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RESEARCH ARTICLE

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Inhibition of the facilitative sugar transporters (GLUTs) by tea extracts and catechins

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Abstract

Tea polyphenolics have been suggested to possess blood glucose lowering properties by inhibiting sugar transporters in the small intestine and improving insulin sensitivity. In this report, we studied the effects of teas and tea catechins on the small intestinal sugar transporters, SGLT1 and GLUTs (GLUT1, 2 and 5). Green tea extract (GT), oolong tea extract (OT), and black tea extract (BT) inhibited glucose uptake into the intestinal Caco-2 cells with GT being the most potent inhibitor (IC_{50}: 0.077 mg/mL), followed by OT (IC_{50}: 0.136 mg/mL) and BT (IC_{50}: 0.56 mg/mL). GT and OT inhibition of glucose uptake was partial non-competitive, with an inhibitor constant (K_i) = 0.0317 and 0.0571 mg/mL, respectively, whereas BT was pure non-competitive, K_i = 0.36 mg/mL. Oocytes injected to express small intestinal GLUTs were inhibited by teas, but SGLT1 was not. Furthermore, catechins present in teas were the predominant inhibitor of glucose uptake into Caco-2 cells, and gallated catechins the most potent: CG > ECG > EGCG ≥ GCG when compared to the non-gallated catechins (C, EC, GC, and EGC). In Caco-2 cells, individual tea catechins reduced the SGLT1 gene, but not protein expression levels. In contrast, GLUT2 gene and protein expression levels were reduced after 2 hours exposure to catechins but increased after 24 hours. These in vitro studies suggest teas containing catechins may be useful dietary supplements capable of blunting postprandial glycaemia in humans, including those with or at risk to Type 2 diabetes mellitus.

KEYWORDS

catechins, facilitative glucose transporters (GLUTs), glucose uptake, sodium-dependent glucose transporter (SGLT1), tea extract

Abbreviations: BBM, brush border membrane; BT, black tea extract; C, (+)-catechin; CG, (+)-catechin gallate; EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin gallate; GC, (+)-gallocatechin; GT, green tea extract; GCG, (+)-gallocatechin gallate; HPLC, high performance liquid chromatography; OT, oolong tea extract; PZ, Phlorizin; TF, theaflavin.

Dejiang Ni and Zeyi Ai have contributed equally to this work and should be considered as co-first authors.

1 | INTRODUCTION

Tea is produced from the buds and leaves of the plant *Camellia sinensis* (L.) O. Kuntze and is one of the most widely consumed beverages in the world. According to different manufacturing processes, tea is generally classified into three types: non-fermented tea, partially fermented tea, and fully fermented tea.[1] Green tea is a non-fermented type and contains 20%-42% catechins (dry weight), which are flavonoid polyphenols that are considered to have number of potential health benefits, including a reduced risk of certain cancers, cardiovascular disease, and Type 2 diabetes mellitus (T2DM).[2] During black tea (fermented tea) manufacture, catechins in fresh leaves are oxidized into quinones by polyphenol oxidase and condensed to form theaflavins and thearubigins,[3] which results in a significant decrease of catechins (3%-10% of dry weight). Oolong tea is partially fermented, and so catechin content is intermediate between green and black teas (8%-20% of the total dry matter).

Diabetes mellitus is a chronic metabolic disorder characterized by a persistent increase in blood glucose concentrations above normal values (ie, hyperglycemia). Poor glycemic control in people with diabetes is associated with an increased risk in micro- and macrovascular complications, such as retinopathy and cardiovascular disease, respectively.[4,5] T2DM is a serious public health issue both in developed and developing countries, and it has become a global epidemic with more than a doubling of people with this metabolic disorder in the last 25 years.[6] The first line of treatment of T2DM patients, in addition to changes to dietary and exercise regimens, is the use of oral antihyperglycemic agents, such the biguanide metformin (the most commonly used), sulfonylureas, α-glucosidase, and dipeptidyl peptidase-4 inhibitors, and glucagon-like peptide 1, an incretin mimetic.[7] However, although these medications are known to ameliorate glycemic control, a range of side effects can occur including nausea, gastrointestinal symptoms (eg, diarrhea, bloating), and increased risk of liver disease and bladder cancer.[8] Moreover, there are substantial concerns about the increase in global health expenditure associated with diabetes and affordability of medication, especially in developing countries.[9] In response to these concerns, there has been an increased interest in investigating the use of antidiabetic components of foods and drinks as dietary adjuncts for the management and prevention of T2DM.[10-12] The blood glucose lowering property of tea phenolics has received particular attention.

The consumption of tea flavonoids and other polyphenolic compounds are reported to have health benefits in individuals with or at risk of T2DM, for example, by improving glucose tolerance, enhancing insulin sensitivity, and reducing oxidative stress.[13,14] The effects of tea on postprandial glycaemia has been attributed to tea polyphenolics, mainly the catechins ((+) catechin, (−) epicatechin, (−) epicatechin gallate, (−) epigallocatechin, and (−) epigallocatechin gallate),[15] which are reported to inhibit the digestion kinetics of available carbohydrates (eg, starch) and glucose absorption in the proximal gut.[2,16-18] Tea and individual catechins are effective inhibitors of intestinal α-amylase and α-glucosidase, delaying starch and disaccharide digestion, respectively.[19-21] Epicatechin gallate (ECG) is also a potent inhibitor of glucose uptake into intestinal cells and a competitive inhibitor of the Na^+ dependent glucose transporter (SGLT1), which is expressed on the apical membrane of small intestinal enterocytes.[22,23] However, the effects of tea catechins on the facilitative sugar transporters expressed in the small intestine are less well understood. GLUT2 is a facilitative sugar transporter expressed on the basolateral membrane of the enterocyte and is responsible for the exit of glucose and fructose from the enterocyte into portal blood. Under conditions of high simple sugar feeding and in diabetes, GLUT2 is also expressed on the apical membrane, contributing to increased intestinal sugar absorptive capacity.[24] In experimental diabetes, GLUT1 has also been detected on the apical membrane of enterocytes.[25] The facilitative fructose transporter, GLUT5, is also present in the small intestine, expressed on the brush border membrane (BBM) and responsible for the absorption of dietary fructose from the lumen into the enterocyte.[26] Thus, the small intestinal facilitative sugar transporters are also potential therapeutic targets for lowering blood sugar levels.

In the present study, we investigated the acute effects of green, oolong, and black tea extracts on reducing glucose uptake in human intestinal Caco-2 cells, a well-established in vitro model of the human enterocyte, which expresses many of the known intestinal sugar transporters, including SGLT1, GLUT1, GLUT2, GLUT3, and GLUT5.[27-29] Xenopus oocytes, which express individual intestinal sugar transporters, were also used to identify the transporters inhibited by the tea samples. In Caco-2 cells, we also studied the short- and long-term effects of individual tea catechins on SGLT1 and GLUT2 gene and protein expression profiles.

