JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Helichrysum and Grapefruit Extracts Inhibit Carbohydrate Digestion and Absorption, Improving Postprandial Glucose Levels and Hyperinsulinemia in Rats

Ana Laura de la Garza,[†] Usune Etxeberria,[†] María Pilar Lostao,[†,§] Belén San Román,[#] Jaione Barrenetxe,[†] J. Alfredo Martínez,*[,†,§] and Fermín I. Milagro[†,§]

[†]Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Spain

[§]Physiopathology of Obesity and Nutrition, CIBERobn, Carlos III Health Research Institute, Madrid, Spain

[#]Discovery Laboratory, Research Department, Biosearch S.A., 18004 Granada, Spain

ABSTRACT: Several plant extracts rich in flavonoids have been reported to improve hyperglycemia by inhibiting digestive enzyme activities and SGLT1-mediated glucose uptake. In this study, helichrysum (*Helichrysum italicum*) and grapefruit (*Citrus* × *paradisi*) extracts inhibited in vitro enzyme activities. The helichrysum extract showed higher inhibitory activity of α-glucosidase (IC_{50} = 0.19 mg/mL) than α-amylase (IC_{50} = 0.83 mg/mL), whereas the grapefruit extract presented similar α-amylase and α-glucosidase inhibitory activities (IC_{50} = 0.42 mg/mL and IC_{50} = 0.41 mg/mL, respectively). Both extracts reduced maltose digestion in noneverted intestinal sacs (57% with helichrysum and 46% with grapefruit). Likewise, both extracts inhibited SGLT1-mediated methylglucoside uptake in Caco-2 cells in the presence of Na^+ (56% of inhibition with helichrysum and 54% with grapefruit). In vivo studies demonstrated that helichrysum decreased blood glucose levels after an oral maltose tolerance test (OMTT), and both extracts reduced postprandial glucose levels after the oral starch tolerance test (OSTT). Finally, both extracts improved hyperinsulinemia (31% with helichrysum and 50% with grapefruit) and HOMA index (47% with helichrysum and 54% with grapefruit) in a dietary model of insulin resistance in rats. In summary, helichrysum and grapefruit extracts improve postprandial glycemic control in rats, possibly by inhibiting α-glucosidase and α-amylase enzyme activities and decreasing SGLT1-mediated glucose uptake.

KEYWORDS: *α-amylase, α-glucosidase, Caco-2 cells, flavonoids, SGLT1*

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disorder that affects different organs and tissues and is characterized by chronically elevated levels of blood glucose due to insulin resistance.[1] The increasing incidence of T2DM, one of the world's most common chronic diseases, is primarily associated with lifestyle-related obesity and sedentarism and is modulated by the genetic background.[2,3] Considering that postprandial hyperglycemia is an early detected symptom in T2DM related to overconsumption of carbohydrate-rich foods, consumption of phytochemicals occurring in natural products could be a possible strategy to control T2DM.[4]

Phytochemicals are non-nutritive plant compounds that have health-protective properties. One of the most relevant families of phytochemicals with proven health benefits is the polyphenols. They are found in many plants including vegetables, fruits, and grains,[5] being flavonoids the most prevalent phenolic compounds. Epidemiological data strongly suggest that diets rich in flavonoids are generally associated with a preventive role against the development of chronic diseases.[6−8]

This situation has led to an increased interest in finding specific natural dietary sources that could be used for treating diabetes. In this sense, numerous studies have investigated flavonoid-rich plant extracts that could reduce or suppress intestinal glucose uptake through the inhibition of digestive enzyme activities.[9−16]

In mammals, dietary carbohydrates are hydrolyzed by pancreatic α-amylase and intestinal α-glucosidase enzymes. Hence, the inhibition of these enzymes is an interesting strategy for the control of postprandial hyperglycemia.[8,17,18] Other approaches are directed to develop agents that inhibit intestinal glucose uptake.[17] Thus, in the intestine, glucose is absorbed mainly by two transporters, depending on the luminal glucose concentration. At low concentrations, glucose is transported across the brush border membrane against a concentration gradient by the sodium-dependent glucose transporter 1 (SGLT1). At higher concentrations, glucose is transported mainly by the low-affinity facilitated transporter, glucose transporter 2 (GLUT2).[19] Dietary flavonoids, given their relative safety and low incidence of adverse gastrointestinal side effects,[15] are candidate agents for managing postprandial hyperglycemia due to their interactions with the intestinal α-glucosidase and pancreatic α-amylase and the inhibition of glucose uptake.[17]

In this context, there are more than 500 species of the *Helichrysum* genus distributed around the world.[20] Plants of this genus have been found to possess antimicrobial, antiallergic, antioxidant, and anti-inflammatory properties.[20,21] The biological activities of *Helichrysum* plants have been attributed to several classes of flavonoids detected in different parts of the plant, such as kaempferol-3-*O*-glucoside and other flavanones.[22]

Received: May 17, 2013
Revised: October 15, 2013
Accepted: November 22, 2013
Published: November 22, 2013

dx.doi.org/10.1021/jf4021569 | *J. Agric. Food Chem.* 2013, 61, 12012−12019

Although several studies on helichrysum and its flavonoid content have been published, there is little information about its potential antihyperglycemic properties.[22,23] On the other hand, grapefruit is an excellent source of many nutrients and phytochemicals that contribute to a healthy diet.[24] *Citrus* flavonoids have evidenced antioxidant, anticancer, anti-inflammatory, chemopreventive, and cardioprotective activities.[25] Many of the flavonoids present in grapefruit, such as hesperidin, naringenin, and kaempferol, exhibit antidiabetic activities. In fact, Shen et al.[14] have demonstrated the inhibitory effects of *Citrus* flavonoids on starch digestion, whereas Pu et al.[26] reported the antihyperglycemic activity of naringenin isolated from *Citrus sinensis* in an animal model.

Thus, the aim of this study was to evaluate the antidiabetic potential of helichrysum and grapefruit extracts by determining their postprandial antihyperglycemic effect and their inhibitory activities on α-amylase, α-glucosidase, and SGLT-1 glucose transporter.

MATERIALS AND METHODS

Chemicals. Rats were fed a standard pelleted chow diet from Harlan Ibérica (Teklad Global, Barcelona, Spain; ref 2014) or a high-fat sucrose (HFS) diet from Research Diets (New Brunswick, NJ, USA; ref D12451). Helichrysum (*Helichrysum italicum*) and grapefruit (*Citrus* × *paradisi*) extracts, as well as acarbose , were provided by Biosearch S.A. (Granada, Spain). Porcine pancreatic α-amylase, α-glucosidase (*Saccharomyces cerevisiae*), *p*-nitrophenyl α-D-glucopyranoside (*p*NPG), maltose, glucose, and phloridzin were purchased from Sigma Chemicals, USA. Starch (162.14 g/mol) was purchased from Panreac. The Caco-2 cell line PD7 clone was kindly provided by Dr. Edith Brot-Laroche. The radiolabeled product [^{14}C]-α-methyl-glucoside (303 mCi/mL) was purchased from Perkin-Elmer (Life Sciences, Boston, MA, USA).

