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***In vivo* and *in vitro* antidiabetic effects of aqueous cinnamon extract and cinnamon polyphenol-enhanced food matrix**

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Abstract

Cinnamon has a long history of medicinal use and continues to be valued for its therapeutic potential for improving metabolic disorders such as type 2 diabetes. In this study, a phytochemically-enhanced functional food ingredient that captures water soluble polyphenols from aqueous cinnamon extract (CE) onto a protein rich matrix was developed. CE and cinnamon polyphenol-enriched defatted soy flour (CDSF) were effective in acutely lowering fasting blood glucose levels in diet-induced obese hyperglycemic mice at 300 and 600 mg/kg, respectively. To determine mechanisms of action, rat hepatoma cells were treated with CE and eluates of CDSF at a range of 1–25 µg/ml. CE and eluates of CDSF demonstrated dose-dependent inhibition of hepatic glucose production with significant levels of inhibition at 25 µg/ml. Furthermore, CE decreased the gene expression of two major regulators of hepatic gluconeogenesis, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. The hypoglycemic and insulin-like effects of CE and CDSF may help to ameliorate type 2 diabetes conditions.

Keywords

Cinnamomum burmannii; cinnamon; diabetes; fasting blood glucose; glucose production

1. Introduction

Botanicals are a valuable source of therapeutics for combating escalating epidemics such as obesity, cardiovascular disease and diabetes. Over 346 million people have diabetes, of which, type 2 diabetes mellitus (T2DM) makes up 90% of these cases (World Health Organization, 2011). T2DM patients live in a chronic state of hyperglycemia due to progression of pancreatic β -cell dysfunction and insulin resistance (Muoio & Newgard, 2008). Development of T2DM can be due to environmental factors as well as genetics. Modification of diet and exercise are effective for prevention and management of T2DM

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Disclosure statement

IR has equity in Nutrasorb LLC, which has interest in developing polyphenol sorption technology.

(Kahn, Hull, & Utzschneider, 2006); (Hu, 2010). Dietary botanical supplements have demonstrated potential in disease prevention and health promotion (Graf, Raskin, Cefalu, & Ribnicky, 2010). Considering the ease of relative accessibility of dietary botanical supplements compared to prescription pharmaceuticals, scientific research supporting the efficacy and safety of botanical therapies is of paramount priority.

Cinnamon powders from the bark of *Cinnamomum* species have long been used in Ayurvedic and traditional Chinese medicines as an anti-diabetic (Leela, 2008; Xie, Zhao, & Zhang, 2011). Within the past decade, several clinical trials evaluating the effects of cinnamon powder or cinnamon extract on symptoms of diabetes have been conducted with mixed outcomes (Qin, Panickar, & Anderson, 2010). However, meta-analysis of cinnamon clinical studies limited to randomized, placebo controlled trials that measured fasting blood glucose (FBG), showed overall significant effects on lowering FBG (Davis & Yokoyama, 2011). Furthermore, the evaluation of human trials that used only aqueous cinnamon extracts showed a greater level of significance for lowering blood glucose (Davis & Yokoyama, 2011). Thus, the form in which cinnamon is administered may be important since extracts (aqueous and/or organic solvent extraction) and powders (pulverized bark material) would provide different compositions of phytochemicals with different levels of bioavailability.

A number of cinnamon phytochemicals, such as cinnamic acid, cinnamaldehyde and proanthocyanidins (PACs) have demonstrated bioactivities in cellular pathways that lead to improved glucose balance *in vivo*. PACs are one of the phytochemical classes that have demonstrated hypoglycemic effects of cinnamon (Qin et al., 2010). PACs, also known as condensed tannins, are polymers of flavan-3-ols (epicatechin and/or catechin) that are singly linked between C-4 to C-6 or C-4 to C-8 (B-type) or doubly linked with additional ester linkage between C2 and C7 (A-type) (Serrano, Puupponen-Pimi, Dauer, Aura, & Saura-Calixto, 2009). Cinnamon PACs have been reported to regulate expression of insulin signaling and glucose transport genes in adipocytes (Cao, Graves, & Anderson, 2010). Additionally, aqueous cinnamon extracts stimulated glucose uptake and glycogen synthesis in adipocytes (Jarvill-Taylor, Anderson, & Graves, 2001). A- and B-type PACs from cinnamon lowered blood glucose concentrations in streptozotocin-induced diabetic mice and increased the consumption of extracellular glucose in normal and insulin resistant hepatoma cells (Lu et al., 2011). The incorporation of cinnamon powder into a rodent diet reversed high-fat high-fructose impaired expression levels of insulin-signaling genes in the liver and muscle of rats (Couturier et al., 2011).

Although cinnamon bark powder contains beneficial phytochemicals, cinnamon is commonly used in small quantities as a flavouring agent, where the dose of bioactives is unlikely to be measurably effective in humans. Conversely, large doses or chronic ingestion of cinnamon powder may also provide an increased dose of oil components that may cause adverse effects, such as inflammation and hyperkeratosis of the fore stomach (Bickers et al., 2005). An aqueous extraction method reduces the exposure to cinnamon oil components as they are not efficiently extracted (Huang, Ho, Fu, & Pan, 2006; Anderson et al., 2004). A novel process that transfers, concentrates and preserves polyphenols from berry juices to produce a phytochemically enhanced functional food ingredient was recently reported by our group (Roopchand et al., 2012). The high content of water soluble bioactive polyphenols found in cinnamon bark can be similarly captured onto a protein rich soy flour matrix. The cinnamon polyphenol enhanced matrix would provide a protein rich dietary source of cinnamon bioactive phytochemicals.

In this study, we developed a phytochemically-enhanced functional food ingredient by capturing water soluble polyphenols from aqueous cinnamon extract (CE) onto a defatted

soy flour matrix (DSF). We investigated the hypoglycemic effects of CE and cinnamon polyphenol-enriched defatted soy flour (CDSF) orally administered to diet-induced obese hyperglycemic mice *in vivo* and hepatic glucose production *in vitro*. Additionally, to determine the mode of action we evaluated the effects of cinnamon extract on the expression of key gluconeogenesis genes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) H4IIE rat hepatoma cells.