2 | MATERIALS AND METHODS

2.1 | Materials

Green, oolong, and black teas were purchased from Changchen Tea Factory in Hubei Province, Anxi Jinghe Tea Company in Fujian Province and Chenyuan Tea Factory in Yunnan Province, China, respectively. The teas were all grown in the same region and harvested between April 23rd and 25th. Differences in catechin content are therefore solely due to the manufacturing process, not the harvest location, the time of year or different growth conditions.[30] They were identified as three-grade tea according to the Tea

Quality Standard of China (GB/T 14456-93, GB/T19598-2006, and GB/T 13 738.1-2008, respectively). Stock cultures of Caco-2 cells (TC7 clone-human adenocarcinoma) were obtained from Dr Edith Brot-Laroche, INSERM, Paris, France. The standards of (−)-epicatechin (EC), (+)-catechin (C), (−)-epigallocatechin (EGC), (+)-gallocatechin (GC), (−)-epicatechin gallate (ECG), (+)-catechin gallate (CG), (−)-epigallocatechin gallate (EGCG), (+)-gallocatechin gallate (GCG), and theaflavin (TF) were purchased from Sigma-Aldrich Chemical Reagent Co. Ltd., UK; Dulbecco's Modified Eagle Medium (DMEM), non-essential amino acids, and trypsin were obtained from Invitrogen Ltd., UK; Fetal Bovine Serum was purchased from PAA Laboratories GmbH, Austria; Penicillin/streptomycin was purchased from Hyclone Laboratories Inc; Plasmocin was obtained from Corporate Headquarters, USA; Biodegradable Scintillation Solution was obtained from Atlanta, Georgia, USA. D-[^{14}C] glucose was purchased from Perkin Elmer, Boston, USA. Radio immunoprecipitation assay (RIPA) lysis buffer and phenylmethanesulfonyl fluoride (PMSF) were obtained from Beyotime (Shanghai, China). BCA protein assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Polyvinylidene Fluoride (PVDF) membranes were obtained from Atto Co. (Tokyo, Japan). Rabbit SGLT1 antibody and rabbit GLUT2 antibody were purchased from Abcam (Cambridge, UK). Enhanced chemiluminescence (ECL) reagents, mouse β-actin antibody, goat anti-rabbit, and goat anti-mouse secondary antibody were all obtained from Proteintech Group (Wuhan, China). Rat GLUT1 (rGLUT1) was obtained from GW Gould (Dartmouth Medical School, Hanover, New Hampshire, USA) in the vector pSP64T containing a 1.58 kb insert and an ampicillin gene). Human GLUT2 and rat GLUT5 were obtained as a plasmid construct from GI Bell and CF Burant (University of Chicago, USA). Rat SGLT1 (Clone ID: 7129017) was obtained from Open biosystems (Thermo Scientific, GmBH). All other chemicals used were analytical grade.

2.2 | Methods

2.2.1 | Preparation of the tea extracts

The tea extracts (TEs) were prepared according to the method as described previously.[31] Briefly, 100 g of each tea sample was ground (milled) and subjected to three successive extractions with 1200 mL of distilled water at 100°C. In every extraction, the tea was left to infuse for 10 minutes at 60°C. The three aqueous extracts were pooled and filtered through filter paper. The filtrates were concentrated using a vacuum rotary evaporator at 45°C, and then, freeze-dried. The tea extract powders were stored at −80°C before being dissolved in water/1% of DMSO.

2.2.2 | Determination of catechins in TEs

Catechins were determined by high performance liquid chromatography (HPLC) at Finlay Tea Extracts Ltd, UK, as described by in our previous work.[32] Briefly, the tea extract powders (0.2 g) were resolved with 10 mL of 70% methanol (vol/vol), then, the solution was filtered through a 0.45 μm membrane. The separation was performed on an ODS reversed-phase column (4.6 mm i.d. × 250 mm, TC-C18, Agilent Technologies Inc, CA, USA) in an Agilent LC1260 system (Agilent Technologies Inc, CA, USA) with a mobile phase (vol/vol) of 9% acetonitrile containing 0.2% of ethylene diamine tetraacetic acid (EDTA) and 2% of acetic acid, at a flow-rate of 1.0 mL/min. Catechins were detected using a diode array detector (DAD) at a wavelength of 278 nm.

2.2.3 | Cell culture

Caco-2 cells were cultured according to the method described previously.[33] Briefly, stock cultures of Caco-2 cells were seeded in 25 cm^2 plastic flasks and cultured in a 95% of air/5% of CO_2 atmosphere in DMEM, supplemented with 10% of (vol/vol) fetal bovine serum, 1% of penicillin/streptomycin, 1% of non-essential amino acids, 1% of L-glutamine, and 0.25 mg plasmocin. All experiments were carried out on cells between passage numbers 43 and 47 and the cells were seeded at a density of 4×10^4 cells/cm^2 into 24-well plates (Costar, UK, Buckinghamshire, UK) and were grown for 16-20 days.

2.2.4 | Glucose uptake by Caco-2 TC7 cell monolayers

Caco-2 cells were grown to confluence. The cells were permitted to come to room temperature for 15-30 minutes, then, washed three times with Krebs buffered salt solution (KBSS, pH 7.2-7.4; 137 mM of NaCl, 4.7 mM of KCl, 1.2 mM of K_2HPO_4, 1.5 mM of $CaCl_2$, 1.2 mM of $MgSO_4$, and 10 mM of HEPES). Caco-2 cells were incubated for 10 minutes at room temperature with tea extracts (dissolved in KBSS) or polyphenols (dissolved in DMSO, and then, diluted with KBSS) prior to experiments. Uptake was initiated by the addition of either the control or test solutions containing tracer D-[^{14}C] glucose and the reaction was terminated after 2 minutes by aspiration of the uptake media (KBSS) followed by the addition of ice-cold phosphate-buffered saline (PBS). The monolayer was washed three times with ice-cold PBS, and then, solubilized in 0.5 mL of 200 mM NaOH for 1 hours at 37°C prior to scintillation counting. Aliquots of 400 μL were added to 2.5 mL of scintillation solution for radioactivity determination (LS 6500 Multi-Purpose Scintillation

Counter, USA) and the remainder used for protein measurement by Coomassie Brilliant Blue G (Protein Quantification Kit-Rapid, Sigma-Aldrich Chemical Reagent Co., Ltd., UK).

