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ABSTRACT

Intestinal α-glucosidase and α-amylase break down nutritional poly- and oligosaccharides to monosaccharides and their activity significantly contributes to postprandial hyperglycemia. Competitive inhibitors of these enzymes, such as acarbose, are effective antidiabetic drugs, but have unpleasant side effects. In our ethnopharmacology inspired investigations, we found that wild strawberry (*Fragaria vesca*), blackberry (*Rubus fruticosus*), and European blueberry (*Vaccinium myrtillus*) leaf extracts inhibit α-glucosidase and α-amylase enzyme activity *in vitro* and are effective in preventing postprandial hyperglycemia *in vivo*. Toxicology tests on H9c2 rat embryonic cardiac muscle cells demonstrated that berry leaf extracts have no cytotoxic effects. Oral administration of these leaf extracts alone or as a mixture to normal (control), obese, prediabetic, and streptozotocin-induced diabetic mice attenuated the starch-induced rise of blood glucose levels. The efficiency was similar to that of acarbose on blood glucose. These results highlight berry leaf extracts as candidates for testing in clinical trials in order to assess the clinical significance of their effects on glycemic control.

Introduction

Type 2 diabetes mellitus (T2DM) is a complex and progressive disease that requires continuous medical care, with multifactorial risk reduction strategies, including strict control of the blood glucose level. T2DM progresses from an early asymptomatic stage characterized by insulin resistance to mild postprandial hyperglycemia and finally to diabetes requiring pharmacological therapy [1]. Impaired glucose tolerance may occur years before the elevated fasting plasma glucose level is observed and is defined as a 2-h postprandial plasma glucose (PPG) level of 7.8–11 mM following a 75-g oral glucose tolerance test [2]. In healthy people, PPG peaks ~ 30 to 60 min after a meal, with maximum levels < 7.8 mM, and then the glucose level normalizes to the preprandial level after 2–3 h [3]. Elevated blood glucose levels are associ-

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ated with protein glycation and production of reactive oxygen species (ROS), which are responsible for most of the cardiovascular and renal complications of the disease [4–6]. Glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors are considered first-line treatments to reduce PPG levels [7], however, inhibition of intestinal α-glucosidase and α-amylase enzyme activities may also be part of the therapeutic strategy [8]. Inhibition of these enzymes leads to a significant delay of carbohydrate breakdown, absorption of monosaccharides, and attenuation of postprandial hyperglycemia [9]. Acarbose, a widely used antidiabetic drug that inhibits pancreatic α-amylase and intestinal α-glucosidase [10] has undesirable gastrointestinal and liver toxic side effects [11–13]. For this reason, there is an increasing demand to find effective and safe therapeutic tools to control postprandial hyperglycemia.

Natural products are valuable sources for antidiabetic drug research since many of the widely used medicines (e.g., metformin or acarbose) were developed from or based on secondary metabolites of living organisms. Plants with folk medicinal use are a focus, since empirical knowledge on clinical effects is a reliable starting point [14]. Clinical trials investigating postprandial glycemic responses revealed the potential of food-related polyphenols to reduce blood glucose levels [15].

One of the most prominent plants in the ethnopharmacological history of diabetes is European blueberry or bilberry (*Vaccinium myrtillus* L., Ericaceae). Bilberry leaf was a frequently used herbal remedy for diabetes before the discovery of insulin therapy [16]. In animal experiments, the extract of bilberry leaf ameliorated glycohemoglobin (HbA1c) and blood glucose levels compared to diabetic controls [17], without affecting insulin level [18]. The antidiabetic activity might be partly related to α-glucosidase inhibitory activity [19].

Polyphenol-rich extracts from bilberry fruits have also been found to be effective inhibitors of α-glucosidase [20]. The antidiabetic activity of the berries was confirmed in individuals with metabolic syndome [21]. Even a single dose significantly reduced postprandial glycemia in volunteers with T2DM [22].

The name blackberry refers to the edible fruits produced by many species of the genus *Rubus* (Rosaceae). The beneficial effects of blackberries on diabetes have been studied in animal experiments [14] and even in humans, but occasionally without the proper specification of the analyzed species [23]. *Rubus fruticosus* L. ex Dierb. leaves have traditionally been used in folk medicine for several purposes, including diabetes. It has been shown that water and butanol extracts of the berry leaves are active in T2DM [24]. In one experiment, different extracts exerted a hypoglycemic effect in normoglycemic and alloxan-induced diabetic animals as well [25]. However, in a study with streptozotocin (STZ)-induced diabetic mice, hyperglycemia and hypoinsulinemia were not affected by blackberry after 28 days of treatment. These animals received a 6.25% blackberry leaf-containing feed along with a blackberry leaf decoction [26].

Strawberry (*Fragaria* sp., Rosaceae) is gaining importance as a functional food due to its beneficial effect on blood glucose levels. In animal experiments, the supplementation of a diet with different strawberry extracts significantly reduced high glucose levels [27, 28]. In a randomized, double-blind, placebo-controlled trial, supplementing a diet with a strawberry extract decreased glycohemoglobin (HbA1c) after 6 weeks of treatment [29]. However, scarce data are available on the effects of other plant parts. It should be noted that all of the abovementioned results were obtained by using garden strawberry (*F.* × *ananassa*), which is a taxonomicaly distinct hybrid species from wild strawberry (*Fragaria vesca* L., Rosaceae). The leaves of the latter have been used in European folk medicine as a tea, both internally and extrenally with several indications, including diabetes [30]. However, no experimental data supports the antidiabetic effects of *F. vesca* leaves.