2. Materials and methods

2.1. Materials

Dexamethasone, 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt (cAMP), DMEM (Dulbecco's modified eagle medium), sodium lactate, sodium pyruvate, and *trans*-cinnamic acid were purchased from Sigma (St. Louis, MO). High glucose DMEM, horse serum, fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Sodium bicarbonate was obtained from Acros Organics (Morris Plains, NJ) and methanol were obtained from EMD Sciences (San Diego, CA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was purchased from TCI (Portland, OR). Isopropanol and hydrochloric acid were from Fisher (Fair Lawn, NJ). Catechin and cinnamtannin B1 were purchased from Enzo Life Sciences (Plymouth Meeting, PA) and procyanidin B2 was obtained from Chromadex (Irvine, CA). All water used in the experiments was purified using a Millipore water purification system with a minimum resistivity of 18.2 MΩ·cm (Bedford, MA) unless otherwise noted.

2.2. Cinnamon extract

Fifty g of *Cinnamomum burmannii* bark powder (Frontier Natural Products Co-op, Norway, IA) was extracted with 1 L water for 1 h in a rotating flask in a water bath heated to 60 °C (method modified from Sheng, Zhang, Gong, Huang, & Zang, 2008 and Anderson et al., 2004). The aqueous cinnamon suspension was centrifuged for 15 min at 1699 rcf to pellet and remove the solids from the aqueous cinnamon solution. Ethanol was added to the aqueous cinnamon solution to 75% ethanol in order to precipitate polysaccharides. The aqueous ethanol solution was vacuum filtered through Whatman No.1 paper to remove the polysaccharide precipitate and the solvent was removed under reduced pressure by rotary evaporation. The extracts were frozen at -80 °C, lyophilized and stored at -20 °C.

2.3. Sorption of cinnamon polyphenols to soy flour matrix

Method of sorption of cinnamon polyphenols to soy flour was adapted from Roopchand et al., (2012). Cinnamon extract (CE) was dissolved in water (5 g/l) and mixed with 30 g defatted soy flour (DSF; Hodgson Mill Inc., IL). A 5 g/l concentration of CE was used to maximize solubility to allow for uniform interaction and reproducible sorption onto the soy flour matrix to produce cinnamon-DSF (CDSF). The suspension was adjusted to pH 4.5 with citric acid, mixed for 5 min on a stir plate followed by centrifugation at 1699 rcf for 15 min. The supernatant was decanted and the CDSF pellet was frozen and lyophilized. This process was repeated 4 times and aliquots of supernatant and starting cinnamon solution were used for total polyphenol (TP) and total PAC analysis. The sorption of phytochemicals to DSF was estimated by subtracting the TP or PAC content of the initial CE solution by the content in the supernatant collected after mixing with DSF and dividing by the dry mass of CDSF. To confirm the binding of polyphenols to DSF, 2 g of CDSF powder was extracted with 20 ml of 1% acetic acid in methanol:acetone:water (40:40:20) by sonication for 5 min and repeated 5 times. The extracts were pooled and dried down by rotary evaporation, lyophilized and stored at stored at -20 °C. In another set of CDSF samples, extraction of the CDSF was extended to 20 rounds of extraction to improve the recovery of PACs from the matrix. After the first 12 rounds of extraction performed as described above, the efficiency

of PAC extraction with a 5 min sonication method diminished. Therefore, three 1 h incubations with shaking, one 10 h incubation with shaking and four overnight incubations with shaking were added to further rounds of extractions to remove PACs.

2.4. Colorimetric analysis of total polyphenols and proanthocyanidins

The TP content was measured by a modified Folin-Ciocalteu method (Sharma et al., 2010; Singleton, Orthofer, & Lamuela-Raventos, 1999). Briefly, Folin-Ciocalteu phenol reagent (Sigma) was mixed 1:1 with 50% methanol. The 1N Folin-Ciocalteu reagent was added to 200 μ l of diluted sample and incubated at room temperature for 10 min. Next, 300 μ l 2M Na_2CO_3 was added, mixed by vortex, and incubated in a 40 °C water bath for 20 min. Samples were cooled on ice and centrifuged for 30 s at 7000 rcf. The supernatant was transferred to a 96-well plate and absorbance was measured at 760 nm in triplicate on a Synergy HT Multi-Detection Microplate Reader (Bio-Tek, Winooski, VT). Serial dilutions of a gallic acid standard were used to generate a calibration curve using linear regression. The results were expressed as mean gallic acid equivalents of at least three independent experiments $\pm$ SEM.

The PAC content was measured using a 4-dimethylaminocinnamaldehyde (DMAC, Sigma) method as described (Prior et al., 2010). Briefly, DMAC (0.1 %) was freshly dissolved in acidified ethanol ($\text{HCl}:\text{H}_2\text{O}:\text{EtOH}$, 12.5:12.5:75) and 210 μ l were added to 70 μ l standard or sample dilutions with a multichannel pipette in a 96-well plate. The plate was incubated at 25 °C for 20 min protected from light and the absorbance was measured at 640 nm on a microplate reader. Serial dilutions of procyanidin A2 (Extrasynthese, Genay Cedex, France) were used to generate a linear calibration curve for PACs and results were expressed as mean procyanidin A2 equivalents of at least 3 independent experiments $\pm$ SEM. All samples for colorimetric analyses were diluted to obtain absorbance readings within the linear range of standard dilutions, and between 0.2 and 0.8.