2.2.5 | Oocyte isolation and injection

Batches of *Xenopus laevis* oocytes were provided by the European Xenopus Resource Centre of the University of Portsmouth (UK). Oocytes were isolated from *Xenopus laevis* and injected with mRNA using established methods.[34] Briefly, mature adult female frogs were anesthetized with 3-amino benzoic acid ethyl ester (2 g/750 mL) in ice water. Frog ovaries were resected, and their ovarian lobes opened and incubated in OR-2 without calcium (5 mM of HEPES, 82.5 mM of NaCl, 2.5 mM of KCl, 1 mM of $MgCl_2$, 1 mM of Na_2HP0_4, 100 μg/mL of gentamicin, pH 7.8) with collagenase type IV (2 mg/mL) for 30 minutes at 23°C. Individual oocytes were isolated and transferred to OR-2 containing 1 mM of $CaCl_2$ and maintained at 18-20°C until injection with mRNA. SGLT1 and GLUT cRNA were prepared by cutting plasmid vectors with appropriate restriction enzymes followed by in vitro transcription utilizing SP6, T7, or T3 mMessage, mMachine (Ambion). cRNA was loaded into capillary pipettes generated using a micropipette puller (P-77, Sutter, Novato, CA), and oocytes were injected using a pressure-controlled injector (Drummond Nanoject 11, USA). Injection volumes were between 30 and 50 nL, and cRNA concentration 0.5-1 mg/mL. Postinjected oocytes were incubated at 20°C in OR-2 containing 1 mM of pyruvate with daily media changes. Experiments were performed on 3-5 days after oocytes were injected with mRNA.

2.2.6 | Oocyte transport protocol

Transport of [^{14}C] D-glucose, D-fructose and 2-[1,2- 3H] deoxy-D-glucose (26.2 mCi/mmol) was examined using groups of 10-20 oocytes injected to express SGLT1 and GLUTs or water injected sham oocytes at 23°C in OR-2 containing 0.5-1 μCui/mL of labeled sugar with added non-labeled sugar for 10 minutes. After incubation in uptake medium, oocytes were immediately washed four times with ice cold PBS solution. Phlorizin, tea extract, and individual catechins were added to the incubation medium as described in the results section. Individual oocytes were dissolved in 500 μL of 10% SDS and internalized radioactivity measured using scintillation spectrometry.

2.2.7 | Quantitative real time RT-PCR

The Caco-2 cells were seeded in 6-well plates (Corning Costar Co., NY, USA) at a density of 4×10^4 cells/cm^2 and were cultured for 21 days. After incubation with different tea catechins (200 μM in full culture medium) for 2 hours or 24 hours, the total RNA from the Caco-2 cells was isolated with a Total RNA Kit (Aidlab, Beijing, China). First-strand cDNA was synthesized using a cDNA synthesis Kit (Aidlab, Beijing, China). The gene expression of SGLT1 and GLUT2 was examined by quantitative real-time PCR with the SYBR Green qPCR Mix (Aidlab, Beijing, China) in an ABI 7500 Real-Time System (Applied Biosystems, USA). The reaction was carried out in a 10 μL RT-reaction mixture containing 5 μL of 2× SYBR Green qPCR Mix, 0.2 μL of forward and reverse primers (10 μmol/μL), and de-ionized water to a final volume of 10 μL, and was performed under the following cycling conditions: an initial denaturing step of 10 minutes at 95°C, followed by 40 cycles consisting of 5 seconds at 95°C and 34 seconds at 60°C.

All of the qPCR experiments were repeated three times. The data were analyzed using the $2^{-\Delta\Delta CT}$ method.[35] All of the primers were synthesized by Sangon Biotech (Shanghai, China), and their sequences are depicted in Table 1.

2.2.8 | Western blot

After incubation with each of the tea catechins (200 μM in full culture medium) for 2 hours or 24 hours, 5 to 8×10^6 Caco-2 cells were washed three times in precooled PBS buffer. The total lysate was prepared on ice by a 150 μL RIPA lysis buffer containing 1% of (vol/vol) PMSF. The lysates were centrifuged at 13,000 rpm for 20 minutes at 4°C, then, the protein-containing supernatant was collected, and the protein content determined using the BCA protein assay kit. Western blotting was performed according to standard techniques, as previously described.[1] Briefly, the proteins were separated by SDS-polyacrylamide gel electrophoresis and were then transferred to a PVDF membrane in transfer buffer (containing 192 mM of glycine, 20 mM of Tris, and 20% of methanol) at 4°C for 120 minutes at a constant 200 mA current.

TABLE 1 Primer sequences for qPCR assays

Gene	Forward primer	Reverse primer
SGLT1	TCGCCATTTCTTTCATCACCATCG	GTGCTGCTCTAGCCCACAAAATAG
GLUT2	ACTCAACCAGCATTTTTCAGACGG	GAAGCTGACAAAGAGGAAGATGGC
β-actin	CAAGATCATTGCTCCTCCTGA	AGTCCGCCTAGAAGCATTTG

After incubation, the membrane was blocked in Tris-buffered saline (TBST, containing 1.5 mM of Tris-base, 8.5 mM of Tris-HCl, 150 mM of NaCl, and 0.1% of SDS, pH 7.5) supplemented with 5% of low-fat milk for 120 minutes. Then, the membrane was incubated with primary antibodies against SGLT1 or GLUT2 (1:2000) or β-actin (1:40 000) at 4°C overnight. After being washed with TBST, the membrane was incubated with peroxidase-conjugated secondary antibodies for 60 minutes at room temperature. The protein blots were displayed using ECL reagents and quantified using BandScan software.

2.3 | Kinetic analysis of inhibition of glucose transport by tea extracts and by catechins

Data for the rates of 1, 3, and 6 mM of glucose uptake into Caco-2 cells in the presence and absence of 0-1 mg/mL of green tea extract were analyzed by fitting to the Eadie-Hofstee (EH) and Hanes-Wolfe (HW) linear transformation of the Michaelis-Menten equation. For EH plots, v is plotted against v/[S] where v is the reaction velocity and [S] is the concentration of glucose. The slope of such plots is equal to -K_m so that if plots obtained at different concentrations of inhibitor are parallel, that is, K_m is unchanged, the inhibition is non-competitive. For HW plots, [S]/v is plotted against [S] and an intercept on the [S] axis is - K_m. A common intercept (ie, an unchanged K_m value) of plots with increasing slope is indicative of non-competitive inhibition. Preliminary values of the kinetic parameters (V_{max}, K_m, K_i, and beta) were also calculated in SigmaPlot using an exploratory non-linear regression analysis function for non-competitive inhibition (partial). The nature of Green tea inhibition of glucose transport was studied more fully in further experiments by obtaining data that could be analyzed by modified Dixon plots [36] and plots for partial non-competitive inhibition.[37]

Data for the rates of 1 mM of glucose uptake into CaCo-2 cells in the presence of green, oolong, and black tea extracts ranging in concentration between 0 and 2 mg/mL and pure catechins (0-0.1 mg/mL; equivalent to 0-200 μM) were analyzed in Sigma Plot, and a best fit curve for each extract and pure catechin plotted using the non-linear regression analysis equation $y = \min + (\max - \min)/1 + (x/IC_{50})$-hillslope four parameter logistic curve.