Only a few studies addressed the antidiabetic actvity of berries' mixtures. For instance, a mixture of strawberry-blueberry powders significantly reduced body weight gain, visceral adiposity, and insulin resitance of Wistar rats receiving a high-fat, high-sucrose diet [31]. Polyphenols from strawberries and cranberries improved insulin sensitivity in nondiabetic, insulin-resistant human subjects [32].

Furthermore, there is some indication showing that berry leaves might be more potent antidiabetics than fruits [24]. In the present study, we aimed for the assessment of three berry leaves for their potential to inhibit α-amylase and α-glucosidase enzymes *in vitro* and to reduce corn starch-induced blood glucose elevation in control, prediabetic, and diabetic mice. Bilberry was chosen based on its confirmed efficacy in other animal models, while blackberry was studied to gain more evidence on its equivocal efficacy. This is the first report on the antidiabetic effect of *F. vesca*.

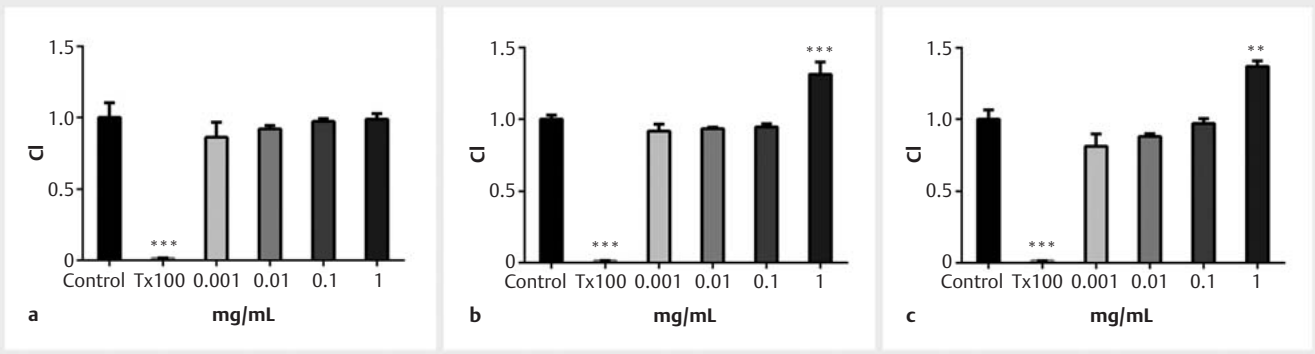
Results

First, the major constituents of the analyzed berry leaf extracts were identified based on the detection of $[M + H]^+$, $[M + Na]^+$, and $[M - H]^-$ molecular ions in full scan negative and positive ionization modes as well as according to their fragmentation patterns presented on the MS^2 spectra obtained in HPLC-HRMS experiments. The identified flavonoids and their amounts showed remarkable differences within the measured leaf extracts (**Table 1S**, Supporting Information). 3-O-Methylquercetin, apigenin-7-glucuronide, hispidulin, and taxifoline-3-*O*-acetate were presented only in the extract of *R. fruticosus*, while epigallocatechin and luteolin-5-*O*-rutinoside were detected only in the *V. myrtillus* extract. Avicularin, epicatechin, gallocatechin, hyperoside, and quercetin were found in the highest amount in the bilberry leaves, while the quercetin-3′-*O*-glucuronide and quercetin-3-rutinoside content of the blackberry leaves were the highest. Furthermore, the highest amount of quercetin-3-sambubioside, taxifoline, and catechin were detected in the strawberry extract, where the catechin concentration was 12 and 4 times higher than in the case of blackberry and bilberry extracts, respectively.

Next, we investigated if berry leaf extracts inhibit glycoside hydrolase activity *in vitro*. Serial dilutions of water-soluble fractions of *V. myrillus*, *R. fruticosus*, and *F. vesca* were tested for inhibition of α-amylase and α-glucosidase. Acarbose was used as a positive control. Berry leaf extracts dose dependently antagonized human α-amylase and α-glucosidase activity. The extract of *F. vesca* had the lowest IC_{50} value for both glycoside hydrolases, while IC_{50} values of *V. myrillus* and *R. fructicosus* were significantly higher (▶ **Ta-**

▶ **Table 1** *In vitro* IC_{50} and inhibition % values of different berry leaf extracts for α-amylase and α-glucosidases. Mean ± SD.

	IC_{50} (μg/mL)		Inhibition %	
	α-amylase	α-glucosidase	α-amylase	α-glucosidase
Vaccinium myrtillus	25.46 ± 1.18	30.46 ± 0.98	81.54 ± 8.3	85.45 ± 12.3
Rubus fruticosus	27.27 ± 1.90	25.62 ± 0.85	83.63 ± 11.5	87.65 ± 9.1
Fragaria vesca	8.84 ± 0.90	7.67 ± 0.19	96.35 ± 7.6	97.21 ± 7.4
Acarbose	0.006 ± 0.0015	2.24 ± 0.01	98.3 ± 5.8	98.6 ± 4.8