2.5. Animals and diet

All experiments with animals were carried out according to guidelines approved (# 04-023) by the Institutional Animal Care and Use Committee at Rutgers University. Five-week-old male C57Bl/6J mice were purchased from Jackson Labs and housed in cages (five mice to a cage) at a temperature of 25 °C with 12-h light-dark cycle. Initially, the mice were given *ad libitum* access to food and water. After acclimatization for one week, the diet was switched from chow (Purina, No.5015) to a very high fat diet (VHFD) containing 60% kcal fat (D12492, Research Diets, New Brunswick, NJ) to induce obesity, insulin resistance, and hyperglycemia (Surwit et al., 1995). Weekly food intake per cage and individual body weight measurements were determined throughout the study. All mice were introduced to intragastric feeding prior to treatment. During this time, a gavage administration of 0.2 ml of double distilled water was performed daily for 2–3 days prior to the scheduled experiments.

After twelve weeks on VHFD, mice were randomly divided into fasting blood glucose (FBG)-balanced experimental groups. Following a 4 h fasting period, FBG was measured prior to treatment and 6 h post treatment. The biological activity of CE was tested using an oral ingestion of 0.25 ml per 50 g body weight of water (vehicle control), Metformin[®] (300 mg/kg), CE (500, 300, or 100 mg/kg) or CDSF (600 mg/kg) ($n= 8-15$). Blood glucose levels were measured using an AlphaTrak handheld glucometer (Abbott Labs Inc., Abbott Park, IL) using test strips. Paired t-tests were used to determine significant differences between FBG levels before and after 6 h administration of each treatment.

2.6. UPLC-MS phytochemical analysis

CE solutions and CDSF eluate samples were separated and analyzed by a UPLC/MS system including the Dionex® UltiMate 3000 RSLC ultra-high pressure liquid chromatography system, consisting of a workstation with Dionex®'s Chromeleon v. 6.8 software package, solvent rack/degasser SRD-3400, pulseless chromatography pump HPG-3400RS, autosampler WPS-3000RS, column compartment TCC-3000RS, and photodiode array detector DAD-3000RS. After the photodiode array detector the eluent flow was guided to a Varian 1200L (Varian Inc., Palo Alto, CA) triple quadrupole mass detector with ESI interface, operated in negative ionization mode. The voltage was adjusted to -4.5 kV, the heated capillary temperature was 280°C , and the sheath gas was compressed air, zero grade. The mass detector was used in scanning mode from 65 to 1500 amu. Data from the Varian 1200L mass detector was collected, compiled and analyzed using Varian's MS Workstation, v. 6.9, SP2. The compounds were separated on a Phenomenex® C8 reverse phase column, size 150×2 mm, particle size $3\text{ }\mu\text{m}$, pore size $100\text{ }\text{\AA}$. The mobile phase consisted of 2 components: Solvent A (0.5% ACS grade acetic acid in double distilled de-ionized water, pH 3–3.5), and Solvent B (100% ACN). The mobile phase flow was 0.20 ml/min , and a gradient mode was used for all analyses. The initial condition of the gradient were 95% A and 5% B; for 30 min the proportion was 5% A and 95% B which was kept for the next 8 min, and during the following 4 min the ratio was brought to the initial condition. An 8 min equilibration interval was included between subsequent injections. The average pump pressure using these parameters was typically around 4300 psi for the initial conditions. Samples (2, 10 and $25\text{ }\mu\text{l}$) and standards ($2\text{--}5\text{ }\mu\text{l}$) were injected onto the UPLC column conditioned at room temperature. Catechin (5, 10, and $20\text{ }\mu\text{g/ml}$), procyanidin B2 (5, 20, 40, and $80\text{ }\mu\text{g/ml}$), cinnamtannin B1 (40, 250, 500, and $1000\text{ }\mu\text{g/ml}$), and *trans*-cinnamic acid (31.25, 125, and $500\text{ }\mu\text{g/ml}$) were used as external standards for compound identification and generation of linear calibration curves by plotting the peak area against the concentration for quantification.

2.7. Cell culture and treatment

H4IIE rat hepatoma cells (CRL-1548, American Type Culture Collection, Manassas, VA) were plated in 24-well tissue culture plates (Greiner Bio One, Frickenhausen, Germany) and grown at 37°C in a humidified incubator with 5% CO_2 to near confluence in high glucose DMEM containing 2.5% (v/v) horse serum, 2.5% (v/v) fetal bovine serum, 100 I.U./ml penicillin and 100 mg/l streptomycin (Cellgro, Manassas, VA). Cells were then washed two times with phosphate buffered saline (PBS) and incubated in serum-free media (glucose-free DMEM, pH 7.4, containing 20 mM sodium lactate, 2 mM sodium pyruvate and 4.4 mM sodium bicarbonate) overnight (18 h). Cells were treated in triplicate with 50 nM insulin, 2 mM Metformin®, media only, vehicle (water), CE ($1\text{--}25\text{ }\mu\text{g/ml}$ as noted), CDSF eluate ($1\text{--}25\text{ }\mu\text{g/ml}$ as noted) or DSF eluate ($25\text{ }\mu\text{g/ml}$) in serum-free media for 8 h. The glucose concentration in the culture medium was determined at the end of incubation with Amplex® Red Glucose Assay Kit (Invitrogen) and the protein content measured by the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). The cellular glucose output was standardized to total protein content and normalized to control. Results are the means $\pm$ SEM of three independent experiments.

2.8. Gene expression

H4IIE cells were grown to confluence as described above and washed two times with PBS before adding fresh high glucose DMEM or high glucose DMEM supplemented with 500 nM dexamethasone and 0.1 mM 8-CTPcAMP (Dex-cAMP) to induce PEPCK and G6Pase gene expression. Cells treated with Dex-cAMP were also treated with 10 nM insulin, 2 mM metformin, vehicle (water) or cinnamon extract (25, 10, 5 and $1\text{ }\mu\text{g/ml}$) for 8 h. The treatments were performed in triplicate in four independent experiments.