For non-competitive inhibition, the equation for the modified Dixon plot[36] is: $v0/vi = (1 + [I]/K_i)$ where $v0$ and vi are the rates in the absence and presence of inhibitor concentrations [I], respectively, and K_i is an inhibition constant. When inhibition is non-competitive the $v0/vi$ plots intersect on the x axis at $-Ki$. IC_{50} values are readily estimated from $v0/vi$ plots and when K_i and IC_{50} values are similar this provides good evidence that the mode of inhibition is pure non-competitive.

Partial non-competitive inhibition occurs when the protein-inhibitor complex is still partially active. For such behavior, Dixon $1/v$ against [I] and $v0/vi$ against [I] plots are hyperbolic (non-linear). Linear analysis is possible, however, by the use of Whiteley plots, $v0/(v0-vi)$ against 1/[I].[37] For such plots, the intercept on the X axis equals $-\beta/K_i$ where $\beta = V_{imax}/V_{max}$; and V_{imax} is the maximum rate at infinite concentrations of inhibitor.

2.3.1 | Statistical analysis

Statistical analysis was performed using the appropriate statistical software (SAS Institute, NC). All the data were expressed as mean ± standard deviation (SD). Pearson's correlation analysis of tea catechin levels and the calculated IC_{50s} for tea extracts was undertaken in Excel. The statistical significances of the difference between the various groups were analyzed using Statistical Analysis with One-way ANOVA and the Bonferroni test for multiple comparison test correction. For comparison between the two groups, Student's t test was used. Significant differences were accepted at $P < .05$.

3 | RESULTS

3.1 | Effect of tea extracts on the glucose uptake in Caco-2 cells

The Caco-2 cell system is a colon carcinoma cell line that is a well-established model of the human enterocyte. During cell culture the cells differentiate to produce a BBM and basolateral membrane that express many of the nutrient transporters found in the gut in vivo. SGLT1 has been detected on the apical membrane and GLUT5 and GLUT2 on both the basolateral and apical membrane.[38] GLUT1 and GLUT3 are also expressed in the cancer cell[27] and may also be contributing to the detected uptake of sugar, although during differentiation their levels have been shown to decrease. When Caco-2 cells are cultured in flasks the cells will differentiate between days 10-21. During this time a basement (or basolateral) membrane will develop and be in contact with the plastic flask and the apical membrane will develop and be in contact with the media. Tight junctions will also develop effectively sealing the apical membrane from the basolateral membrane. Measured uptake of glucose is therefore via SGLT1 and GLUTs expressed on the apical membrane.

The kinetics of D-glucose uptake into the Caco-2 cells was investigated by varying the D-glucose concentrations (1 mM, 3 mM, and 6 mM), in the absence or presence of GT (0-1 mg/mL). SGLT1 has a K_m of 0.1-0.6 mM, GLUT1 and GLUT3 a K_m of 1-5 mM, and GLUT2 a K_m of 15-17 mM[39] and so the glucose concentration range we used is sufficient

to provide a guide to the basis of the inhibition in Caco-2 cells expressing a mixture of SGLT1 and GLUTs. The Eadie-Hofstee and Hanes-Wolfe plots (Figure 1A,B) indicated clearly that the K_m was not affected by the presence of the inhibitor indicating that the type of inhibition was non-competitive. The results were also analyzed by non-linear regression (Figure 1C); however, the limited number of substrate concentrations means that the values obtained for the kinetic parameters K_m and K_i should not be overinterpreted. The approximate values provided by the non-linear analysis were 19 mM and 0.022 mg/mL for K_m and K_i, respectively. The calculated K_m is consistent with GLUT2 K_m of 15-17 mM, although SGLT1 and other GLUTs will be contributing toward the calculated transport of glucose.

The green tea extract (GT), oolong tea extract (OT), and black tea extract (BT) all inhibited glucose uptake into the intestinal Caco-2 cells in a dose-dependent manner (Figure 2). The inhibition curves for GT and OT plateaued at the highest tea concentrations suggesting that the inhibition may be partial, that is, some transport activity remains even when the transporters are fully "saturated" with respect to the inhibitor. Dixon type plots of GT and OT inhibition data became non-linear as the concentration of inhibitor increased (data not shown) indicating inhibition was partial non-competitive. Whiteley plot analysis of the GT and OT data for partial non-competitive inhibition (Figure 2A,B inserts) reveals a better fit of the data. In contrast, the Dixon type plots of BT inhibition data were reasonably linear (Figure 2C insert), which indicated that inhibition by black tea is fully non-competitive. The K_i and IC_{50} values (mg/ml) calculated from the various plots were, 0.032 and 0.077, 0.057 and 0.136, and 0.36 and 0.56 for GT, OT, and BT, respectively. Green tea extract is the most potent inhibitor of glucose uptake in vitro, followed