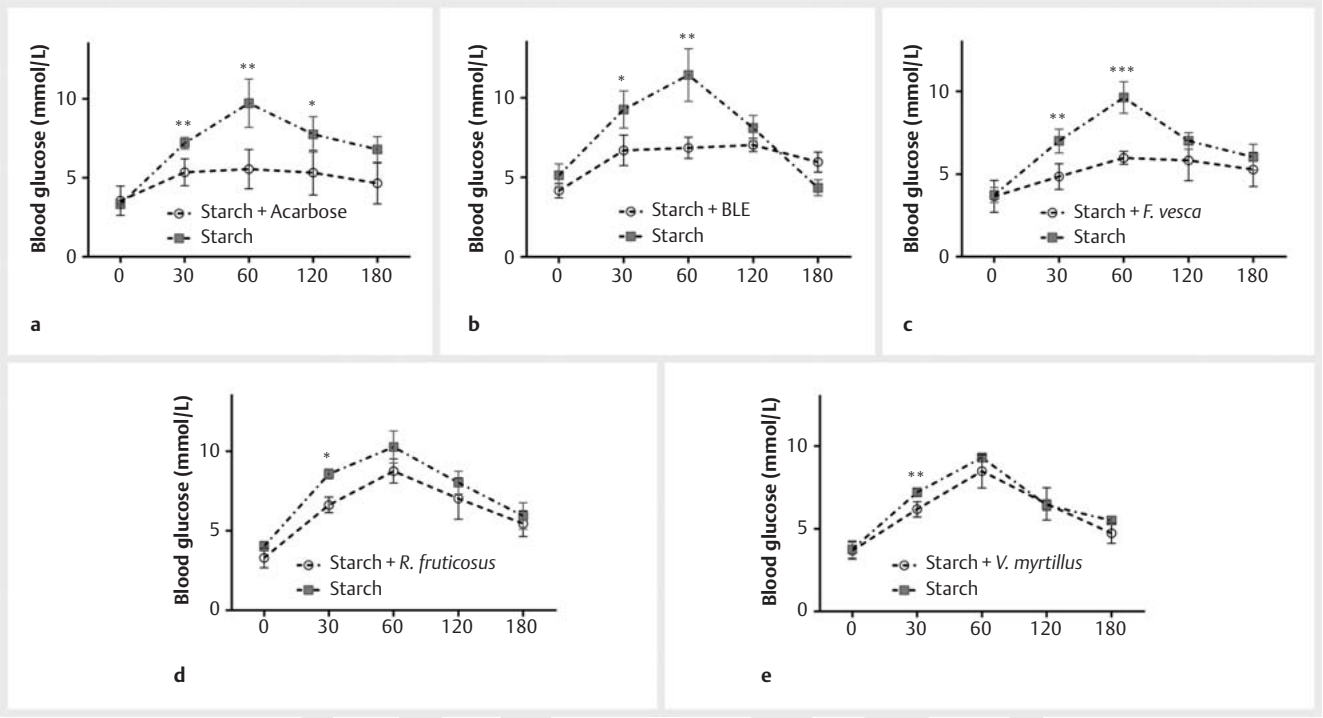
▶ **Fig. 1** Results of the RT-CES cytotoxicity assay. Mean ± SD values of cell index (CI) of blueberry leaf extract-treated (**a**), blackberry leaf extract-treated (**b**), and wild strawberry leaf extract-treated (**c**) H9c2 rat embryonal cardiac muscle cells. Cell culture medium was used as a positive control and cell culture medium supplemented with 0.1 % of Triton X100 was used as a negative control. Each measurement was determined from three independent experiments. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test; $^{**}p < 0.010$, $^{***}p < 0.001$ vs. control.

ble 1). Of note is that the IC_{50} values of each herb extracts were in a similar range for both α-amylase and α-glucosidase activity, while the IC_{50} value of acarbose for α-glucosidase was 370-fold higher than for α-amylase (▶ **Table 1**). Wild strawberry extract showed the highest inhibitory effect on α-amylase and α-glucosidase activity, with 96 and 97 % inhibition at a concentration of 5 mg/mL, respectively (▶ **Table 1**). These results showed that the tested extracts effectively inhibited both human α-amylase and α-glucosidase activity *in vitro*.

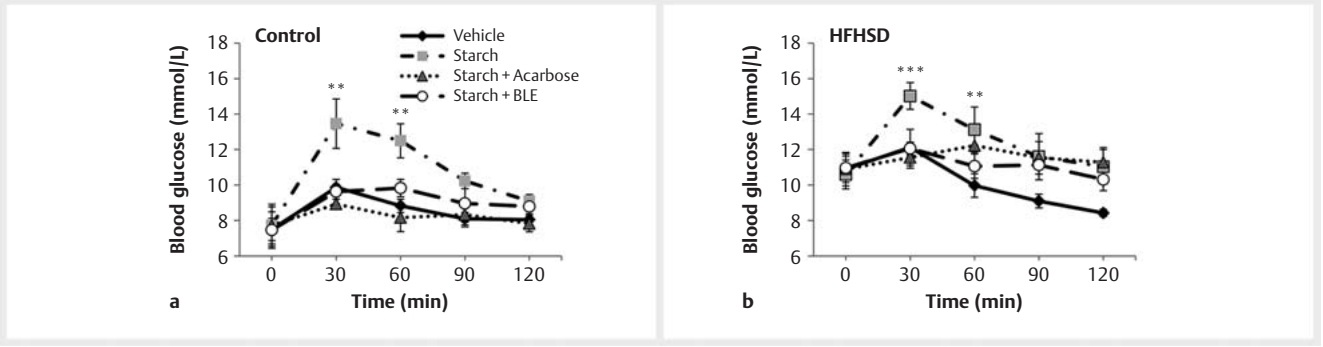
To measure the cytotoxicity of the extracts, a real-time cell electronic sensing (RT-CES) assay was used on H9c2 rat embryonal cardiac muscle cells. The cell index (CI) of the wells treated with *V. myrtillus* extract indicated no significant cytotoxic effect at any concentrations applied (▶ **Fig. 1 a**). The effect of extracts from *R. fructicosus* and *F. vesca* on H9c2 cells was not significant at concentrations of 0.001, 0.010, and 0.100 mg/mL, however the highest concentration used (1 mg/mL) increased the CI significantly (▶ **Fig. 1 b, c**). Collectively, our results showed that none of the plant extracts have cytotoxic effect at the concentration range administered.