2.9. Total RNA extraction, purification, and cDNA synthesis

Total RNA was extracted from H4IIE cells using Trizol reagent (Invitrogen) following the manufacturer's instructions. RNA was checked for purity and quantified spectrophotometrically by absorbance measurements at 260 and 280 nm using the NanoDrop system (NanoDrop Technologies). RNA was then treated with Deoxyribonuclease I (Invitrogen), following the manufacturer's guidelines. A ratio of OD 260/280 = 2.0 was considered to be good quality RNA. The cDNAs were synthesized with 3 µg of RNA for each sample, using ABI High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The thermal cycle program was set as follows: 10 min, 25 °C; 60 min, 37 °C; 60 min, 37 °C; 5 sec, 85 °C, and final hold at 4 °C.

2.10. Quantitative PCR and data analysis

The synthesized cDNAs were diluted fivefold and 5 µl of the dilution was used for PCR with 12.5 µl of Power SYBR Green PCR master mix (Applied Biosystems), 6 µM of each primer and 7 µl BPC grade water (Sigma) to a final reaction volume of 25 µl. The primer sequences were as follows: β -actin: forward primer 5'-GGG AAA TCG TGC GTG ACA TT-3', reverse primer 5'-GCG GCA GTG GCC ATC TC-3'; PEPCK: forward primer 5'-GCA GAG CAT AAG GGC AAG GT-3', reverse primer 5'-TTG CCG AAG TTG TAG CCA AA-3'; G6Pase: forward primer 5'-TCT ACC TTG CGG CTC ACT TTC-3', reverse primer 3'-GAA AGT TTC AGC CAC AGC AAT G-5'. Quantitative PCR amplifications were performed on an ABI 7300 Real-Time PCR System (Applied Biosystems) with the following thermal cycler profile: 2 min, 50 °C; 10 min, 95 °C; 15 min, 95 °C; 1 min, 60 °C for the dissociation stage; 15 sec, 95 °C; 1 min, 60 °C; 15 sec, 95 °C.

PEPCK and G6Pase mRNA expressions were analyzed using the comparative $\Delta\Delta C_t$ method and normalized with respect to the average C_t value of β -actin. The Dex-cAMP treatment (positive control) served as the calibrator for $\Delta\Delta C_t$ analysis and was assigned a value of 1.0. All experimental samples were run in triplicate and each reaction included no-RT and no-template controls.

2.11. Cell viability

The effect of the treatments on cell viability was measured by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT; TCI, Portland, OR) assay (Mosmann, 1983). MTT was dissolved in cell-based assay buffer (Cayman Chemical, Ann Arbor, MI) at 5 mg/ml and filtered through a 0.22 µm membrane (Corning, Corning, NY). After 8 h of treatment with extracts, MTT was added to each well (0.5 mg/ml) and incubated for 3 h. The media were carefully aspirated and the formazan product was dissolved in 0.1 N HCl isopropanol; the absorbance was read at 570 nm. All treatments were performed in triplicate and three independent experiments were performed under each media condition.

2.12. Statistical analysis

The data are expressed as mean $\pm$ SEM and were analyzed by one-way ANOVA. Post hoc analyses performed using Dunnett's test for multiple comparisons to a control or paired t-test. All statistical procedures were performed with SAS 9.3 software (Cary, IN). A two-tailed p -value < 0.05 was considered statistically significant.

3. Results

3.1. Cinnamon extract phytochemistry

Aqueous cinnamon extract contained a high level of polyphenols and proanthocyanidins (Table 1). An average yield of 10.9% extract (w/w) was recovered from cinnamon powder. The CE contained 441 mg/g TP, measured by the Folin-Ciocalteu method, and 220 mg/g PACs, measured by the DMAC method. These values would correspond to 46 mg/g TP and 23 mg/g PACs in cinnamon powder. Individual PACs and cinnamic acid were identified and quantified by HPLC-MS using authentic standards. Cinnamon extract contained mean concentrations of 100 ± 38 μ g/g catechin, 1.52 ± 0.18 mg/g procyanidin B2, 5.47 ± 0.38 mg/g cinnamtannin B1 and 6.03 ± 0.56 mg/g *trans*-cinnamic acid. The sum of the monomer, dimer and trimer, quantified by UPLC-MS indicate that the aqueous extract contains 7.1 mg/g PAC, however, this value is much lower than that of the concentration of total PACs from the DMAC method as standards are not readily available to account for all PACs present in the extract. This can be partly explained by the presence of compounds with equivalent negative ion masses but differing retention times to procyanidin B2 (m/z 577, [M-H]); Fig 1a) and cinnamtannin B1 (m/z 863, [M-H]), Fig. 1b), which are likely isomers that have previously been reported in *C. burmannii* (Anderson et al., 2004). Additional masses corresponding to larger PAC oligomers have also been reported in *C. burmannii* (Anderson et al., 2004) with which the DMAC reagent would react and take into account.

3.2. Binding of cinnamon polyphenols to defatted soy flour

Cinnamon extract polyphenols were sorbed onto DSF to produce CDSF containing an estimated content of 65 mg/g TP and 35 mg/g PAC from CE. These compound classes showed an efficient reproducible binding, capturing $88 \pm 4\%$ TP and $93 \pm 2\%$ PAC present in CE. To confirm the presence of cinnamon phytochemicals in the CDSF matrix, polyphenols and PACs were removed by solvent extraction of CDSF. In addition to the colorimetric assays, the peak areas in the extracted single ion chromatograms corresponding to catechin and epicatechin (m/z 289, [M-H]), procyanidin B2 (m/z 577, [M-H]), cinnamtannin B1 (m/z 863, [M-H]), and cinnamic acid (m/z 147, [M-H]), identified and quantified by UPLC-MS, showed a clear reduction in PAC and cinnamic acid from CE solution after sorption indicating that they had been transferred to the soy flour matrix. The sorption efficiency was determined by calculating the amount of the compound in the initial solution by the amount of the compound left in the solution after the sorption process. For procyanidin B2, $87 \pm 3.4\%$ was bound to the DSF matrix and for cinnamic acid only $12 \pm 5.5\%$ was sorbed. For the trimer cinnamtannin B1, the two peaks corresponding to m/z 863, merged into one peak in the solution after sorption. By combining the two major m/z 863 peaks, we calculate that $83 \pm 0.5\%$ of trimer was sorbed. For catechin, the concentration was below the limit of quantification after sorbing since the concentration of catechin was low in the initial solution, relative to the other compounds. However, noting that the percent sorption was calculated by subtracting the peak areas of catechin, $55.1 \pm 0.1\%$ was sorbed onto the DSF matrix.