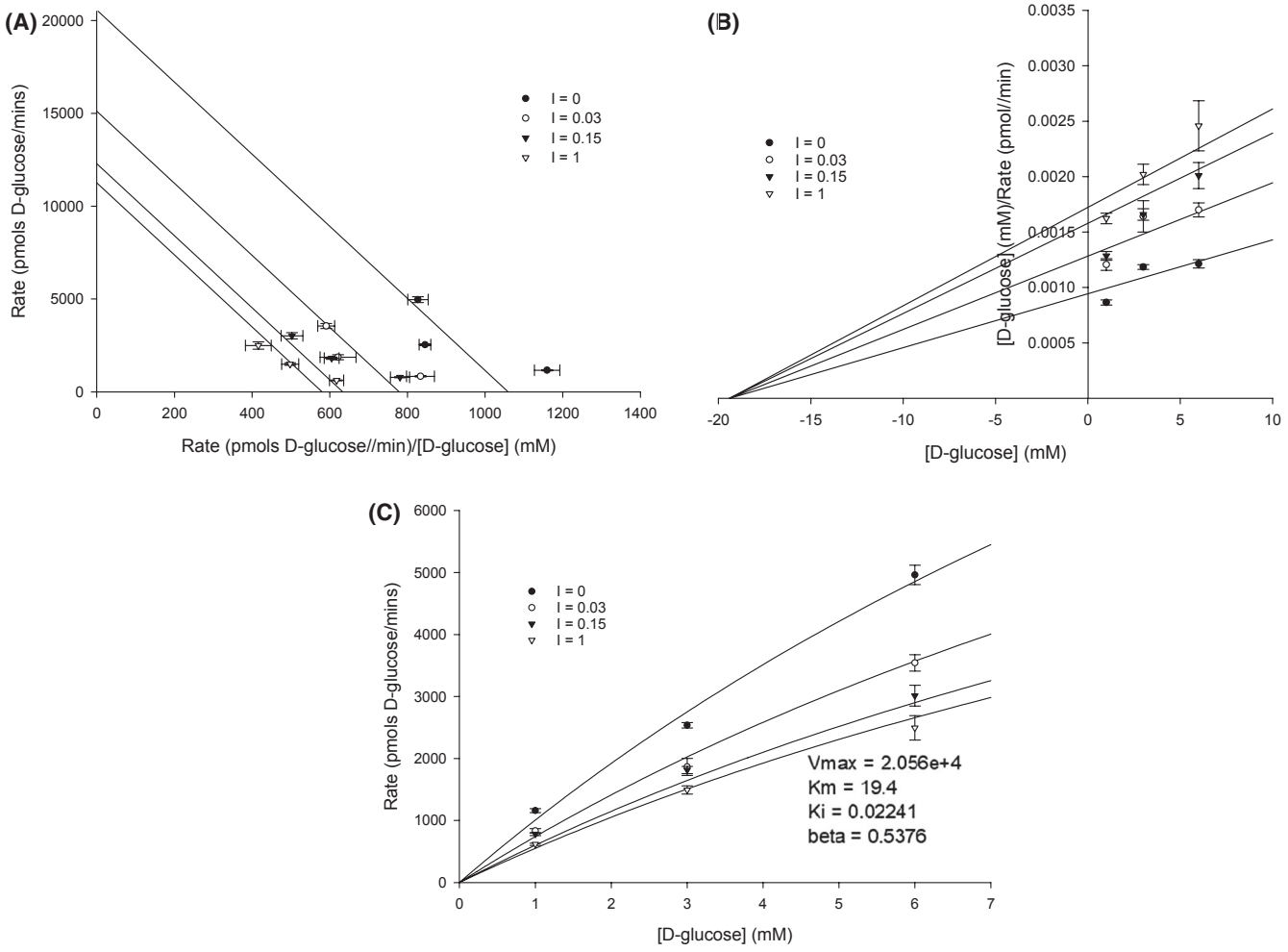

FIGURE 1 Concentration-dependent uptake of glucose (1-6 mM) by Caco-2 cells in the presence of GT at a concentration of 0 mg/mL (●), 0.03 mg/mL (○), 0.15 mg/mL (▼), or 1.0 mg/mL (▽). Caco-2 cells were incubated for 2 minutes in an uptake medium containing 1-6 mM D-[^{14}C] glucose at room temperature in the presence of GT. A, Eadie-Hofstee B, Hanes-Wolfe plots, and C, Non-linear regression analysis of glucose uptake; data are presented as the mean ± SD (N = 4). Each plot was generated from the mean value of individually fitted parameters. Best fit and kinetic constants were obtained from the exploratory kinetics function in SigmaPlot using the non-competitive inhibition (partial) equation