To test the effect of berry leaf extracts *in vivo*, first, normal control CD1 mice were fasted overnight and then challenged with corn starch alone or combined with one of the following single berry leaf extracts: *F. vesca, R. fructicosus*, or *V. myrtillus* [400 mg/kg body weight (bw)]. Acarbose served as a positive control (10 mg/kg bw). The baseline blood glucose level after overnight fasting was 4.51 ± 0.44 mM. Following oral administration of corn starch, blood glucose levels increased significantly, peaked 60 min after treatment (9.73 ± 1.53 mM), and gradually declined to the baseline level by 180 min post-administration (▶ **Fig. 2**). Control mice that were given tap water only did not display any significant blood glucose elevation. Acarbose (10 mg/kg bw) completely prevented an increase of the starch-induced blood glucose level. *F. vesca* leaf extract significantly prevented an increase of the starch-induced blood glucose level at 30 and 60 min (▶ **Fig. 2**). On the other hand, *R. fructicosus* and *V. myrtillus* leaf extracts significantly inhibited a blood glucose increase following starch administration at 30 min (▶ **Fig. 2**). The combined (1 : 1 : 1) mixture of berry leaf extracts (BLEs) (1200 mg/kg bw) significantly inhibited a blood glucose increase following corn starch administration at the 30 and 60 min time points. (▶ **Figs. 2** and **3**).

Next, we tested the effect of the BLEs in the prediabetic model. By providing C57BL6 mice with a Western style [high fat/high sucrose (HFHS)] diet for 5 months resulted in significant weight gain (27.0 ± 1.1 g vs. 2.5 ± 0.8 g) and impaired glucose tolerance. As expected, the blood glucose concentration of prediabetic mice following 5 h fasting was higher compared to lean controls at the beginning of the experiment (10.84 ± 0.17 and 7.61 ± 0.15 mM, respectively; $p < 0.01$). The blood glucose level was further elevated upon starch administration, peaking at 14 mM (at 30 min) then gradually decreasing to that of the measured value at time point 0. However, when starch was coadministered with acarbose



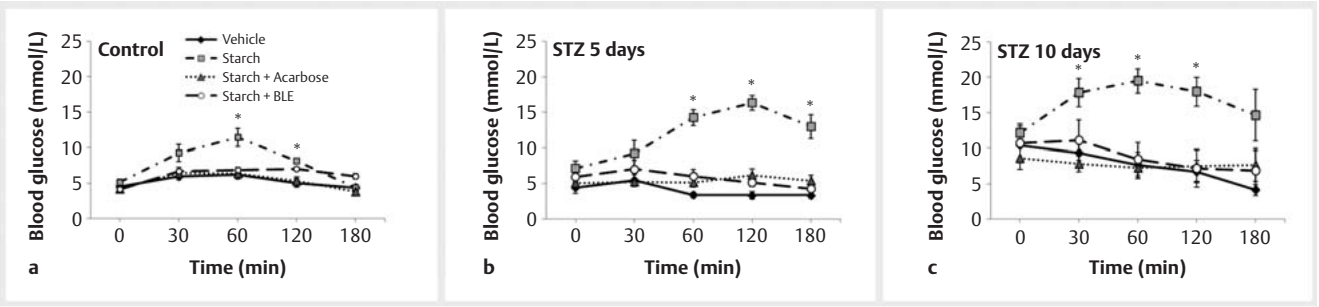
▶ **Fig. 2** Blood glucose levels in control CD1 mice. Time course (0–180 min) of blood glucose levels. Mean ± SEM values. Nondiabetic, control animals were fasted overnight before oral administration of corn starch (1 g/kg bw) alone or in combination with acarbose (10 mg/kg bw) (**a**), a mixture of berry leaf extract (BLE, 1200 mg/kg bw) (**b**), *F. vesca* (**c**), *R. fructicosus* (**d**), or *V. myrtillus* (**e**) leaf extracts (400 mg/kg bw). Each measurement was determined from three independent experiments. X-axis: time in minutes. Y-axis: blood glucose in mmol/L. Data were analyzed by multiple t-test; $^{*}p < 0.050$, $^{**}p < 0.010$, $^{***}p < 0.001$.



▶ **Fig. 3** Blood glucose levels in control and prediabetic C57BL6 mice. Time course (0–120 min) of blood glucose levels. **a** Mean ± SEM values. Mice were kept on a high-fat, high-sugar (HFHS) diet or on a control diet for 5 months. Animals were fasted for 5 h before oral administration of vehicle or corn starch (1 g/kg bw) alone or in combination with acarbose (10 mg/kg bw) or berry leaf extract (BLE, 1200 mg/kg bw) at time point 0. **b** Each measurement was determined from three independent experiments. Data were analyzed by multiple t-test; $^{**}p < 0.010$ vs. starch + BLE, $^{***}p < 0.001$ vs. starch + BLE.

(10 mg/kg bw) or BLE (1200 mg/kg bw), the blood glucose concentration was significantly lower than that of strach administration only. The glucose concentration of prediabetic mice treated with acarbose or BLE was comparable, indicating that plant extracts were effective in alleviating postprandial hyperglycemia (▶ **Fig. 3**).