The CDSF eluates contained 290 mg/g TP and 159 mg/g PAC, confirming their presence in CDSF. Recovery of PAC and TP by solvent extraction after 6 rounds of extractions of CDSF was less than 50%. However, after extension of the extraction of CDSF to 20 rounds, 80% of the PACs were recovered. The presence of color on the matrix indicated that some PAC compounds remain tightly bound to the matrix and could not be completely extracted.

3.3. Diet induced obese hyperglycemic mice

After 12 weeks of *ad libitum* access to VHFD, mice developed FBG levels above 200 mg/dl. Acute oral administration of cinnamon extract demonstrated a dose dependent decrease in

FBG in the diet induced obese and hyperglycemic mouse model. Metformin® (300 mg/kg) and CE (500 and 300 mg/kg) showed significant differences between FBG levels before and after treatment by paired t-test ($n = 8-15$ mice per treatment) (Fig. 2a). Relative to initial FBG levels, 500, 300, and 100 mg/kg CE reduced the mean FBG by 18.9%, 14.6% and 0.3%, respectively, after 6 h. In another set of experiments, CDSF 300 mg/kg also showed a significant decrease in FBG, whereas DSF, treated with acidified water to mimic CDSF production, did not significantly lower FBG (Fig. 2b). Compared to initial FBG levels, 300 mg/kg CE, 600 mg/kg CDSF, and 600 mg/kg DSF reduced the mean FBG by 17.8%, 16.2% and 3.5%, respectively, after 6 h. Although there is a difference in percent reduction of FBG between 500 and 300 mg/kg CE, there was not a significant difference (18.9% and 14.6%, respectively). We hypothesize that the higher dose of 500 mg/kg does not further reduce the FBG because the effect of CE has reached a plateau and will not further reduce FBG in this acute test.

3.4. Glucose production

H4IIE rat hepatoma cells were cultured to measure the effects of cinnamon extract on glucose production in glucose-free media. Addition of cinnamon extract or CDSF eluate in cell culture media dose dependently inhibited the production of glucose by hepatocytes, although, only 25 $\mu\text{g/ml}$ was statistically significant (Fig. 3). However, in a separate set of H4IIE cells all concentrations of CE lowered the glucose production to a statistically significant degree (Fig 4a). Both sets of H4IIE cells showed a dose dependent trend, however improved the statistical significance in the second set of cells was most likely due smaller standard error of measurements from newly reconstituted cells. The eluates of CDSF also demonstrated dose dependent responses in decreasing glucose production by H4IIE cells whereas the eluates of acidified water treated DSF did not demonstrate an effect. The treatment of cells with isolated compounds in the concentration range of CE alone did not show significant reduction in glucose production. While cinnamic acid showed no reduction in cellular glucose production, treatment with procyanidin B2 (50 and 250 ng/ml) and cinnamtannin B1 (0.5 and 1 $\mu\text{g/ml}$) reduced glucose production on average to 75.4% and 77.5% of media control, respectively.

3.5. Gene expression

Changes in gene expression levels of key gluconeogenesis enzymes were measured to determine possible mechanisms by which CE inhibits hepatic glucose production (Fig 4a). H4IIE rat hepatoma cells were treated with Dex-cAMP to stimulate gluconeogenesis and expression of PEPCK and G6Pase genes. Treatment with CE inhibited the expression of PEPCK but was significant only at 25 $\mu\text{g/ml}$ (Fig. 4b). G6Pase gene expression was inhibited in a dose-dependent pattern by CE with significant differences in gene expression at 10 and 25 $\mu\text{g/ml}$ (Fig. 4c).

3.6. Cell viability

Cell viability assays were performed to confirm that CE was not cytotoxic. Cells were incubated with MTT reagent for an additional 3 h after 8 h treatments. There was no cytotoxicity with CE treatment (Fig. 5). At least three independent cell viability experiments were performed under each of the two *in vitro* media conditions.

4. Discussion

Cinnamon spice has a long history of medicinal use, is treasured globally, and continues to be valued for its therapeutic potential. In this study, aqueous cinnamon extract was effective in lowering FBG in diet induced hyperglycemic mice. The inhibition of hepatic glucose production through suppression of PEPCK and G6Pase gene expression is suggested as a

mechanism by which CE reduces FBG. In addition, we produced a phytochemically-enhanced functional food ingredient that efficiently captured water soluble polyphenols from CE which also demonstrated antidiabetic effects *in vitro* and *in vivo*. CE and CDSF both demonstrated hypoglycemic properties that may be beneficial for improving T2DM conditions.

A diet induced diabetic mouse model was selected for the study as it mimics the progression of diabetes due to chronic obesity and genetic susceptibility (Surwit, Kuhn, Cochrane, McCubbin, & Feinglos, 1988). The glucose lowering effects of CE and CDSF showed acute effectiveness on chronically high FBG levels due to T2DM. Previous animal studies using streptozotocin or fructose induced diabetic rodent models demonstrated enhanced insulin sensitivity, decreased blood glucose concentration, and improved glucose utilization with cinnamon treatment for 2 or 12 weeks (Couturier et al., 2011; Lu et al., 2011). Follow up *in vivo* studies may focus on the prevention and amelioration of diabetes symptoms with long term administration of CE or CDSF in the diet. Additionally, the DSF matrix would preserve cinnamon bioactives and provide protein when incorporated into diet formulations.