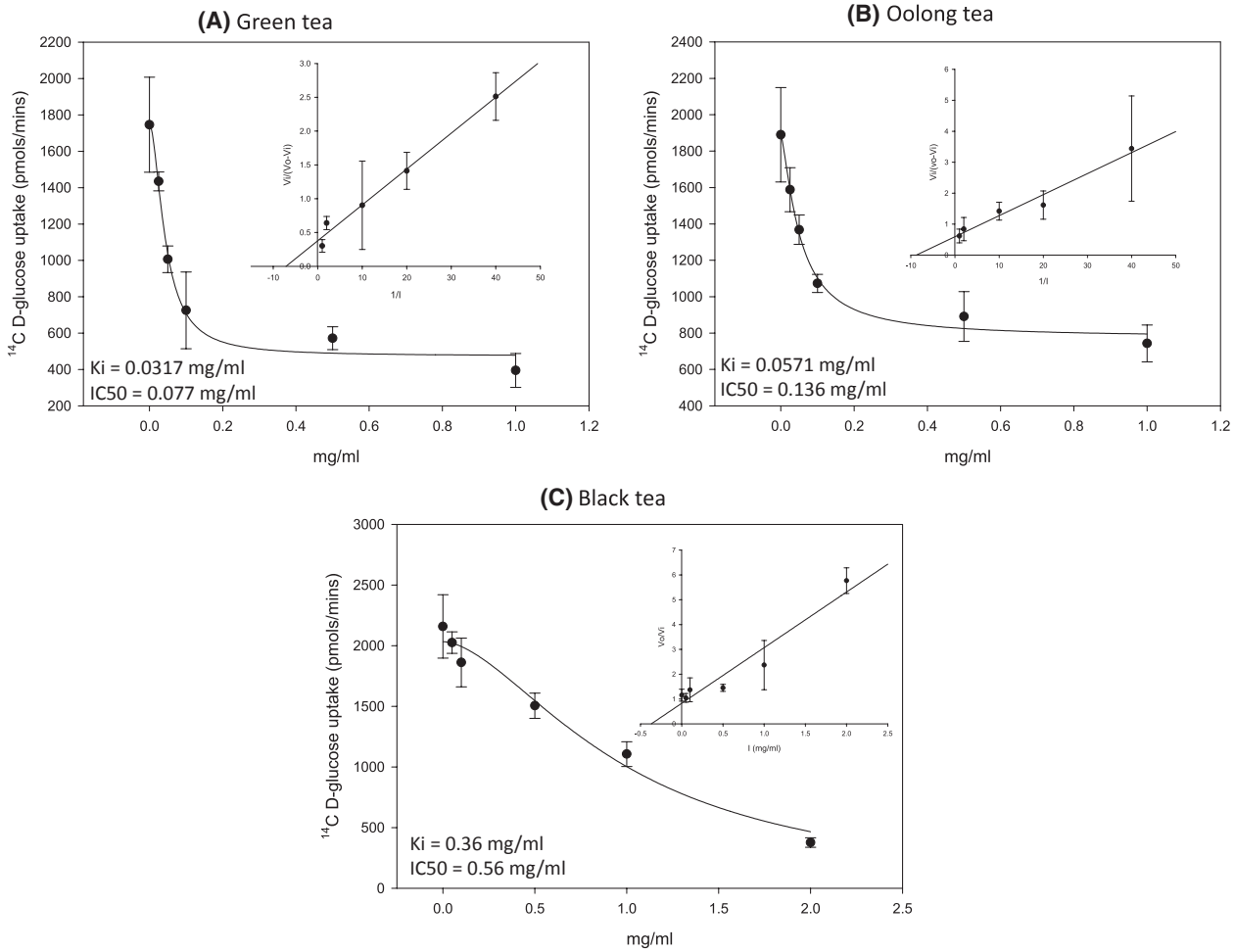

FIGURE 2 Effect of tea extracts on D-[^{14}C] glucose uptake in Caco-2 cells. Caco-2 cells were preincubated with green (Figure 2A), oolong (Figure 2B) or black tea (Figure 2C) extracts for 10 minutes, then, incubated for 2 minutes in an uptake medium containing 1 mM D-[^{14}C] glucose at room temperature in the presence of GT, OT, or BT. GT and OT were tested at a range of concentrations of 0 to 1.0 mg/mL and BT were tested at a range of concentrations of 0-2.0 mg/mL as indicated in the figure. Best fit curves were plotted in SigmaPlot using the non-linear regression analysis equation $y = \min + (\max - \min)/1 + (x/\mathrm{IC}_{50})$-hillslope. Results are expressed as means ± SD (N = 6). The inserts present data plotted according to the modified Dixon method or for partial non-competitive inhibition (see Methods) and were used to calculate K_i and IC_{50s}

by oolong, and then, black tea. In close agreement with the calculated Whiteley and modified Dixon plot data, non-linear regression analysis of the GT, OT, and BT yielded IC_{50s} equal to 0.0438 mg/mL (SE 0.0069), 0.0522 mg/mL (SE 0.009), and 0.9839 mg/mL (SE 0.53), respectively. The differences in K_i found for the various tea types is consistent with likely loss of certain inhibitory compounds during preparation of black tea.

3.2 | Effects of tea extract on sugar uptake into oocytes injected to express SGLT1 and facilitative sugar transporters (GLUTs)

Caco-2 cells express SGLT1 and GLUTs[38] and is therefore a mixed kinetic/transporter model of the human enterocyte. To study the effects of tea extracts on individual small intestinal sugar transporters, we used the *Xenopus Laevis* oocyte system. In oocytes injected to express SGLT1, D-glucose uptake was enhanced when compared to non-injected sham oocytes (Figure 3A) indicating functional expression of the SGLT1 transporter protein in the plasma membrane. As expected, phlorizin (PZ), the classic inhibitor of Na^+-dependent glucose transport completely abolished D-glucose uptake in SGLT1-injected oocytes but had no effect on D-glucose uptake in sham oocytes. DMSO (1%), the solvent used to dissolve tea extracts, did not significantly affect D-glucose uptake into sham or SGLT1 injected oocytes, indicating the solvent had minimal effect on diffusion or SGLT1 transport activity. Green tea inhibited SGLT1 mediated D-glucose uptake when compared to D-glucose alone, but not in the presence of 1% of DMSO (the true control) (Figure 3B). Black tea had no effect on SGLT1 mediated D-glucose uptake in the presence of 1% of DMSO or its absence (D-glucose). Thereafter, the effects of tea extracts on sugar uptake were compared against 1% of DMSO.