By comparison, the blood glucose profile of C57BL6 mice kept on normal rodent chow was also evaluated. The blood glucose concentration of the nondiabetic C57BL6 control mice worked out similarly to that of the prediabetic mice using the same experimental design. The only difference was that the glucose level measured in the nondiabetic mice was in the physiological range at the beginning and end of the experiment (▶ **Fig. 3**).



▶ **Fig. 4** Blood glucose levels in control and STZ-diabetic CD1 mice. Time course (0–180 min) of blood glucose levels. **a** Mean ± SEM blood glucose values. Mice received a vehicle (control) or streptozotocin (STZ) injection and were tested 5 (**b**) and 10 (**c**) days after STZ/vehicle treatment. Animals were fasted overnight before oral administration of the vehicle or corn starch (1 g/kg bw) alone or in combination with acarbose (10 mg/kg bw) or berry leaf extract (BLE, 1200 mg/kg bw) at time point 0. Each measurement was determined from three independent experiments. X-axis: time in minutes. Data were analyzed by multiple t-test; $^{*}p < 0.05$ vs. starch + BLE.

Next, we investigated if BLE is able to suppress starch-induced blood glucose elevations in STZ-induced diabetic mice. Single-dose STZ treatment resulted in an elevation of blood glucose levels in normal chow-fed mice 5 and 10 days after treatment (29.36 ± 4.85 mM after 5 days and 31.18 ± 3.86 mM after 10 days). Basal blood glucose levels after overnight fasting were comparable in the control and STZ-treated mice 5 days after STZ injection, however, significantly elevated levels were measured 10 days after STZ injection, indicating progressive loss of insulin-producing cells and impaired glucose homeostasis (▶ **Fig. 4**). Soluble corn starch treatment resulted in significant and long-lasting hyperglycemia in all STZ-treated mice. The peak glucose values of starch-treated, STZ-diabetic mice were higher than that in nondiabetic animals (16.4 ± 1.8 mM, STZ 5 days; 19.5 ± 3.0 mM STZ 10 days; vs. 11.4 ± 1.5 mM, nondiabetic). When corn starch was coadministered with acarbose or BLE, blood glucose levels did not elevate significantly at any time point of sampling, indicating inhibition of the starch-degrading enzyme activity. Based on the present data, BLE (1200 mg/kg bw) was as effective as acarbose (10 mg/kg bw) in preventing starch-induced elevation of blood glucose in control, prediabetic, and as well as in diabetic mice.

Subchronic (3 days, 2× daily) administration of BLE (1200 mg/kg) to STZ-diabetic mice did not reduce basal blood glucose levels (28.56 ± 3.31 mM vs. 29.65 ± 3.96 mM).

Discussion

Here we report that BLEs efficiently attenuate soluble corn starch-induced "postprandial" blood glucose elevation in normal mice and hyperglycemia in prediabetic and STZ-induced diabetic mice. This effect is likely due to the inhibition of intestinal α-amylase and α-glycosidase enzymes by water-soluble extracts made from *V. myrillus, R. fruticosus*, and *F. vesca* leaves. However, the issues of unambiguous causality between hydrolase inhibition and antidiabetic action by the BLE and of other potential underlying mechanisms of action need further exploration in the future.

Lasting postprandial hyperglycemia is a prominent sign in prediabetes and in diabetic patients. Ingested complex carbohydrates and oligosaccharides are digested by glycosidases such as α-amylase and α-glycosidases within the small intestine, providing simple sugars for absorption. This glucose load results in an elevation of the blood glucose level, which provokes appropriate insulin responses to keep glycemia in the homeostatic range. However, impaired insulin secretion and glucose intolerance associated with metabolic X syndrome and that seen in prediabetic/diabetic patients result in prolonged hyperglycemia and contribute to oxidative stress-related diabetic complications [33]. Therefore, reduction of postprandial hyperglycemia via inhibition of intestinal glucosidases is a viable treatment strategy in diabetes [9, 34].

Here we confirm that acarbose, a pseudotetrasaccharide antidiabetic drug, inhibits α-amylase and α-glycosidase activity *in vitro* and attenuates corn starch-induced elevation of blood sugar levels *in vivo*. However, acarbose has common gastrointestinal side effects, such as diarrhea, flatulence, and stomach pain and there are case reports on increased incidences of renal tumors, serious hepatic injury, and acute hepatitis. By contrast, α-amylase and α-glycosidase inhibitors from medicinal plants have less adverse effects than the existing drugs [35].

Based on the IC_{50} values, acarbose is a much more potent inhibitor of α-amylase than any of the BLEs. Therefore, in acarbose-treated animals, more unabsorbed complex carbohydrates remain in the gut, which are substrate for colonic bacterial fermentation and result in gas production that is responsible for most of its side effects.

There is a long list of plant-derived antidiabetic natural products (for review see [36]), and part of those are α-glycosidase/amylase inhibitors [36–40]. *In vitro* α-glucosidase inhibitory activity (IC_{50}) of the BLEs tested by us is in the mid-µg/mL range, significantly lower than that reported for black tea leaves (28 mg/mL) [38] or ginger (3 mg/mL) [41], but higher than those of ayurvedic antidiabetic medicinal plants *Salacia oblongata* (4 µg mL) [42] and *Punica granatum* (1.8 µg/mL) [43], while comparable to that reported on mulberry leaf extract [37].