The data presented suggests that CE decreased FBG through inhibition of hepatic glucose production by suppressing the gene expression of two major regulators of hepatic gluconeogenesis, PEPCK and G6Pase. The expression of PEPCK and G6Pase are regulated by insulin, however, in patients with T2DM, insulin resistance results in dysregulation of hepatic gluconeogenesis leading to a hyperglycemic state (Barthel & Schmoll, 2003). PEPCK is a rate limiting transcriptionally regulated enzyme in hepatic gluconeogenesis. G6Pase converts glucose-6-phosphate to glucose in the final step of gluconeogenesis. Treatment of cell culture media with CE demonstrated concentration dependent suppression of G6Pase expression correlating with decreased glucose production (Fig. 3, 4a and 4c). While, in Fig. 3 only CE at 25 µg/ml had statistical significance and in Fig. 4 all CE concentrations had some level of statistical significance, both illustrate a dose dependent reduction in glucose production. The differences were due biological variations from newly reconstituted cells from frozen stocks in the second set of experiments with less variation within treatments. PEPCK expression was only inhibited at 25 µg/ml CE and did not demonstrate concentration-dependent inhibition of gene expression (Fig. 4b). Cell viability assays indicated that treatment concentrations used were not cytotoxic (Fig. 5). Reduction of these two enzymes has been associated with improved insulin resistance and suggests CE extract would attenuate symptoms of T2DM.

Cinnamaldehyde has been reported to improve the enzyme activities of pyruvate kinase and PEPCK in liver and kidney as well as enhance insulin release from pancreatic islets and glucose uptake through glucose transporter (GLUT4) translocation in skeletal muscle tissues of streptozotocin-induced diabetic rats (Anand, Murali, Tandon, Murthy, & Chandra, 2010). In our cinnamon extract, we did not detect ions corresponding to cinnamaldehyde by UPLC-MS analysis. Compared to *C. cassia* (1.99%) or *C. zeylanicum* bark (2.56%), cinnamaldehyde concentration was lower in *C. burmannii* bark (0.054%) by hot methanol extraction (Archer, 1988). Additionally, the aqueous extraction method used in this study would not efficiently extract volatile oils such as cinnamaldehyde. There is a possibility that cinnamaldehyde may be present below the detection limit and delivery of 25 µg/ml CE may have contained sufficient cinnamaldehyde levels to inhibit PEPCK gene expression. However, other CE components may have been responsible for inhibition of PEPCK and G6Pase gene expression as well. Further detailed chemical fractionation would be required to determine the specific bioactive components responsible for these interactions.

Cinnamic acid was another major phenolic component of CE that may have also contributed to *in vivo* hypoglycemic effects. In TNFα induced insulin resistant hepatocytes, cinnamic

acid stimulated glucose uptake and alleviation of insulin resistance through promotion of insulin receptor tyrosyl phosphorylation and up-regulation of insulin signal associated protein expression (Huang, Shen, & Wu, 2009). Although there were high levels of cinnamic acid in the aqueous extract, cinnamic acid alone did not demonstrate any effect on glucose production in hepatoma cells, whereas procyanidin B2 and cinnamtannin B1 showed modest but reproducible inhibition of glucose production. However, cinnamic acid may have contributed to the *in vivo* hypoglycemic effect of CE and CDSF.

PACs are abundant dietary antioxidants implicated in health improvement and prevention of cardiovascular disease, diabetes and cancer (Crozier, Jaganath, & Clifford, 2009). Although PACs are ubiquitously present in edible plants, dietary consumption of PACs is low. The mean daily intake of PACs by people in the United States above 2 years of age was estimated to be 57.7 mg per person; the main sources were apple, chocolate and grape (Gu et al., 2004). Cinnamon provided a much greater source of PACs, (8.1 g/100 g food) compared to apple (141 mg/100 g fresh weight), however cinnamon is commonly used as a flavoring agent rather than readily edible food. The development of a dietary food ingredient that captures PACs onto a protein rich soy flour matrix provides a palatable delivery of bioactive compounds, 35 mg/g PACs and 65 mg/g TPs. Production of CDSF from CE is straightforward, requiring mixing with water, centrifugation to separate solids, and freeze drying. The sorption of TPs and PACs to defatted soy flour occurs most likely through electrostatic interactions without covalent or ionic bond formation and PACs are known for their ability to bind and precipitate macromolecules such as proteins and carbohydrates (Bravo, 1988). The difficulty of complete PAC recovery after rigorous solvent extractions from CDSF is an indication that the binding of PACs involves stronger interactions to be investigated. An additional benefit of the sorbing process is that the DSF matrix protects the stability of polyphenols. Incubation of blueberry-DSF at 4, 25 and 37 °C for 22 weeks showed no change in concentration of total polyphenols extracted from the matrix (Roopchand et al., 2012).

Assessment of randomized, placebo controlled clinical trials with aqueous cinnamon extracts that measured FBG, showed significant effects on lowering FBG (Davis & Yokoyama, 2011). The use of an aqueous extract may provide a higher dose and/or increased bioavailability of bioactive components. On the whole, data from this study suggest that supplementation with aqueous cinnamon extract or cinnamon polyphenol enriched food matrixes show promise in managing glucose homeostasis. Further research is needed to optimize the production of CDSF and evaluate potential protective and synergistic effects from the DSF on cinnamon bioactives. In this study, CE and CDSF supplementation demonstrated benefits in short term improvement of glucose metabolism *in vivo* and *in vitro*. The data warrants continued investigation into the benefits of long term CE and CDSF supplementation on the promotion of health and prevention of metabolic-related diseases.