Plant Extract Preparation. Initial enzymatic in vitro assays were performed with plant extracts produced by accelerated solvent extraction (ASE) using a Dionex ASE200 equipment (Dionex Corp., USA). Plant samples (1−5 g) were pulverized, mixed with washed sea sand (Panreac Química S.A.U., Spain), and introduced into the extraction cells, where 30 mL of the appropriate solvent at 50 °C was added: methanol/water (3:1) and methanol/water (1:1) for *Helichrysum italicum* and *Citrus* × *paradisi*, respectively. After 3 h of incubation, liquid extracts were filtered, concentrated, and dried by spray-drying. Solid extracts were stored at room temperature.

UPLC-MS/MS Analyses. The extracts were previously dissolved in a methanol/water (50:50, v/v) solution at a concentration of 10 mg/mL. Analyses of helichrysum and grapefruit extracts were performed by ultraperformance liquid chromatography (UPLC) and positive ion electrospray ionization (ESI) source Z-spray with MS/MS. The MS was operated in negative mode to analyze the phenolic compounds. The data were acquired in selected reaction monitoring (SRM). The ionization source working conditions were as follows: capillary voltage, 3 kV; source temperature, 150 °C; cone gas flow rate, 80 L/h; desolvation gas flow rate, 800 L/h; desolvation temperature, 400 °C.[27] An ABEH C_{18} (100 mm, 2.1 mm × 1.7 μm) column, thermostated at 30 °C, was used. The solvents were (A) 0.2% acetic acid and (B) acetonitrile. The absorbance was recorded at 278 and 339 nm.

In Vitro α-Amylase Activity Assay. The α-amylase activity was determined using porcine pancreatic α-amylase solution (EC 3.2.1.1) in the absence (control) and presence of acarbose, helichrysum, and grapefruit extracts. The reaction mixture consisted of 40 μL of extracts at different concentrations (range from 0.02 to 1.5 mg/mL), α-amylase solution (5 μg/mL in 0.1 M sodium phosphate buffer at pH 7.0), and 40 μL of 1% (w/v) starch solution and was incubated for 10 min at 37 °C. After the incubation period, the reaction was stopped with 20 μL of 1 M HCl. The reaction mixture was then diluted after the addition of 100 μL of iodine solution (5 mM I_2, 5 mM KI), and absorbance was measured at 580 nm (Multiskan Spectrum, Thermo Scientific, USA). Inhibitory activity was expressed as the relative absorbance difference (%) of the test natural extracts to the absorbance change of the control sample. [28]

In Vitro α-Glucosidase Activity Assay. The α-glucosidase activity was determined by measuring the release of *p*-nitrophenol from *p*NPG, as described elsewhere.[29] Briefly, 1 mg of α-glucosidase was dissolved in 100 mL of 0.1 M sodium phosphate buffer at pH 6.8, containing 200 mg of bovine serum albumin. The reaction mixture consisted of 10 μl extracts at different concentrations (range from 0.01 to 0.5 mg/mL), premixed with 490 μL of sodium phosphate buffer and 250 μL of 0.5 mM *p*NPG, and incubated at 37 °C for 5 min. After incubation, 250 μL of α-glucosidase solution was added and incubated at 37 °C for 15 min. The reaction was stopped with 200 μL of 2 M Na_2CO_3. The absorbance was measured at 400 nm on a Multiskan Spectrum spectrophotometer (Thermo Scientific, USA) and compared to the control, which contained 250 μL of PBS instead of natural extracts. Acarbose, a marketed antidiabetic drug that inhibits glycoside hydrolases, resulting in a decrease of postprandial hyperglycemia,[30] was used as a positive control of α-glucosidase inhibition.

Intestinal Digestion and Absorption Studies. Male Wistar rats (weight 220−250 g) were obtained from the Applied Pharmacology Research Center (CIFA) of the University of Navarra, Pamplona, Spain. After a 15 h overnight fast, rats were anesthetized by intraperitoneal injection of a mixture (4:1) of ketamine chlorohydrate (Ketolar; Merial SA, Barcelona, Spain) and medetomidine chlorohydrate (Domtor; Pfizer Orion, Espoo, Finland), at a dose of 0.25 mL/100 g bw. After anesthesia, laparotomy was performed, and a segment (20 cm) of jejunum was quickly excised, rinsed with ice-cold saline solution (NaCl 0.9%), and cut into eight segments of 2.5 cm each.

One end of the intestinal segment was tied and filled with 0.4 mL of Krebs−Ringer−Tris buffer (KRT) composed of 1 M NaCl, 1 M KCl, 1 M Cl_2Ca, 0.5 M PO_4H_2K, 0.5 M SO_4Mg, 1 M Tris, and 1 M HCl and containing 200 mM starch or 100 mM maltose. Different conditions were tested: without α-amylase or glucosidase enzyme (as blanks), with the respective enzymes (0.5%), and with the enzymes plus acarbose (0.5%), plus helichrysum extract (2%) or plus grapefruit extract (1%). After tying the other end of the sacs, they were placed in an Erlenmeyer containing 2.5 mL of the same KRT buffer. Sacs were incubated at 37 °C under continuous shaking and gassed with O_2; those with starch for 30 min and those with maltose for 7.5 min. After the incubation period, KRT buffer inside and outside the intestinal sacs was collected to measure glucose concentration using the HK-CP kit (ABX Pentra, Montpellier, France) adapted for Pentra C200 analyzer (HORIBA ABX, Montpellier, France). The experiment was approved by the Animal Research Ethics Committee of the University of Navarra (04/2011).

Methylglucoside Uptake in Human Caco-2 Cells. Caco-2 cells were maintained in a humidified atmosphere of 5% CO_2−95% at 37 °C and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1% penicillin (1000 U/mL), 1% streptomycin (1000 μg/mL), and 1% amphoterycin (250 U/mL). Once the cells reached 80% confluence, they were dissociated with 0.05% trypsin−EDTA and subcultured on 2.5 or 7.5 cm^2 plastic flasks at a density of 2.5×10^4 cells cm^{-2}. For uptake studies, the cells were seeded at 6×10^4 cells cm^{-2} density in 24-well culture plates. Culture medium was replaced every 2 days. Cell confluence was confirmed by microscopic observance. Experiments were performed 17−21 days post seeding.[31]

For the glucose uptake measurements, the Caco-2 cells were preincubated in serum and glucose-free DMEM, 2 h before the beginning of the experiment. After a washing with PBS, 0.5 mL of buffer containing 0.1 mM α-methylglucoside (MG) with traces of ^{14}C MG (0.2 μCi/mL) was added to the cells. Substrate uptake was measured for 15 min in the presence and in the absence of helichrysum (0.6 mg), grapefruit (0.24 mg), or the SGLT1 inhibitor phloridzin (0.5 mM).

