diabetes. J. Med. Plants Res. 7, 257–266.
Sami, W., Ansari, T., Butt, N.S., Rashid, M., Hamid, A., 2017. Effect of diet on type 2 diabetes mellitus: a review introduction. Int. J. Health Sci. (Qassim). 11, 65–71.
Sancho, R.A.S., Pastore, G.M., 2012. Evaluation of the effects of anthocyanins in type 2 diabetes. Food Res. Int. 46, 378–386.
Sarkar, D., Orwat, J., Hurburt, T., Woods, F., Pitts, J.A., Shetty, K., 2016. Evaluation of phenolic bioactive-linked functionality of blackberry cultivars targeting dietary management of early stages type-2 diabetes using *in vitro* models. Sci. Hortic. 212, 193–202.
Shai, J.,L., Magano, R.,S., Lebelo, L.,S., Mogale, M.,A., 2011. Inhibitory effects of five medicinal plants on rat alpha-glucosidase: comparison with their effects on yeast alpha-glucosidase. J. Med. Plants Res. 5, 2863–2867.
Spínola, V., Llorent-Martínez, E.J., Castilho, P.C., 2018a. Polyphenols of *Myrica faya* inhibit key enzymes linked to type II diabetes and obesity and formation of advanced glycation end-products (in vitro): Potential role in the prevention of diabetic complications. Food Res. Int (In Press, Corrected Proof). https://doi.org/10.1016/j.foodres.2018.10.010.
Spínola, V., Pinto, J., Castilho, P.C., 2018b. Hypoglycemic, anti-glycation and antioxidant in vitro properties of two *Vaccinium* species from Macaronesia: a relation to their phenolic composition. J. Funct. Foods 40, 595–605.
Skrovankova, S., Sumczynski, D., Mlcek, J., Jurikova, T., Sochor, J., 2015. Bioactive compounds and antioxidant activity in different types of berries. Int. J. Mol. Sci. 16, 24673–24706.
Suryanarayana, P., Kumar, P.A., Saraswat, M., Petrash, J.M., Reddy, G.B., 2004. Inhibition of aldose reductase by tannoid principles of *Emblica officinalis*: implications for the prevention of sugar cataract. Mol. Vis. 10, 148–154.
Tan, Y., Chang, S.K.C., 2017. Digestive enzyme inhibition activity of the phenolic substances in selected fruits, vegetables and tea as compared to black legumes. J. Funct. Foods 38 (Part B), 644–655.
Velickovic, I., Grujic, S., Dzamic, A., Krivosej, Z., Marin, P., 2015. *In vitro* antioxidant activity of dewberry (*Rubus caesius* L. var. *aquáticus* Weihe. & Nees.) leaf extracts. Arch. Biol. Sci. 67, 1323–1330.
Wang, W., Yagiz, Y., Buran, T.J., Nunes, C.D.N., Gu, L., 2011. Phytochemicals from berries and grapes inhibited the formation of advanced glycation end-products by scavenging reactive carbonyls. Food Res. Int. 44, 2666–2673.
Xiao, S., Castro, R., Maciel, D., Gonçalves, M., Shi, X., Rodrigues, J., Tomás, H., 2016. Fine tuning of the pH-sensitivity of laponite–doxorubicin nanohybrids by polyelectrolyte multilayer coating. Mater. Sci. Eng. C 60, 348–356.
Xie, Y., Chen, X., 2013. Structures required of polyphenols for inhibiting advanced glycation end products formation. Curr. Drug Metabol. 14, 414–431.
Yeh, W., Hsia, S., Lee, W., Wu, C., 2017. Polyphenols with antiglycation activity and mechanisms of action : a review of recent findings. J. Food Drug Anal. 25, 84–92.
Zhang, L., Li, J., Hogan, S., Chung, H., Welbaum, G.E., Zhou, K., 2010. Inhibitory effect of raspberries on starch digestive enzyme and their antioxidant properties and phenolic composition. Food Chem. 119, 592–599.
Zia-Ul-Haq, M., Riaz, M., De Feo, V., Jaafar, H.Z.E., Moga, M., 2014. *Rubus fruticosus* L.: constituents, biological activities and health related uses. Molecules 19, 10998–11029.