The capacity of strawberry and blueberry fruits or leaves to inhibit α-glucosidase and α-amylase activity has been shown [19, 20, 44, 45], and our study is the first that demonstrates that leaf extracts of *Fragaria, Vaccinium*, and *Rubus* are also efficient *in vitro* inhibitors of α-glucosidase and α-amylase enzyme activitiy. Leaf extracts are not only cheap alternatives of fruit extracts as inhib-

itors of enzymes, but also contain higher amounts of phenolic compounds, which are related to antihyperglycemic effects.

BLEs improve glycemic control in healthy control, prediabetic, and diabetic mice. A diet rich in carbohydrates and fat results in excessive weight gain and impaired glucose tolerance in mice, as seen in the prediabetic state in humans. At this stage, the basal, non-fasted glucose levels are already elevated, peaking at the highest concentration after a carbohydrate load, and returning to near baseline after challenge. By contrast, the pancreatic islet function in STZ-treated mice is significantly impaired, as seen in diabetic patients, and is characterized by a highly elevated baseline concentration and long lasting challenge-induced blood glucose levels. Intragastric administration of water-soluble corn starch results in blood glucose elevation in all groups of fasted mice, however, the peak of the glycemia was highest in the STZ-diabetic group. Even in these diabetic animals, BLE alleviates starch-induced blood glucose levels comparable to those obtained by acarbose, which is not significantly different from those that are seen in animals treated with vehicle only.

A combination of our present *in vitro* and *in vivo* data suggests that the antihyperglycemic effect of BLEs is related to inhibition of intestinal glucosidase and amylase, however, inhibition of endogenous glucose production, stimulation of glucose uptake and/or potentiation of insulin secretion and action should also be considered. For instance, bilberry (*V. myrtillus*) extracts not only inhibit α-glucosidase but also ameliorate hyperglycemia and improve insulin sensitivity via activation of the AMP-activated protein kinase and upregulation of glucose transporter 4 in white adipose tissue and skeletal muscle. This activation is accompanied by suppression of glucose production and fat content in the liver [46]. Leaf extract from *R. fructicosus* decreased basal (unstimulated) blood glucose levels in normal and STZ-treated rats, suggesting inhibition of intestinal glucose absorption [47]. The water extract of strawberry fruit also inhibits both α-amylase and α-glucosidase activities in rats [40], while there is a clinical trial reporting improved insulin sensitivity following strawberry polyphenol administration [32].

BLEs and their major biologically active ingredients are generally well tolerated and relatively free of toxic compounds at the doses tested. The reported LD_{50} value of *R. fructicosus* is 8.1 g/kg bw, much higher than that reported for many toxic plants [47]. Our *in vitro* studies using the RT-CES test on H9c2 rat embryonal cardiac muscle cells also indicate that different BLEs are not cytotoxic in the dose range up to 1 mg/mL. Furthermore, we did not observe any signs of acute *in vivo* toxicity following BLE administration in mice.

Numerous phytoconstituents, as potential inhibitors of mammalian α-amylase and α-glucosidase, primarily polyphenolic compounds, have been isolated from plants [35]. Our experiments revealed polyphenolic compounds as potential major components of the tested extracts, however, further analytical studies are required to identify the active components. The high inhibitory effect of the selected mixture (BLE) may be attributed to its synergistic effects. This suggests that the bioactive component might not be a single molecule, but rather several compounds are required to efficiently inhibit both α-amylase and α-glucosidase activity.

The pathobiological changes linking an increased postprandial blood glucose level and cardiovascular disease consist primarily of elevated ROS production and endothelial dysfunction leading to atherothrombotic propensities [5]. Therefore, regulation of the postprandial blood glucose level is a crucial factor in the treatment of diabetes-related cardiovascular complications [6]. Furthermore, previous studies have demonstrated that the water-soluble extract of strawberry and blueberry leaves contains a high level of compounds with antioxidant properties, such as flavonoids, suggesting that introducing the selected medicinal plants might act as potent suppressors of the ROS-related pathological consequences in diabetic patients as well.

In conclusion, a combination of selected BLEs with high inhibitory potential against α-glucosidase and with a moderate *in vitro* inhibitory effect on α-amylase results in a mixture that is able attenuate the "postprandial" elevation of blood glucose levels in healthy control, prediabetic, and diabetic mice. The inhibitory potential of BLEs is comparable to that of acarbose, a leading antidiabetic drug with uncomfortable side effects. Considering the IC_{50} values in the *in vitro* tests, and also the doses (400/1200 mg/kg) applied in the *in vivo* experiments, the use of the analyzed BLEs might contribute to the blood glucose control achieved with medicines in prediabetes, metabolic X syndrome, and type 2 diabetes. Since the extracts analyzed by us were prepared with hot water, the consumption of berry leaf teas seems to be a healthy habit in risk reduction due to a high blood glucose level.

Materials and Methods

Preparation of extracts

Dried leaves from wild strawberry (*F. vesca* L., Fitodry Ltd, Tiszaföldvár, Hungary, Lot: 22422 12 062015), blackberry (*R. fruticosus*, Gyógyfű Ltd, Sóskút, Hungary, Lot: 20-3-79-27.11.2015, certificate number: CF-360/2014, and European blueberry (*V. myrtillus*, Gyógyfű Ltd, Sóskút, Hungary, lot: 20-3-7-09.11.2015, certificate number: M-0007104) were used.

Dried leaves were mechanically shredded, and then 3.0 g were boiled in 300 mL water for 20 min then cooled down to room temperature, filtered, and the solution was lyophylized. The lyophylized powder (5 mg) was reconstituted in 1 mL HPLC grade water, and centrifuged at 10 000 rpm to eliminate any water-insoluble material.