Methylglucoside uptake was stopped with ice-cold free-substrate buffer followed by aspiration. Cells were again washed twice with ice-cold buffer and finally solubilized in 500 μL of 1% Triton X-100 in 0.1 N NaOH. Samples (100 μL) were taken to measure radioactivity on a Wallac 1409 liquid scintillation counter (Wallac Oy, Turku, Finland). Methylglucoside uptake values were corrected for protein concen-

tration, as determined by the Bradford method (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA).

Oral Tolerance Tests. Twenty-five male Wistar rats from CIFA of the University of Navarra (Pamplona, Spain), with an initial average weight of 210 g ± 12, were randomly distributed into five groups (n = 5/group) and fasted for 15 h, with free access to water. These rats were orally administered by gastric intubation (5 mL/kg bw) either water, vehicle, or natural extracts as follows: (1) control, water; (2) vehicle, starch, maltose, or glucose, 2 g/kg bw in a 30% w/v solution; (3) acarbose, vehicle in a 30% w/v solution and 5 mg/kg bw acarbose; (4) helichrysum, vehicle in a 30% w/v solution and 1 g/kg bw helichrysum; and (5) grapefruit, vehicle in a 30% w/v solution and 0.5 g/kg bw grapefruit. Glycemia was measured before (0 min) and after the oral administration (30, 60, 90, 120, and 180 min) by venous tail puncture using a glucometer and blood glucose test strips (Optium Plus, Abbott Diabetes Care, Witney Oxon, UK). The glucose content was expressed as milligrams per deciliter, and the areas under the curve (AUC) were calculated according to the formula

$$\mathrm{AUC}_{0-180\mathrm{min}} = 30 \times [G_{30} + G_{60} + G_{90} + G_{120} + (G_0 + (G_{180} \times 2)/2]$$

Glucose and Insulin Levels in Insulin-Resistant Rats. Thirty-eight male Wistar rats from CIFA of the University of Navarra (Pamplona, Spain), with an initial average weight of 260 g ± 11, were kept in an isolated room exposed to a temperature between 21 and 23 °C, controlled humidity (50 ± 10%), and a 12 h:12 h artificial light/dark cycle with water and food ad libitum. The experimental protocol was approved by the Animal Research Ethics Committee of the University of Navarra (04/2011).

The animals were randomized into two groups: control (n = 8) and HFS (n = 30). During 22 days, rats had ad libitum water and food access (standard chow diet and HFS diet, respectively). Subsequently, the rats fed the HFS diet were divided into three groups: HFS nonsupplemented (n = 10), HFS supplemented with helichrysum extract (2 g/kg bw) (n = 10), and HFS supplemented with grapefruit extract (1 g/kg bw) (n = 10). After 35 days (5 weeks) of supplementation, rats were sacrificed, trunk blood was collected, and serum was obtained for analysis of glucose and insulin. Glucose was measured using the HK-CP kit (ABX Pentra, Montpellier, France) adapted for the Pentra C200 analyzer (HORIBA ABX, Montpellier, France), whereas insulin was quantified with a specific ELISA kit following the protocol described by the manufacturer (Linco Research, St. Charles, MO, USA). Insulin resistance was evaluated by the homeostasis model of insulin resistance (HOMA-IR) formula:

$$[\text{serum glucose level (mmol/L)} \times \text{insulin level (μU/mL)}/22.5]$$

Statistical Analysis. All of the results are expressed as the mean ± standard deviation of the mean (SD). Statistical significance of differences among the groups was evaluated using a one-way ANOVA test followed by Dunnett's post hoc test or the nonparametric Kruskal–Wallis test followed by the U Mann–Whitney test. A level of probability of $p < 0.05$ was set as statistically significant. All analyses were performed using SPSS 15.0 packages of Windows (Chicago, IL, USA).

The concentration giving 50% inhibition (IC_{50}) was calculated by nonlinear regression. The dose–response curve was obtained by plotting the percentage inhibition versus concentration.

RESULTS

Composition of Helichrysum and Grapefruit Extracts. The quantification of the phenolic compounds in the two extracts (helichrysum and grapefruit) was performed by UPLC-MS/MS and is reported in Table 1. The major phenolic compound in helichrysum extract was kaempferol-3-*O*-glucoside (13.4 g/kg dried weight), but also high concentrations of chlorogenic acid-3-*O*-glucoside (2.5 g/kg dried weight), naringenin-7-*O*-glucoside (3.9 g/kg dried weight), and naringenin diglycoside (1.2 g/kg dried weight) were found. The most abundant phenolic compounds found in grapefruit extract were kaempferol rutinoside (54.2 g/kg dried weight), which is a flavonol, and naringenin-7-*O*-rutinoside (5.2 g/kg dried weight), a flavanone.

Table 1. Quantification (mg/kg extract) of Phenolic Compounds in Helichrysum and Grapefruit Extracts

phenolic compound	helichrysum	grapefruit
phenolic acids		
gallic acid	7.3	10.9
caffeic acid	67.3	20.2
chlorogenic acid	1039	109
chlorogenic acid-3-*O*-glucoside	2515	40.2
flavonoids		
flavanones		
naringenin	230	1000
naringenin-7-*O*-glucoside	3892	219
naringenin diglycoside	1188	108
naringenin-7-*O*-rutinoside (narirutin)	37	5234
naringenin-4′-glucoside-7-rutinoside	0.03	83
hesperidin	11	711
flavonols		
kaempferol	7	nd[a]
kaempferol-3-*O*-glucoside	13375	6
kaempferol rutinoside	434	54193
myricetin glucoside	652	7
flavones		
metoxiluteolin	0.8	0.03
flavanols		
epigallocatechin	nd	nd
epigallocatechin-3-*O*-gallate	29	4

[a]nd, not detected.

***α*-Amylase and *α*-Glucosidase Inhibitory Activities.** Helichrysum and grapefruit extracts showed in vitro *α*-amylase and *α*-glucosidase inhibitory activities. For example, the IC_{50} values in *α*-glucosidase inhibition assays were 0.19 mg/mL for helichrysum and 0.41 mg/mL for grapefruit (Table 2). Helichrysum reduced *α*-glucosidase activity by 9.3% and grapefruit by 15.5% at a concentration of 0.1 mg/mL, whereas acarbose-induced inhibition was 92.7%. As shown in Table 2, both extracts also inhibited *α*-amylase activity in vitro. However, helichrysum extract showed higher IC_{50} values for *α*-amylase than for *α*-glucosidase.