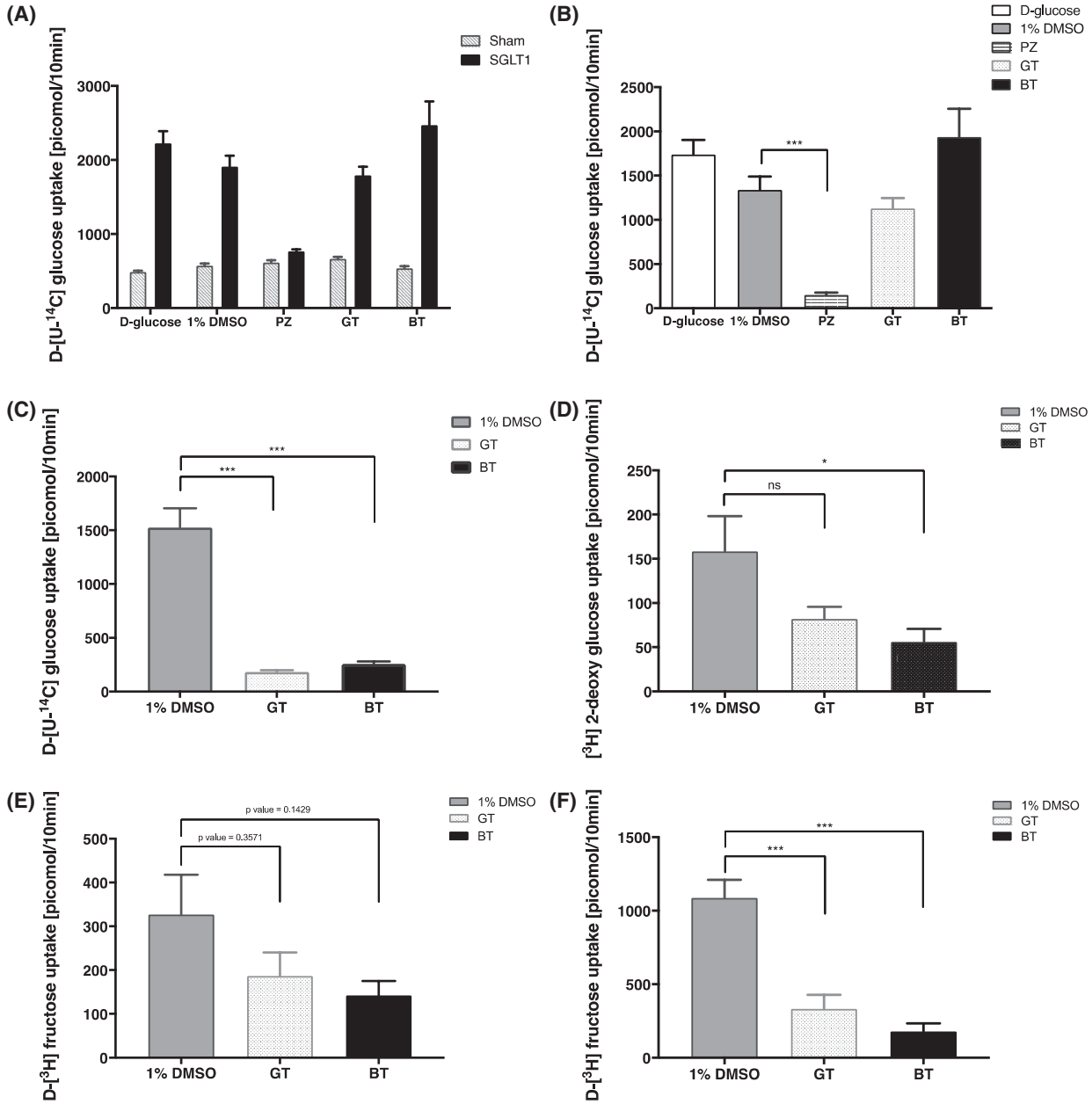

FIGURE 3 Inhibition of sugar transporters by green and black tea extracts (GT and BT, respectively). A, D-glucose uptake into oocytes injected to express SGLT1 and sham (non-injected) oocytes. B, SGLT1 mediated D-glucose uptake, calculated by subtraction of sham oocyte data from SGLT1 injected oocyte data. Uptake tested in oocytes at 10 minutes with D-[U-^{14}C] glucose (0.5 μCui/mL) and non-labeled D-glucose (10 mM). C, GLUT1 mediated D-glucose uptake. D, GLUT2 mediated 2-deoxy D-glucose uptake. E, GLUT2 mediated D-fructose uptake. F, GLUT5 mediated D-fructose uptake. Uptake tested in oocytes at 10 minutes with D-[U-^{14}C] fructose (0.5 μCui/mL) and non-labeled D-fructose (10 mM). D-glucose: Uptake media without any additional supplement. PZ: Uptake media supplemented with 0.5 mM Phlorizin/1% of DMSO. 1% DMSO: Uptake media with 1% DMSO. GT and BT: Uptake media with green tea or black tea extracts (1 mg/mL) dissolved in 1% of DMSO. B-F are all sham corrected transport data. Sham uptake of ^{14}C D-glucose and ^{3}H D-fructose were approx. 500 pmols/10 minutes and ^{14}C 2-DG 100-150 pmols/10 minutes *Indicates results are significantly different between the test samples and control at $P < .05$ level; ** and *** indicate significance at $P < .01$ and $P < .001$, respectively. NS indicates results are not significantly different between the test samples and control at $P > .05$

In oocytes injected to express GLUT1, GLUT1 mediated D-glucose uptake was inhibited by green and black tea when compared to D-glucose uptake in the presence of solvent (1% of DMSO) (Figure 3C). Previous studies have shown GLUT2 mediated D-glucose transport in oocytes to be poor, possibly because GLUT2 has a lower affinity for glucose when compared to other GLUTs. Uptake of sugar into oocytes expressing GLUT2 can however be improved by using 2-deoxy D-glucose, a non-metabolizable glucose analog that once inside the cell is rapidly phosphorylated and trapped.[40]

In oocytes injected to express GLUT2, green, and black tea inhibited 2-DG and D-fructose uptake (Figure 3D,E, respectively) but only reached statistical significance with black teas inhibition of 2-DG. In contrast to GLUT2, D-fructose uptake mediated by GLUT5 expressed in oocytes was significantly inhibited by green and black teas (Figure 3F).

3.3 | Effect of catechins in tea extracts on the glucose uptake in Caco-2 cells

Based on the HPLC analysis of the catechin contents and composition of the tea extracts, the dominant polyphenols in the tea samples were determined, the results of which are presented in Table 2. The catechins consisted of EGCG, GCG, EGC, GC, ECG, EC, and C, and the total contents in GT, OT, and BT were 22.13%, 14.39%, and 2.24%, respectively. Furthermore, linear regression analysis showed the capacity of tea extracts to inhibit glucose uptake was significantly correlated to total catechin content (r value for IC_{50} D-[^{14}C] glucose uptake versus total catechin content was −0.959) implying that catechins could be the predominant inhibitors in tea extracts. The inhibitory capacity of tea extracts also showed good correlation with individual catechins, except for catechin ($r = -0.394$). Gallic acid levels in tea extracts did not correlate with potency of the extracts to inhibit glucose transport as shown by the positive correlation coefficient ($r = 0.975$).

As shown in Figure 4A-D, the inhibitory activity of catechins was observed for eight types of catechins (C, EC, GC, EGC, GCG EGCG, CG, and ECG). Inhibition of D-glucose uptake into CaCo-2 cells by C, EC, GC, and EGC was weak (Figure 4A,B) and so inhibition constants could not be accurately calculated using Dixon plots. However, CG, ECG, GCG, and EGCG were more potent inhibitors of glucose transport (Figure 4C,D) and so the K_i and IC_{50} values were calculated using the modified Dixon plots.[36] Kinetics analysis showed good agreement between the K_i and IC_{50} values suggesting inhibition of uptake by the catechins was pure non-competitive. In close agreement with the calculated Dixon plot data, SigmaPlot non-linear regression analysis of the GCG, EGCG, CG, ECG best fit curves yielded IC_{50} values of 0.076, 0.091, 0.0134, and 0.0143 mg/mL, respectively.

3.4 | Effects of black tea polyphenolics and polysaccharides on glucose uptake in Caco-2 cells

Although GT was the most potent inhibitor of glucose transport in Caco-2 cells, BT also inhibited glucose transport despite low levels of catechins. The inhibitory activity of BT could be attributed to some other bioactive present, such as flavonols, flavones, anthocyanins, phenolic acids, and polysaccharides, as previously reported.[41,42] In our experiments, tea polysaccharides, cyanidin, pelargonidin, rutin, and apigenin had no effect on glucose uptake by Caco-2 cells (Figure 5A-C). In 1 mg/mL of our extracts, we estimated that tea polysaccharides (1%-3% of the dry tea) and polyphenols (<0.15%-0.01% of the dry tea) will be in the range of 0.1-30 μg/mL, yet, we saw no inhibition by 0.5-3 mg/mL of tea polysaccharides or by 25-50 μg/mL of individual polyphenols (100 μM) strongly suggesting these low-level compounds are not inhibitors of glucose transport in our extracts. Flavonols

TABLE 2 Content of gallic acid and catechins in green, oolong, and black tea extracts (%) of dry weight and correlation coefficients of analyte content versus IC_{50} D-[^{14}C] glucose uptake

Analyte	Green tea extracts	Oolong tea extracts	Black tea extracts	Correlation Coefficient (r)
Gallic acid	0.20	0.11	0.85	0.975
Gallocatechin (GC)	0.84	0.77	ND	NA
Epigallocatechin (EGC)	4.64	4.28	ND	NA
Catechin (C)	0.77	0.14	0.29	−0.394
Epicatechin (EC)	1.96	1.37	0.48	−0.956
Gallocatechin gallate (GCG)	0.53	0.43	ND	NA
Epigallocatechin gallate (EGCG)	10.64	6.18	0.77	−0.937
Catechin gallate (CG)	0.21	ND	ND	NA
Epicatechin gallate (ECG)	2.54	1.22	0.70	−0.791
Total catechin	22.13	14.39	2.24	−0.959

Note:: ND means not detectable. NA means not applicable. Pearson's correlation analysis: Correlation Coefficient (r).