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References

- Anand P, Murali KY, Tandon V, Murthy PS, Chandra R. Insulinotropic effect of cinnamaldehyde on transcriptional regulation of pyruvate kinase, phosphoenolpyruvate carboxykinase, and GLUT4 translocation in experimental diabetic rats. *Chemico-Biological Interactions*. 2010; 186:72–81. [PubMed: 20363216]

- Anderson RA, Broadhurst CL, Polansky MM, Schmidt WF, Khan A, Flanagan VP, Graves DJ. Isolation and characterization of polyphenol type-A polymers from cinnamon with insulin-like biological activity. *Journal of Agricultural and Food Chemistry*. 2004; 52:65–70. [PubMed: 14709014]
- Archer AW. Determination of cinnamaldehyde, coumarin and cinnamyl alcohol in cinnamon and cassia by high-performance liquid chromatography. *Journal of Chromatography*. 1988; 441:272–216.
- Barthel A, Schmolli D. Novel concepts in insulin regulation of hepatic gluconeogenesis. *Am J Physiol Endocrinol Metab*. 2003; 285:E685–E692. [PubMed: 12959935]
- Bickers D, Calow P, Greim H, Hanifin JM, Rogers AE, Saurat JH, Tagami H. A toxicologic and dermatologic assessment of cinnamyl alcohol, cinnamaldehyde and cinnamic acid when used as fragrance ingredients: The RIFM expert panel. *Food and Chemical Toxicology*. 2005; 43:799–836. [PubMed: 15811570]
- Bravo L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews*. 1988; 56(11):317–333. [PubMed: 9838798]
- Cao H, Graves DJ, Anderson RA. Cinnamon extract regulates glucose transporter and insulin-signaling gene expression in mouse adipocytes. *Phytomedicine*. 2010; 17:1027–1032. [PubMed: 20554184]
- Couturier K, Qin B, Batandier C, Awada M, Hininger-Favier I, Canini F, Anderson RA. Cinnamon increases liver glycogen in an animal model of insulin resistance. *Metabolism*. 2011; 60:1590–1597. [PubMed: 21550075]
- Crozier A, Jaganath IB, Clifford MN. Dietary phenolics: Chemistry, bioavailability and effects on health. *Natural Products Reports*. 2009; 26:1001–1043.
- Davis PA, Yokoyama W. Cinnamon intake lowers fasting blood glucose: Meta-analysis. *Journal of Medicinal Food*. 2011; 14:884–889. [PubMed: 21480806]
- Graf BL, Raskin I, Cefalu WT, Ribnicky DM. Plant-derived therapeutics for the treatment of metabolic syndrome. *Current Opinion in Investigational Drugs*. 2010; 11:1107–1115. [PubMed: 20872313]
- Gu L, Kelm MA, Hammerstone JF, Beecher G, Holden J, Haytowitz D, Prior RL. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *The Journal of Nutrition*. 2004; 134:613–617. [PubMed: 14988456]
- Hu FB. Globalization of diabetes: The role of diet, lifestyle, and genes. *Diabetes Care*. 2010; 34:1249–1257. [PubMed: 21617109]
- Huang DW, Shen SC, Wu JSB. Effects of caffeic acid and cinnamic acid on glucose uptake in insulin-resistant mouse hepatocytes. *Journal of Agricultural and Food Chemistry*. 2009; 57:7687–7692. [PubMed: 19685889]
- Huang, TC.; Ho, CT.; Fu, HY.; Pan, MH. Cytotoxic properties of leaf essential oil and components from indigenous cinnamon (*cinnamomum osmophloeum* Kaneh). Washington, DC: 2006. p. 299-313.
- Jarvill-Taylor KJ, Anderson RA, Graves DJ. A hydroxychalcone derived from cinnamon functions as a mimetic for insulin in 3T3-L1 adipocytes. *Journal of the American College of Nutrition*. 2001; 20:327–336. [PubMed: 11506060]
- Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature*. 2006; 444:840–846. [PubMed: 17167471]
- Leela, NK. Cinnamon and cassia. In: Parthasarathy, VA.; Chempakam, B.; Zachariah, TJ., editors. *Chemistry of Spices*. Wallingford, UK: CAB International; 2008. p. 124-145.
- Lu ZL, Jia Q, Wang R, Wu XM, Wu YC, Huang CG, Li YM. Hypoglycemic activities of A- and B-type procyanidin oligomer-rich extracts from different cinnamon barks. *Phytomedicine*. 2011; 18:298–302. [PubMed: 20851586]
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. 1983; 65:55–63. [PubMed: 6606682]
- Muoio DM, Newgard CB. Molecular and metabolic mechanisms of insulin resistance and β -cell failure in type 2 diabetes. *Nature Reviews Molecular Cell Biology*. 2008; 9:193–205.

- Prior RL, Fan E, Ji H, Howell A, Nio C, Payne MJ, Reed J. Multi-laboratory validation of a standard method for quantifying proanthocyanidins in cranberry powders. *Journal of the Science of Food and Agriculture*. 2010; 90:1473–1478. [PubMed: 20549799]
- Qin B, Panickar KS, Anderson RA. Cinnamon: Potential role in the prevention of insulin resistance, metabolic syndrome, and type 2 diabetes. *Journal of Diabetes Science and Technology*. 2010; 4(3): 685–693. [PubMed: 20513336]
- Roopchand DE, Grace MH, Kuhn P, Cheng DM, Plundrich N, Poulev A, Raskin I. Efficient sorption of polyphenols to soybean flour enables natural fortification of foods. *Food Chemistry*. 2012; 131:1193–1200.
- Serrano J, Puupponen-Pimi R, Dauer A, Aura A, Saura-Calixto F. Tannins: Current knowledge of food sources, intake, bioavailability and biological effects. *Molecular Nutrition & Food Research*. 2009; 53:S310–S329. [PubMed: 19437486]
- Sharma M, Li L, Celver J, Killian C, Kovoor A, Seeram NP. Effects of fruit ellagitannin extracts, ellagic acid, and their colonic metabolite, urolithin A, on wnt signaling. *Journal of Agricultural and Food Chemistry*. 2010; 58:3965–3969. [PubMed: 20014760]
- Sheng X, Zhang Y, Gong Z, Huang C, Zang YQ. Improved insulin resistance and lipid metabolism by cinnamon extract through activation of peroxisome proliferator-activated receptors. *PPAR Research*, Article ID. 2008; 581348:1–9.
- Singleton, VL.; Orthofer, R.; Lamuela-Raventos, RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In: Packer, L., editor. *Methods in enzymology*. New York: Academic Press; 1999. p. 152-178.
- Surwit RS, Feinglos MN, Rodin J, Sutherland A, Petro AE, Opara EC, Rebuffe-Scrive M. Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and A/J mice. *Metabolism: Clinical and Experimental*. 1995; 44:645–651. [PubMed: 7752914]
- Surwit RS, Kuhn CM, Cochrane C, McCubbin JA, Feinglos MN. Diet-induced type II diabetes in C57BL/6J mice. *Diabetes*. 1988; 37:1163–1167. [PubMed: 3044882]
- World Health Organization. Diabetes fact sheet no. 312. 2011. Retrieved August 18, 2011.
- Xie W, Zhao Y, Zhang Y. Traditional chinese medicines in treatment of patients with type 2 diabetes mellitus. *Evidenced-Based Complementary Alternative Medicine*. 2011; 2011:1–13.