Table 2. Inhibitory Activity (IC_{50}) of Helichrysum and Grapefruit Extracts against *α*-Amylase and *α*-Glucosidase, Using Starch and *p*NPG as Substrates, Respectively (n = 2)

	α-amylase (IC_{50})	*α*-glucosidase (IC_{50})
acarbose (μg/mL)	4.17 ± 0.01	6.91 ± 0.01
helichrysum (mg/mL)	0.83 ± 0.05	0.19 ± 0.01
grapefruit (mg/mL)	0.42 ± 0.06	0.41 ± 0.01

Effects of Extracts on in Vitro Intestinal Digestion and Absorption of Carbohydrates. The starch and maltose digestion was evaluated by using noneverted intestinal sacs and measuring glucose levels at both sides of the sacs. In these conditions, the activity of *α*-amylase was not inhibited in the presence of helichrysum or grapefruit extracts (Figure 1A). However, when the glucose levels were measured in the basolateral side of the sacs (glucose liberated from the starch

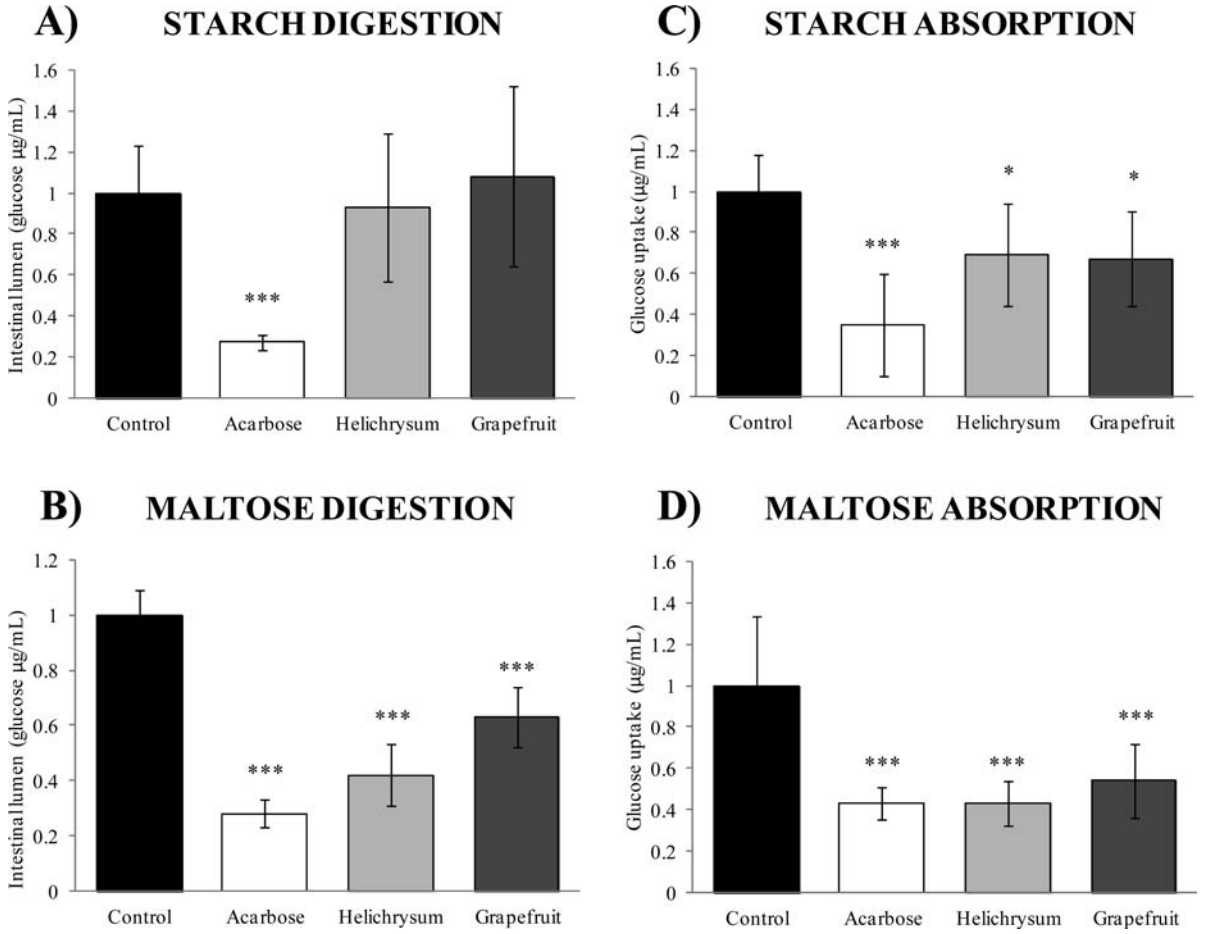

Figure 1. In vitro digestion and absorption studies using noneverted intestinal sacs with 200 mM starch (A, C) and with 100 mM maltose (B, D) as substrates, in the presence and in the absence of helichrysum extract (2%), grapefruit extract (1%), and acarbose (0.5%). Results are expressed as the mean ± SD. Statistical analysis was performed using the ANOVA test, and Dunnett's test was used to analyze differences in the mean of each group with control group (normalized to 1). (A, C) $n = 12$, (B, D) $n = 6$. *, $p < 0.05$; ***, $p < 0.001$.

that was transported from the intestinal lumen to the basolateral side), lower levels of glucose were detected in the presence of both extracts ($p < 0.05$, Figure 1C), suggesting that helichrysum and grapefruit extracts may have inhibitory activities on the intestinal glucose transporters, such as SGLT1. With regard to the intestinal α-glucosidase inhibitory activity, both extracts reduced the amount of glucose liberated from maltose inside the sacs, which means that the two extracts significantly inhibited the activity of intestinal maltase–glucoamylase (Figure 1B). Likewise, in the basolateral side, helichrysum and grapefruit extracts significantly reduced glucose uptake (57 and 46%, respectively) up to similar levels to those found for acarbose (Figure 1D).

Inhibition of SGLT1-Mediated Methylglucoside Uptake in Caco-2 Cells. In response to the above results, it was investigated whether helichrysum and grapefruit extracts could inhibit the uptake of 0.1 mM MG, a specific substrate of SGLT1. In this case, phloridzin (an SGLT1 inhibitor) was used as a positive control. As shown in Figure 2, both phloridzin and helichrysum inhibited MG uptake (56 and 54% respectively), whereas the inhibitory effect of the grapefruit extract was lower (29%). These findings strongly suggest that helichrysum and grapefruit extracts inhibited SGLT1-mediated methylglucoside uptake in enterocytes.

Effects of the Extracts on Postprandial Hyperglycemia in Rats. Oral carbohydrate (starch, maltose, and glucose) tolerance tests analyzed the effects of the extracts on postprandial

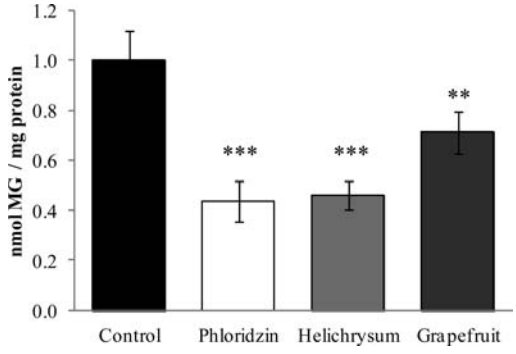

Figure 2. Inhibitory effects of helichrysum (0.6 mg) and grapefruit (0.24 mg) extracts on SGLT1-mediated MG uptake in Caco-2 cells. Results are expressed as the mean ± SD. Statistical analysis was performed using the ANOVA test, and Dunnett's test was used to analyze differences in the mean of each group with control group. Control and phloridzin $n = 6$; helichrysum and grapefruit $n = 15$. **, $p < 0.01$; ***, $p < 0.001$.