Flavonoid profiling of the extracts by HPLC-HRMS analysis

Five milligrams of each lyophilized BLE was weighted into glass vials and extracted with a 1-mL methanol : water mixture (4 : 6, v/v) in an ultrasonic bath (USC 300T; VWR) for 10 min. The samples were then centrifuged (Heraus Pico 17; Thermo Scientific) at 13 000 rpm for 10 min and 700 µL supernatants were transferred into HPLC vials. The measurements were performed on a Dionex UltiMate 3000 UHPLC system (Thermo Scientific) coupled with a Q-Exactive Plus Orbitrap mass spectrometer (Thermo Scientific). The separations were carried out on a Kinetex F5 UHPLC column (150 × 2.1 mm, 2.6 µm; Phenomenex) at a flow rate of 300 µL/min with a gradient elution method. Solvent A was water + 0.1 %

HCOOH (VWR) and solvent B was methanol + 0.1% HCOOH (VWR). The elution was started at 5% B for 1 min and increased to 95% B for 15 min, which was held for 4 min and decreased to 5% for 0.5 min and remained at the same value for 5.5 min. The injection volume was 3 µL and the column temperature was set to 30 °C. Detection of the compounds was carried out in both negative and positive ionization modes with Full MS-ddMS^2 mode. The full scan range was set between 200–1000 *m/z* value, and the resolution was 70 000 (at *m/z* = 200) in both ionization modes. Resolution of the ddMS^2 scans were 17 500 at *m/z* = 200, the isolation window was 0.4 *m/z*, and the normalized collision energy was 60 in the positive and negative modes as well. The intensity threshold of ddMS^2 measurements in the positive mode was 2e5, and 1e5 in the negative ionization mode. The evaluations of the raw files were performed with Compound Discoverer 2.1 software (Thermo Scientific). The maximum mass deviation was 5 ppm for peak identification, retention time alignment, compound grouping, database search, and elemental composition prediction. The extraction solvent was applied for background correction. Hydrogen and sodium adducts were screened in the positive ionization mode and $[M - H]^-$ molecular ions in the negative ionization mode. For the identification of flavonoids, three different types of identification methods were applied. For the first identification method, the raw data files were compared with a standard flavonoid mixture containing the following compounds: apigenin, quercetin-3-glucoside, epicatechin-3-glucoside, quercetin-3′-glucoside, epicatechin, quercetin, and taxifolin. During the standard comparison, retention times, exact masses, and fragmentation patterns were considered. In the second case, the recorded MS^2 spectra of the selected precursor ions were compared to the mzCloud spectral database and identified if the percentage match was higher than 90%. In the third case, the flavonoids were determined based on the characteristic MS^2 fragments reported by Barnaba et al. [48], where flavonoids were identified based on a minimum of five characteristic fragments with a maximum 5 ppm mass difference compared with the theoretical masses. The relative amounts of the identified flavonoids were calculated from the integrated area of the $[M - H]^-$ molecular ions measured in the negative ionization mode.

Inhibition of α-glucosidase activity *in vitro*

Activity of α-glucosidase was determined by the standard method. First, 10 µL α-glucosidase (EC 3.2.1.20; Sigma) (10 U/mL) and 10 µL inhibitor (5 mg/mL) were mixed. Then 50 µL potassium phosphate buffer (67 mM) pH 6.8 at 37 °C were added to the mixture. After incubation at 37 °C for 5 min, 400 µL of 10 mM p-NPG (*p*-nitrophenyl-α-D-glucopyranoside) solution were added. Change in absorbance at 405 nm was measured kinetically with a spectrophotometer in five consecutive times. The inhibition rate was determined as percentage using the following equation:

$$\text{Inhibition } (\%) = \frac{(A1 - A2)}{A1} \times 100$$

where A1 is the absorbance of the control and A2 is the absorbance of the test sample. For those extracts that showed a good inhibition rate, the IC_{50} values were determined using different concentrations of plant extracts.

Human salivary α-amylase inhibition assay

Human salivary α-amylase from human saliva Type IX-A (EC 3.2.1.1, A0521; Sigma) was used. The enzyme did not show α- or β-glucosidase activity. 2-Chloro-4-nitrophenyl-β-D-maltoheptose (CNP-G7) substrate was synthesized using the method previously described [49]. Purity of the product was determined by HPCL and MALDI-TOF measuring 1330.3475 g/mol $[C_{48}H_{74}O_{38}NCl + Na]^+$ and 1346.3214 g/mol $[C_{48}H_{74}O_{38}NCl + K]^+$. For each measurement, the CNP-G7 substrate was prepared freshly in MES buffer at a 5 mM concentration. For the enzyme activity measurement, our new HPLC-based method was used [50]. Briefly, 0.08 U enzyme was mixed with 400 µL substrate and incubated at 37 °C for 5 min, then 20 µL of the reaction mixture was injected to the loop of HPLC every 15 min 4 times. Inhibition of HSA activity was determined by the addition of 8 µL of BLEs (5 mg/mL) or acarbose (5 mg/mL) (positive control) to the reaction mixture. The CNP-G7 and CNP-oligomer products were measured at 302 nm. The amount of CNP-G3 was specified by the area of peak. The rates of the reaction were determined by the representation of the peak area of CNP-G3 measured at four different times. For evaluation, linear regression was used and the slope of the curve gave the enzyme activity. IC_{50} values were determined using different concentrations of plant extracts as the inhibitor.

Cell culture

H9c2 rat embryonal cardiac muscle cells (from ATCC) were cultured in DMEM containing 10% fetal bovine serum and supplemented with 4 mM L-glutamine (Sigma-Aldrich), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained in 100 mm TC dishes (Orange Scientific) in an incubator with humidified air at 37 °C and 5% CO_2.