Highlights

- Aqueous cinnamon extract lowered fasting blood glucose in diet induced obese mice.
- Cinnamon polyphenol-enriched flour lowered blood glucose in diet induced obese mice.
- Cinnamon polyphenols inhibited hepatic glucose production.
- Cinnamon extract decreased gene expression of glucose-6-phosphatase.
- Cinnamon extract decreased gene expression of phosphoenolpyruvate carboxykinase.

1a

Fig. 1.

Representative UPLC-ESI-MS chromatograms and mass spectrographs of [M-H] ion masses from cinnamon extract. (a) Chromatogram of m/z 577, [M-H] ion scan and corresponding mass spectrographs of the two major peaks at A: 8.369 min and B: 9.732 min. Peak B corresponds to procyanidin B2. (b) Chromatogram of m/z 863, [M-H] ion scan and corresponding mass spectrographs of the two major peaks at A: 10.317 min and B: 10.634 min. Peak B corresponds to cinnamtannin B1.

Fig. 2. Effect of acute oral administration of cinnamon extract and cinnamon sorbed to defatted soy flour (CDSF) on fasting blood glucose. (a) Fasting blood glucose (FBG) levels of diet induced hyperglycemic mice prior to (black bars) and 6 hours after (gray bars) oral administration of water vehicle (VEH), Metformin[®] (300 mg/kg), or cinnamon extract (500, 300, or 100 mg/kg). (b) Diet induced hyperglycemic mice were treated with Metformin[®] (300 mg/kg), cinnamon extract (CE, 300 mg/kg), cinnamon-DSF (CDSF, 600 mg/kg) and DSF (600 mg/kg). Values are the means $\pm$ SEM ($n= 8-15$). Significant differences were determined by paired t-tests of FBG before and after treatment *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Fig. 3.

Inhibition of glucose production in H4IIE rat hepatoma cells by cinnamon extract, cinnamon-defatted soy flour (cinnamon-DSF) eluate, and defatted soy flour (DSF) eluate. Cells were serum-starved overnight prior to treatment with Metformin[®] (2 mM), insulin (50 nM), cinnamon extract (25, 6.25, 1.56 µg/ml), cinnamon-DSF (25, 6.25, 1.56 µg/ml), or DSF eluate (25 µg/ml) for 8 h. Cellular glucose output was measured by Amplex Red[®] Glucose Assay, standardized to total protein content and normalized to control (vehicle). Results are the means $\pm$ SEM of three independent experiments. Treatments were compared to control (media alone) and significant differences were determined by Dunnett's multiple comparisons test *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Fig. 4. Inhibition of (a) glucose production (b) PEPCK and (c) G6Pase gene expression in H4IIE rat hepatoma cells by cinnamon extract. Cells were serum-starved overnight prior to treatment with insulin (50 nM) or cinnamon extract (25, 10, 5 or 1 μ g/ml) for 8 h. Cellular glucose output was measured by Amplex Red[®] Glucose Assay and standardized to total protein content and normalized to control. Results are the means $\pm$ SEM of three independent experiments. Expression of PEPCK and G6Pase were stimulated with Dex-cAMP treatment in all wells, except control (white). Dex-cAMP stimulated cells were treated with insulin (10 nM) or cinnamon extract (25, 10, 5 or 1 μ g/ml) for 8 h. The levels of gene expression were measured by qRT-PCR and presented as the fold change ratio of gene expression normalized

to Dex-cAMP control using the comparative $\Delta\Delta C_t$ method. Results are the means $\pm$ SEM of four independent experiments. Treatments were compared to Dex-cAMP control and significant differences were determined by Dunnett's multiple comparisons test *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Fig. 5. Effect of cinnamon extract on H4IIE cell viability. Cells were treated identically to glucose production assay or gluconeogenic gene expression assay followed by 3 h of incubation with MTT reagent. A representative figure is presented showing no differences in cell viability under Dex-cAMP stimulated conditions.

Table 1

Total polyphenol and proanthocyanidin content in cinnamon extract, cinnamon bark powder, cinnamon defatted soy flour and eluate of cinnamon defatted soy flour. Values are expressed as the means of gallic acid (GAE) or procyanidin A2 equivalents (PAE) $\pm$ SEM.

<i>Cinnamomum burmannii</i>	Total Polyphenols GAE mg/g	Proanthocyanidins PAE mg/g
Extract	441 $\pm$ 24	220 $\pm$ 13
Bark Powder	46 $\pm$ 3	23 $\pm$ 1
CDSF	65 $\pm$ 4	35 $\pm$ 2
CDSF Eluate	290 $\pm$ 20	159 $\pm$ 10