hyperglycemia in an in vivo animal model (Figure 3). In the oral starch tolerance test, the administration of both extracts and acarbose induced a significant reduction of blood glucose levels (at different times of the test) when compared with the vehicle group (Figure 3A). In the oral maltose tolerance test, helichrysum induced a significant decrease in postprandial glucose levels when the AUC was measured (Figure 3B), but

dx.doi.org/10.1021/jf4021569 | J. Agric. Food Chem. 2013, 61, 12012–12019

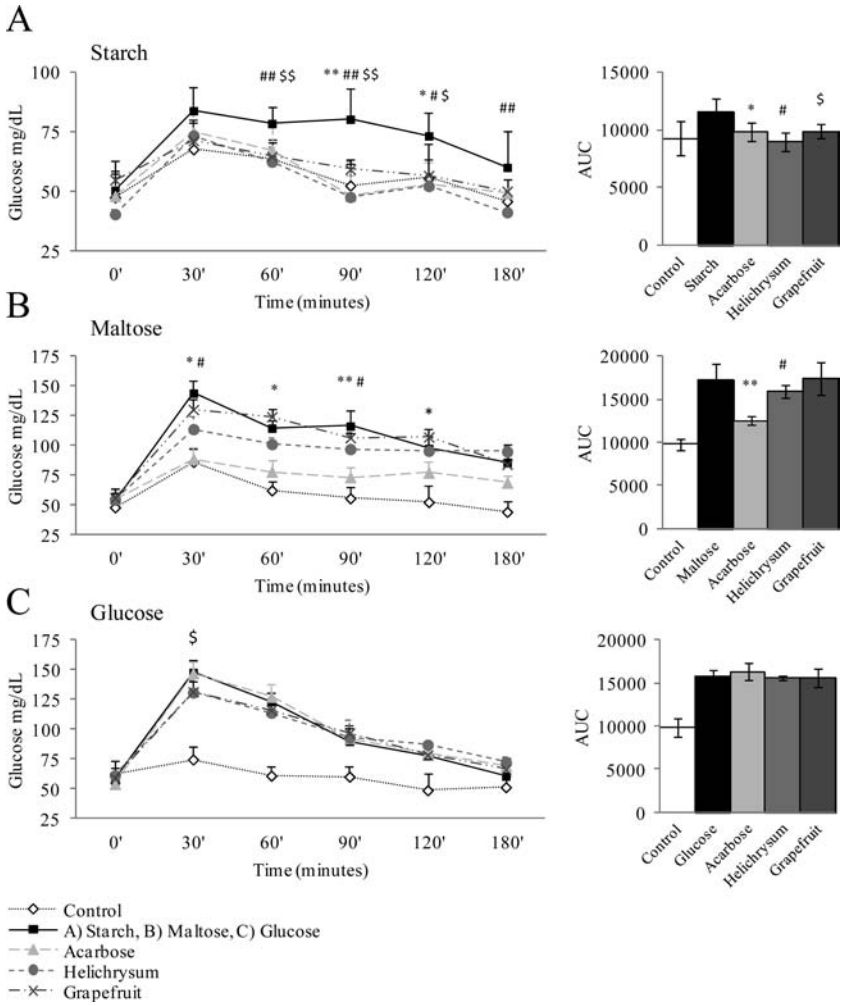

Figure 3. Effects of helichrysum and grapefruit extracts on blood glucose levels after oral (A) starch, (B) maltose, and (C) glucose administration in male Wistar rats. Results are expressed as the mean ± SD. Statistical analysis was performed using nonparametric variable (Kruskal–Wallis) followed by the U Mann–Whitney to test differences in the mean of each group (starch, maltose, and glucose) with vehicle (water) group. $n = 5$. *, $p < 0.05$; **, $p < 0.01$ (acarbose); #, $p < 0.05$; ##, $p < 0.01$ (helichrysum); $, $p < 0.05$; $$, $p < 0.01$ (grapefruit).

Table 3. Serum Glucose and Insulin Levels after 5 Weeks of Supplementation with Helichrysum and Grapefruit Extracts in a Rat Model of Insulin Resistance[a]

	groups			
	control (standard diet)	HFS nonsupplemented	HFS + helichrysum	HFS + grapefruit
glucose (mmol/L)	5.5 ± 0.3	6.1 ± 0.8	5.6 ± 0.5	5.6 ± 0.8
insulin (μU/mL)	12.1 ± 5.2*	24.1 ± 15.2	14.4 ± 4.4*	12.2 ± 4.9*
HOMA index	2.7 ± 1.0**	6.8 ± 4.6	3.6 ± 1.3*	3.0 ± 1.2**

[a]Results are expressed as the mean ± SD. Statistical analysis was performed using the ANOVA test, and Dunnett's test was used to analyze differences in the mean of each group with the nonsupplemented group. *, $p < 0.05$; **, $p < 0.01$.

no significant differences were found in the grapefruit group. Finally, in the oral glucose tolerance test, the administration of acarbose, as expected, had no effect when compared with the vehicle group (Figure 3C). However, helichrysum and grapefruit extract administration slightly decreased plasma glucose levels 30 min after oral administration, being statistically significant ($p < 0.05$) in the grapefruit group (Figure 3C).

Effects of the Extracts on Serum Glucose and Insulin Levels in Insulin Resistant Rats. The dietary supplementation with helichrysum and grapefruit extracts during 5 weeks resulted in a decrease of serum insulin levels in rats with diet-induced insulin resistance (31 and 50%, respectively, Table 3). Furthermore, the HOMA index, which is an indicator of insulin resistance, was significantly lower after the treatment with helichrysum ($p < 0.05$) and grapefruit ($p < 0.01$) extracts (47 and 54%, respectively, Table 3).

DISCUSSION

The results of this study demonstrate that helichrysum and grapefruit extracts are able to improve postprandial glycemic control in rats by reducing glucose absorption in the gastrointestinal tract. Helichrysum extract inhibited the activity of α-glucosidase in in vitro studies, being less effective for α-amylase, and also inhibited α-glucosidase activity in intestinal sacs.

dx.doi.org/10.1021/jf4021569 | J. Agric. Food Chem. 2013, 61, 12012–12019

Grapefruit extract inhibited both digestive enzymes in in vitro studies (with a similar IC_{50}) and also α-glucosidase activity in intestinal sacs. In rats, both extracts induced a reduction of postprandial hyperglycemia after an oral starch tolerance test and a decrease of hyperinsulinemia in insulin-resistant animals.