Real-time cell electronic sensing cytotoxicity assay

An RT-CES 96-well E-plate (Roche) was coated with gelatine solution (0.2% in PBS) for 20 min at 37 °C, then the gelatine was washed twice with PBS solution. Growth media (50 µL) was then dispensed into each well of the 96-well E-plate for background readings by the RT-CES system prior to the addition of 50 µL of cell suspension. Devices containing cell suspensions were kept at room temperature in a tissue culture hood for 30 min prior to insertion into the RT-CES device in the incubator to allow cells to settle. Cell growth was monitored overnight by measurements of electrical impedance every 5 min. The next day, cells were treated with the different BLEs at concentrations of 0.001, 0.010, 0.100, and 1.000 mg/mL. Cell culture medium was used as a positive control and cell culture medium supplemented with 0.1% of Triton X100 was used as a negative control. Continuous recording of impedance measured in wells for 24 h were converted into the CI value. The treated and control wells were further monitored over 24 h by measurements of electrical impedance every 5 min. The raw plate reads for each titration point were normalized relative to the CI status right before treatment. Each treatment was repeated in at least three wells per plate during the experiments.

Animals and diet

Adult male C57BL6 and CD1 mice were bred and housed in groups of 3–5/cage at the minimal disease level of the Medical Gene Technology Unit of the Institute of Experimental Medicine. Animals had free access to food and water and were maintained under controlled conditions (temperature, 21 ± 1 °C; humidity, 65 %; light-dark cycle, 12-h light/12-h dark, lights on at 7 : 00 a. m.). All procedures were conducted in accordance with the guidelines set by the European Communities Council Directive (86/609 EEC) and the protocol was approved by the Institutional Animal Care and Use Committee of the Institute of Experimental Medicine, Budapest Hungary (permit number: PEI/001/35-4/2013; March 6, 2013).

High-fat/high-sucrose diet-induced prediabetic obesity

These experiments were carried out on male C57BL6 mice, which are prone to diet-induced obesity. Animals were fed an HFHS diet for 5 months, starting at the age of 9–11 weeks, by providing a custom-made mixture of standard chow, lard, and sucrose (58 % standard chow, 30 % lard, 12 % sucrose). The energy content of this chow was 4.9 kcal/g and contained 7.4 kcal% protein, 39.2 kcal% carbohydrates, and 47.8 kcal% fat. Control animals received standard rodent chow [VRF1 (P), obtained from Special Diets Services (3.4 kcal/g, 22.5 kcal% protein, 65 kcal% carbohydrates, 12.6 kcal% fat)]. At the end of HFHS diet, at 29–31 weeks of age, the body weight of the animals increased to 52.04 ± 2.01 g compared to 27.45 ± 2.6 g body weight values of the control chow-fed mice.

Streptozotocin-induced diabetes

STZ is toxic to insulin-producing pancreatic beta cells in mammals and results in serious diabetes. STZ (Sigma Aldrich S0130) was freshly dissolved in 100 mM sodium citrate (pH 4.5) and administered intraperitoneally at 200 mg/kg bw dose to adult male CD1 mice (at the age 9–10 weeks, 30.48 ± 2.43 g average bw). Control animals received vehicle injection. STZ-induced diabetes was verified by tail bleeds at 5 days after drug treatment. Mice with non-fasted blood glucose concentrations above 14 mM were considered diabetic.

In vivo experimental design

Acarbose (Glycobay; Bayer), corn starch (Haasy), *F. vesca, R. fructicosus*, and *V. myrtillus* leaf extracts and the mixture BLEs were freshly dissolved in sterile drinking water and administered to fasted mice via oral gavages (200 µL/animal). The BLE was prepared by mixing equal amounts of lyophylized powders from each extract.

Normal (nondiabetic), prediabetic (HFHS diet induced), and diabetic (STZ treated) mice were randomly divided into groups (4–7 animals/group). STZ-induced diabetic mice were tested twice, at 5 and 10 days post-STZ. In experiments on CD1 animals, mice were fasted overnight. In studies on C57BL6 mice (HFHS diet, prediabetic, and control), animals were fasted for 5 h. All fasted animals had free access to water. After fasting, vehicle (sterile drinking water) or starch (1 g/kg bw) was administered via oral gavage. Right after vehicle or starch treatment, animals received a second gavage of acarbose (10 mg/kg bw, positive control) or *F. vesca, R. fructicosus, V. myrtillus* leaf extracts (400 mg/kg bw), and BLE (1200 mg/kg bw). Blood samples were taken from the tail vein at 0 min and at 30, 60, 120, and 180 min after oral gavages. The blood glucose level was measured with a DCont Personal Blood Glucose Meter (77 Elektronika Ltd.).

To determine the effective dose of BLE, a pilot experiment was carried out in which normal CD1 mice were fasted overnight and treated with 1 g/kg bw starch alone or in combination with 10, 200, 400, 600, 1200, and 1600 mg/kg bw BLE or 10 mg/kg bw acarbose.

Statistical analysis

All data are expressed as means ± SEM. Data passing the Kolmogorov-Smirnov normality test were analyzed by one-way ANOVA followed by Tukey's post hoc test. Differences in blood glucose levels between the groups in the *in vivo* experiments were analyzed by multiple t-test. Statistical analysis was performed using GraphPad PRISM version 6 software (GraphPad Software). $P \leq 0.05$ was considered significant.

Supporting information

Data on major components of the analyzed plant samples can be found in **Table 1S**.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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