To precisely understand the mechanism of the digestive enzyme inhibitory activity, we analyzed by HPLC the phenolic compounds present in the extracts, particularly flavonoids and glycosidic flavonoids (Table 1). In previous investigations, several authors have reported that different natural sources containing flavonoids and glycosidic flavonoids are able to inhibit digestive enzyme activities in vitro.[12] In this sense, Goto et al.[9] investigated the effects of tiliroside, a glycosidic flavonoid found in strawberries, and observed an α-amylase inhibitory activity that delayed carbohydrate digestion, as well as a reduction of SGLT1 and GLUT2-mediated glucose uptake in enterocytes. The most abundant flavonoid found in our helichrysum extract was kaempferol 3-*O*-glucoside. In this sense, Pereira et al.[32] showed an in vitro inhibitory effect of kaempferol on α-glucosidase activity, and Matsui et al.[33] also reported in vitro and in vivo the inhibition of α-glucosidase by kaempferol. However, there are few studies about the inhibitory effect of *Citrus* flavonoids on digestive enzyme activities. In this context, Shen et al.[14] found that *Citrus* flavonoids may not be effective as α-amylase and α-glucosidase digestive enzyme inhibitors because of the low percentage of inhibitory activity.

On the basis of these results, we speculate that the presence of flavonoid glycosides might have contributed to the inhibitory effect on digestive enzymes, although the role of other compounds cannot be discarded. Our results showed different responses to natural extracts depending on the assay model. In vitro, helichrysum and grapefruit extracts significantly inhibited α-glucosidase activity, which is in agreement with other results showing that extracts rich in flavonoids can inhibit the activity of this enzyme.[11,13,34] Moreover, other flavonoid-rich extracts can also contribute to reduce glucose uptake by modifying the activity of other carbohydrate-digestive enzymes. For instance, Grussu et al.[10] showed that extracts from berries had α-amylase inhibitory activity in vitro. In contrast, our results showed that both extracts (helichrysum and grapefruit) are more effective inhibiting α-glucosidase than α-amylase in vitro, which is in accordance with the results reported by Rubilar et al.[35] It could be possible that interactions between some compounds occurring in the extracts, such as flavonol and flavanone derivatives, potentiated the inhibitory activity on digestive enzymes. Therefore, the identification of those natural compounds with high α-glucosidase but lower α-amylase inhibitory activity could help to prevent certain side-effects resulted by the nonspecific inhibition of α-amylase and that are mediated by the excessive accumulation of undigested carbohydrates in the large intestine.[11]

To further study the mechanisms responsible for the health properties of our extracts, ex vivo intestinal digestion and absorption studies were performed. We have taken into account three reasons to choose the doses: first, the translation of the doses to human nutrition, which are required to remain within certain limits; second, the doses that other authors have used with similar extracts; third, in vitro testing of different concentrations of 10 μL extracts that served to find the amylase inhibitory activity (range from 0.02 to 1.5 mg/mL) and the glucosidase inhibitory activity (range from 0.01 to 0.5 mg/mL). As the amylase inhibitory activity was lower for the helicrysum extract (Table 2) and the total amount of flavonoids was lower in helichrysum (Table 1), we decided to use higher doses of helichysum extract in the determinations in noneverted intestinal sacs, Caco-2 cells, and rats. The results in noneverted intestinal sacs suggest that the extracts induce a reduction of maltose digestion, which is in accordance with the maltase inhibitory activity shown by both extracts in vitro. On the other hand, a clear inhibition of starch digestion was not observed in the sacs. However, a decrease in starch-derived glucose uptake was found in the same experiment. The effect was lower on maltose than on starch, which could be partially explained because maltase–glucoamylase is an enzyme located in the gut mucosa and because maltose digestion and absorption processes are faster than those of starch. These results suggest that other mechanisms could be involved. In this sense, the results in Caco-2 cells suggest that helichrysum and grapefruit extracts can inhibit SGLT1-mediated glucose uptake. SGLT1 is a low-capacity and high-affinity transporter that can transport glucose against a concentration gradient. Manzano et al.[36] found that apple and strawberry extracts rich in different flavonoids inhibited glucose uptake in Caco-2 cells. Furthermore, Rodríguez et al.[37] reported that kaempferol 3-*O*-α-rhamnoside purified from *Bauhinia megalandra* leaves inhibited glucose absorption in rat isolated intestinal segments and suggested that this flavonol is a competitive inhibitor of intestinal SGLT1 cotransporter.

From our results, it could be hypothesized that both extracts not only inhibit carbohydrate-digestive enzymes but also reduce glucose uptake by decreasing SGLT1-mediated uptake (as demonstrated in Caco-2 cells). Previous studies have shown that phloridzin, a plant derivative glycoside, is a specific and competitive inhibitor of SGLT1 in the intestine.[38,39] In this sense, Lostao et al.[40] reported that, besides phloridzin, other phenylglucosides can act as inhibitors of SGLT1, although it was unclear whether the monosaccharide moiety or the aglycons were responsible of the phenylglucoside interaction with SGLT1. In further studies, Díez-Sampedro et al.[41] demonstrated that the structure of the aglycon determined whether or not a glucoside was a transport substrate, an inhibitor, or a noninteracting sugar. In fact, our results suggest that the high content of glycosylated flavonoids in the extracts would contribute, at least in part, to the inhibition of glucose uptake.

In the in vivo model, helichrysum and grapefruit extracts induced a reduction of postprandial glycemia after a starch overload, but only the helichrysum extract was able to reduce glycemia after oral maltose administration. Thus, although grapefruit showed strong α-glucosidase inhibitory activity in vitro, some interactions occurring in the intestinal tract might explain its lower inhibitory effect in vivo. Perhaps, as α-glucosidase is sensitive to pH, changes induced by grapefruit extract on the intestinal pH may affect the enzyme activity.[11]

The effect of the dietary supplementation of these extracts in a model of insulin resistance in rats revealed an improvement in serum insulin levels and insulin resistance after 5 weeks of treatment. These results are in agreement with Aslan et al.,[22] who demonstrated the blood glucose-lowering effect of helichrysum in diabetic rats. Concerning grapefruit, Jung et al.[42] observed a decrease in blood glucose levels in *db*/*db* mice treated with *Citrus* flavonoids (0.2 g/kg), whereas Molvihill et al.[43] found that naringenin (3% w/w) prevented hyperinsulinemia in mice with insulin resistance.

Overall, these results suggest that helichrysum and grapefruit extracts have a moderate effect on improving insulin resistance by affecting carbohydrate digestion and absorption. Although these results are positive in an animal model, it is important to study

the effects of both extracts in humans, where other factors, such as genetics and microbiota, may be involved.

In the present study, some differences in the inhibitory activities of digestive enzymes and glucose transporters were observed. Variations in extract composition, likewise, the susceptibility of the α-amylase and α-glucosidase enzymes and SGLT1 glucose transporter to different conditions, can explain the differences observed. However, these results could indicate a synergistic action of different compounds occurring in the extracts (including the flavonoids), as was observed when a combination of roselle, chrysanthemum, and butterfly pea extracts with mulberry extract showed additive interaction on intestinal maltase inhibition.[44] Nevertheless, as reported in the same study,[44] the interactions between different phenolic compounds can also reduce inhibition. These differences can also be due to the presence in the extracts of other components that may interact with the glycosylated flavonoids present in greater proportion. Thus, by comparison with other works, it can be speculated that the effects may be due to flavonoids present in the extracts, although the involvement of other compounds cannot be ruled out.

In summary, both extracts (helichrysum and grapefruit) have potential antihyperglycemic properties by acting on carbohydrate-digestive enzymes and SGLT1 glucose transporter. They also improve insulin resistance in rats. Because we have found positive effects of helichrysum and grapefruit extracts in vitro and in vivo, it can be concluded that the combination of natural compounds of the extracts can interact among them, enrich health properties of natural foods, and provide an opportunity to develop a novel class of natural agents to help manage glucose metabolism, which could be included in the diet as a supplement.

AUTHOR INFORMATION

Corresponding Author

*(J.A.M.) Postal address: Department of Nutrition, Food Science and Physiology, University of Navarra, C/Irunlarrea 1, 31008 Pamplona, Navarra, Spain. Phone: +34 948425600. Fax: +34 948425740. E-mail: jalfmtz@unav.es.

Funding

We thank Línea Especial (LE/97) from the University of Navarra (Spain), CIBERobn from Madrid (Spain), Biosearch S.A. within the framework of the CENIT PRONAOS Program granted by the Center for Industrial Technological Development (CDTI, initiative INGENIO 2010) (Spain), and Fundación Marcelino Botín for financial support. A. L. de la Garza holds a predoctoral grant from the Asociación de Amigos, University of Navarra, and also thanks the Department of Education, Universities and Research of Basque Government, for the predoctoral grant given to U. Etxeberria.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Our sincere acknowledgments to Asun Redín and Esther Gimeno for their assistance and María José Motilva from the University of Lleida for her contribution in the characterization of the extracts.

ABBREVIATIONS USED

HFS, high-fat sucrose diet; HOMA-IR, homeostatic model assessment−estimated insulin resistance; IC_{50}, concentratrion estimated to give 50% inhibition; KRT, Krebs−Ringer−Tris; OMTT, oral maltose tolerance test; OSTT, oral starch tolerance test; *p*NPG, *p*-nitrophenyl α-D-glucopyranoside; SGLT1, sodium dependent glucose transporter 1; T2DM, type 2 diabetes mellitus; UPLC, ultraperformance liquid chromatography

REFERENCES

(1) Alberti, K. G.; Zimmet, P. Z. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabetes Med.* **1998**, *15*, 539−553.

(2) Ntzani, E. E.; Kavvoura, F. K. Genetic risk factors for type 2 diabetes: insights from the emerging genomic evidence. *Curr. Vasc. Pharmacol.* **2012**, *10*, 147−155.

(3) la Fleur, S. E.; Luijendijk, M. C.; van Rozen, A. J.; Kalsbeek, A.; Adan, R. A. A free-choice high-fat high-sugar diet induces glucose intolerance and insulin unresponsiveness to a glucose load not explained by obesity. *Int. J. Obes.* **2011**, *35*, 595−604.

(4) Donner, T.; Muñoz, M. Update on insulin therapy for type 2 diabetes. *J. Clin. Endocrinol. Metab.* **2012**, *97*, 1405−1413.

(5) D'Archivio, M.; Filesi, C.; Di Benedetto, R.; Gargiulo, R.; Giovannini, C.; Masella, R. Polyphenols, dietary sources and bioavailability. *Ann. Ist. Super. Sanita* **2007**, *43*, 348−361.

(6) Kris-Etherton, P. M.; Hecker, K. D.; Bonanome, A.; Coval, S. M.; Binkoski, A. E.; Hilpert, K. F.; et al. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *Am. J. Med.* **2002**, *113* (Suppl. 9B), 71S−88S.

(7) Hanhineva, K.; Torronen, R.; Bondia-Pons, I.; Pekkinen, J.; Kolehmainen, M.; Mykkanen, H.; et al. Impact of dietary polyphenols on carbohydrate metabolism. *Int. J. Mol. Sci.* **2010**, *11*, 1365−1402.

(8) Etxeberria, U.; de la Garza, A. L.; Campión, J.; Martínez, J. A.; Milagro, F. I. Antidiabetic effects of natural plant extracts via inhibition of carbohydrate hydrolysis enzymes with emphasis on pancreatic α-amylase. *Expert Opin. Ther. Targets* **2012**, *16*, 269−297.

(9) Goto, T.; Horita, M.; Nagai, H.; Nagatomo, A.; Nishida, N.; Matsuura, Y.; et al. Tiliroside, a glycosidic flavonoid, inhibits carbohydrate digestion and glucose absorption in the gastrointestinal tract. *Mol. Nutr. Food Res.* **2012**, *56*, 435−445.

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

(11) Hogan, S.; Zhang, L.; Li, J.; Sun, S.; Canning, C.; Zhou, K. Antioxidant rich grape pomace extract suppresses postprandial hyperglycemia in diabetic mice by specifically inhibiting α-glucosidase. *Nutr. Metab.* **2010**, *7*, 71−80.

(12) Kim, J. S.; Kwon, C. S.; Son, K. H. Inhibition of α-glucosidase and amylase by luteolin, a flavonoid. *Biosci., Biotechnol., Biochem.* **2000**, *64*, 2458−2461.

(13) Kim, S. H.; Jo, S. H.; Kwon, Y. I.; Hwang, J. K. Effects of onion (*Allium cepa* L.) extract administration on intestinal α-glucosidases activities and spikes in postprandial blood glucose levels in SD rats model. *Int. J. Mol. Sci.* **2011**, *12*, 3757−3769.

(14) Shen, W.; Xu, Y.; Lu, Y. H. Inhibitory effects of Citrus flavonoids on starch digestion and antihyperglycemic effects in HepG2 cells. *J. Agric. Food Chem.* **2012**, *60*, 9609−9619.

(15) Yilmazer-Musa, M.; Griffith, A. M.; Michels, A. J.; Schneider, E.; Frei, B. Grape seed and tea extracts and catechin 3-gallates are potent inhibitors of α-amylase and α-glucosidase activity. *J. Agric. Food Chem.* **2012**, *60*, 8924−8929.

(16) Escandón Rivera, S.; González-Andrade, M.; Bye, R.; Linares, E.; Navarrete, A.; Mata, R. α-Glucosidase inhibitors from *Brickellia cavanillesii*. *J. Nat. Prod.* **2012**, *75*, 968−974.

(17) Williamson, G. Possible effects of dietary polyphenols on sugar absorption and digestion. *Mol. Nutr. Food Res.* **2013**, *57*, 48−57.

(18) Mata, R.; Cristians, S.; Escandón Rivera, S.; Juárez-Reyes, K.; Rivero Cruz, I. Mexican antidiabetic herbs: valuable sources of inhibitors of α-glucosidases. *J. Nat. Prod.* **2013**, *76*, 468−483.

(19) Kellett, G.; Brot Laroche, E. Apical GLUT2: a major pathway of intestinal sugar absorption. *Diabetes* **2005**, *54*, 3056−3062.

(20) Gouveia, S. C.; Castilho, P. C. Characterization of phenolic compounds in *Helichrysum melaleucum* by high-performance liquid chromatography with on-line ultraviolet and mass spectrometry detection. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 1851−1868.
(21) Suzgec, S.; Mericli, A. H.; Houghton, P. J.; Cubukcu, B. Flavonoids of *Helichrysum compactum* and their antioxidant and antibacterial activity. *Fitoterapia* **2005**, *76*, 269−272.
(22) Aslan, M.; Deliorman Orhan, D.; Orhan, N.; Sezik, E.; Yesilada, E. In vivo antidiabetic and antioxidant potential of *Helichrysum plicatum* ssp. *plicatum* capitulums in streptozotocin-induced-diabetic rats. *J. Ethnopharmacol.* **2007**, *109*, 54−59.
(23) Semenya, S.; Potgieter, M.; Erasmus, L. Ethnobotanical survey of medicinal plants used by Bapedi healers to treat diabetes mellitus in the Limpopo Province, South Africa. *J. Ethnopharmacol.* **2012**, *141*, 440−445.
(24) Uckoo, R. M.; Jayaprakasha, G. K.; Balasubramaniam, V. M.; Patil, B. S. Grapefruit (*Citrus paradisi* Macfad) phytochemicals composition is modulated by household processing techniques. *J. Food Sci.* **2012**, *77*, C921−C926.
(25) Park, H. J.; Jung, U. J.; Cho, S. J.; Jung, H. K.; Shim, S.; Choi, M. S. Citrus unshiu peel extract ameliorates hyperglycemia and hepatic steatosis by altering inflammation and hepatic glucose- and lipid-regulating enzymes in db/db mice. *J. Nutr. Biochem.* **2013**, *24*, 419−427.
(26) Pu, P.; Gao, D. M.; Mohamed, S.; Chen, J.; Zhang, J.; Zhou, X. Y.; et al. Naringin ameliorates metabolic syndrome by activating AMP-activated protein kinase in mice fed a high-fat diet. *Arch. Biochem. Biophys.* **2012**, *518*, 61−70.
(27) Ortega, N.; Romero, M. P.; Macia, A.; Reguant, J.; Angles, N.; Morello, J. R.; et al. Obtention and characterization of phenolic extracts from different cocoa sources. *J. Agric. Food Chem.* **2008**, *56*, 9621−9627.
(28) Xiao, Z.; Storms, R.; Tsang, A. A quantitative starch-iodine method for measuring α-amylase and glucoamylase activities. *Anal. Biochem.* **2006**, *351*, 146−148.
(29) Elya, B.; Basah, K.; Mun'im, A.; Yuliastuti, W.; Bangun, A.; Septiana, E. K. Screening of α-glucosidase inhibitory activity from some plants of Apocynaceae, Clusiaceae, Euphorbiaceae, and Rubiaceae. *J. Biomed. Biotechnol.* **2012**, DOI: 10.1155/2012/281078.
(30) de la Garza, A. L.; Milagro, F. I.; Boque, N.; Campión, J.; Martínez, J. A. Natural inhibitors of pancreatic lipase as new players in obesity treatment. *Planta Med.* **2011**, *77*, 773−785.
(31) Fanjul, C.; Barrenetxe, J.; Inigo, C.; Sakar, Y.; Ducroc, R.; Barber, A.; et al. Leptin regulates sugar and amino acids transport in the human intestinal cell line Caco-2. *Acta Physiol.* **2012**, *205*, 82−91.
(32) Pereira, D. F.; Cazarolli, L. H.; Lavado, C.; Mengatto, V.; Figueiredo, M. S.; Guedes, A.; et al. Effects of flavonoids on α-glucosidase activity: potential targets for glucose homeostasis. *Nutrition* **2011**, *27*, 1161−1167.
(33) Matsui, T.; Kobayashi, M.; Hayashida, S.; Matsumoto, K. Luteolin, a flavone, does not suppress postprandial glucose absorption through an inhibition of α-glucosidase action. *Biosci., Biotechnol., Biochem.* **2002**, *66*, 689−692.
(34) Boath, A. S.; Stewart, D.; McDougall, G. J. Berry components inhibit α-glucosidase in vitro: synergies between acarbose and polyphenols from black currant and rowanberry. *Food Chem.* **2012**, *135*, 929−936.
(35) Rubilar, M.; Jara, C.; Poo, Y.; Acevedo, F.; Gutierrez, C.; Sineiro, J.; et al. Extracts of maqui (*Aristotelia chilensis*) and murta (*Ugni molinae* Turcz.): sources of antioxidant compounds and α-glucosidase/α-amylase inhibitors. *J. Agric. Food Chem.* **2011**, *59*, 1630−1637.
(36) Manzano, S.; Williamson, G. Polyphenols and phenolic acids from strawberry and apple decrease glucose uptake and transport by human intestinal Caco-2 cells. *Mol. Nutr. Food Res.* **2010**, *54*, 1773−1780.
(37) Rodriguez, P.; Gonzalez-Mujica, F.; Bermudez, J.; Hasegawa, M. Inhibition of glucose intestinal absorption by kaempferol 3-*O*-α-rhamnoside purified from *Bauhinia megalandra* leaves. *Fitoterapia* **2010**, *81*, 1220−1223.
(38) Alvarado, F. Hypothesis for the interaction of phlorizin and phloretin with membrane carriers for sugars. *Biochim. Biophys. Acta* **1967**, *135*, 483−495.
(39) Masumoto, S.; Akimoto, Y.; Oike, H.; Kobori, M. Dietary phloridzin reduces blood glucose levels and reverses Sglt1 expression in the small intestine in streptozotocin-induced diabetic mice. *J. Agric. Food Chem.* **2009**, *57*, 4651−4656.
(40) Lostao, M. P.; Hirayama, B. A.; Loo, D. D.; Wright, E. M. Phenylglucosides and the Na /glucose cotransporter (SGLT1): analysis of interactions. *J. Membr. Biol.* **1994**, *142*, 161−170.
(41) Díez-Sampedro, A.; Lostao, M. P.; Wright, E. M.; Hirayama, B. A. Glycoside binding and translocation in Na(+)-dependent glucose cotransporters: comparison of SGLT1 and SGLT3. *J. Membr. Biol.* **2000**, *176*, 111−117.
(42) Jung, U. J.; Lee, M. K.; Park, Y. B.; Kang, M. A.; Choi, M. S. Effect of citrus flavonoids on lipid metabolism and glucose-regulating enzyme mRNA levels in type-2 diabetic mice. *Int. J. Biochem. Cell Biol.* **2006**, *38*, 1134−1145.
(43) Mulvihill, E. E.; Allister, E. M.; Sutherland, B. G.; Telford, D. E.; Sawyez, C. G.; Edwards, J. Y.; et al. Naringenin prevents dyslipidemia, apolipoprotein B overproduction, and hyperinsulinemia in LDL receptor-null mice with diet-induced insulin resistance. *Diabetes* **2009**, *58*, 2198−2210.
(44) Adisakwattana, S.; Ruengsamran, T.; Kampa, P.; Sompong, W. In vitro inhibitory effects of plant-based foods and their combinations on intestinal α-glucosidase and pancreatic α-amylase. *BMC Complement. Altern. Med.* **2012**, *12*, 110.