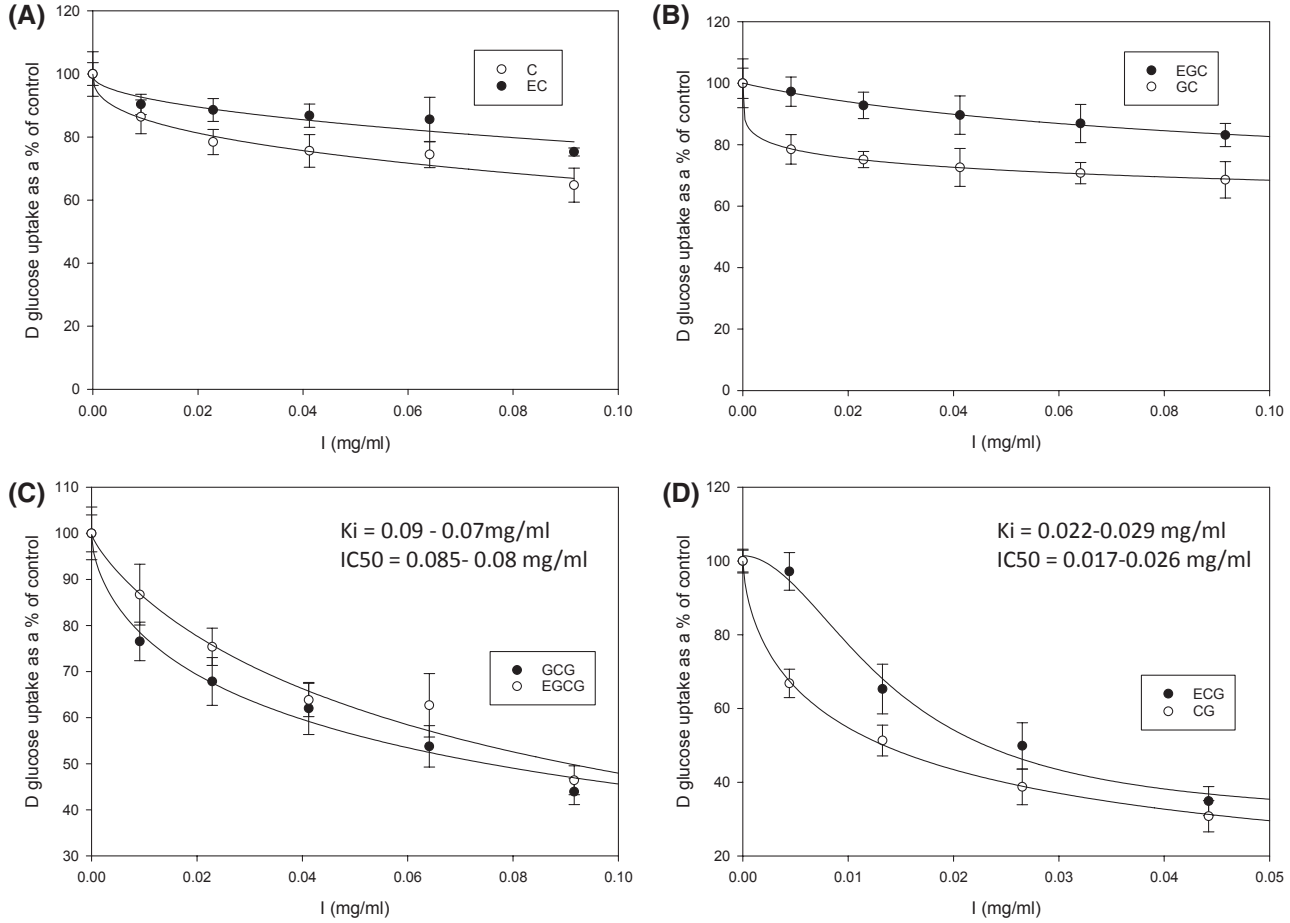

FIGURE 4 Effect of catechins on D-[^{14}C] glucose uptake in Caco-2 cells. A, Effect of EC and C on glucose uptake. B, Effect of EGC and GC on glucose uptake. C, Effect of EGCG and GCG on glucose uptake. D, Effect of ECG and CG on glucose uptake. Caco-2 cells were preincubated with catechins for 10 minutes, and then, incubated for 2 minutes in an uptake medium containing 1 mM D-[^{14}C] glucose at room temperature in the presence of catechins. The catechins were dissolved in DMSO (0.5%), and then, diluted with KBSS; the tested concentration ranges are shown in the figures. Best fit curves were plotted using SigmaPlot for the nonlinear regression analysis equation $y = \min + (\max - \min)/1 + (x/\mathrm{IC50})$-hillslope. Estimates of K_i and IC_{50} values for GCG, EGCG, CG, and ECG were obtained from linear Withycombe Dixon plots (plots not shown)

(mainly quercetin and myricetin) have been reported to inhibit intestinal glucose uptake markedly,[33,38] and this was also observed in the present study (Figure 5D). However, quercetin and myricetin only constitute up to 0.2%-0.4% and 0.07%-0.2% in tea, respectively, and are poorly soluble in water,[43] which suggests that they are unlikely to be the predominant inhibitors in tea.

Phenolic acids are other important active components in tea, with 0.5%-1.4% of gallic acid, 0.3% of chlorogenic acid, and ≤0.1% of caffeic acid.[43] The investigation by Welsch et al showed that caffeic acid and chlorogenic acid inhibited sodium-dependent glucose uptake by rat BBM vesicles.[44] We also found caffeic acid and chlorogenic acid could modestly but significantly reduce Caco-2 cell glucose uptake (Figure 5E). The catechin content in GT is greater than BT, whereas the four theaflavins (TFs) and thearubigins (TRs) are higher in BT than in GT.[45] A previous study reported that TFs inhibit sugar transport while TRs do not show any inhibitory activity.[22] With regard to TFs, in our studies, theaflavin (TF) was found to significantly inhibit glucose uptake by Caco-2 cells, similar to BT (Figure 5F). In 1 mg/mL of our extracts, we estimate TFs will be in the range of 30-60 μg/ml. We used 77 μg/mL of TFs in our study, suggesting TF plays an important role in black tea inhibition of glucose transport.

3.5 | Effect of green tea catechins on SGLT1 and GLUT2 gene and protein expression levels in Caco-2 cells

To evaluate the effect of green tea catechins on glucose transporters at a molecular level, gene and protein expression profiles of SGLT1 and GLUT2 in Caco-2 cells were examined by qRT-PCR and Western blotting. As shown in Figure 6A,B incubating Caco2 cells with green tea catechins for 2 and 24 hours resulted in a significant 50%-75% decrease

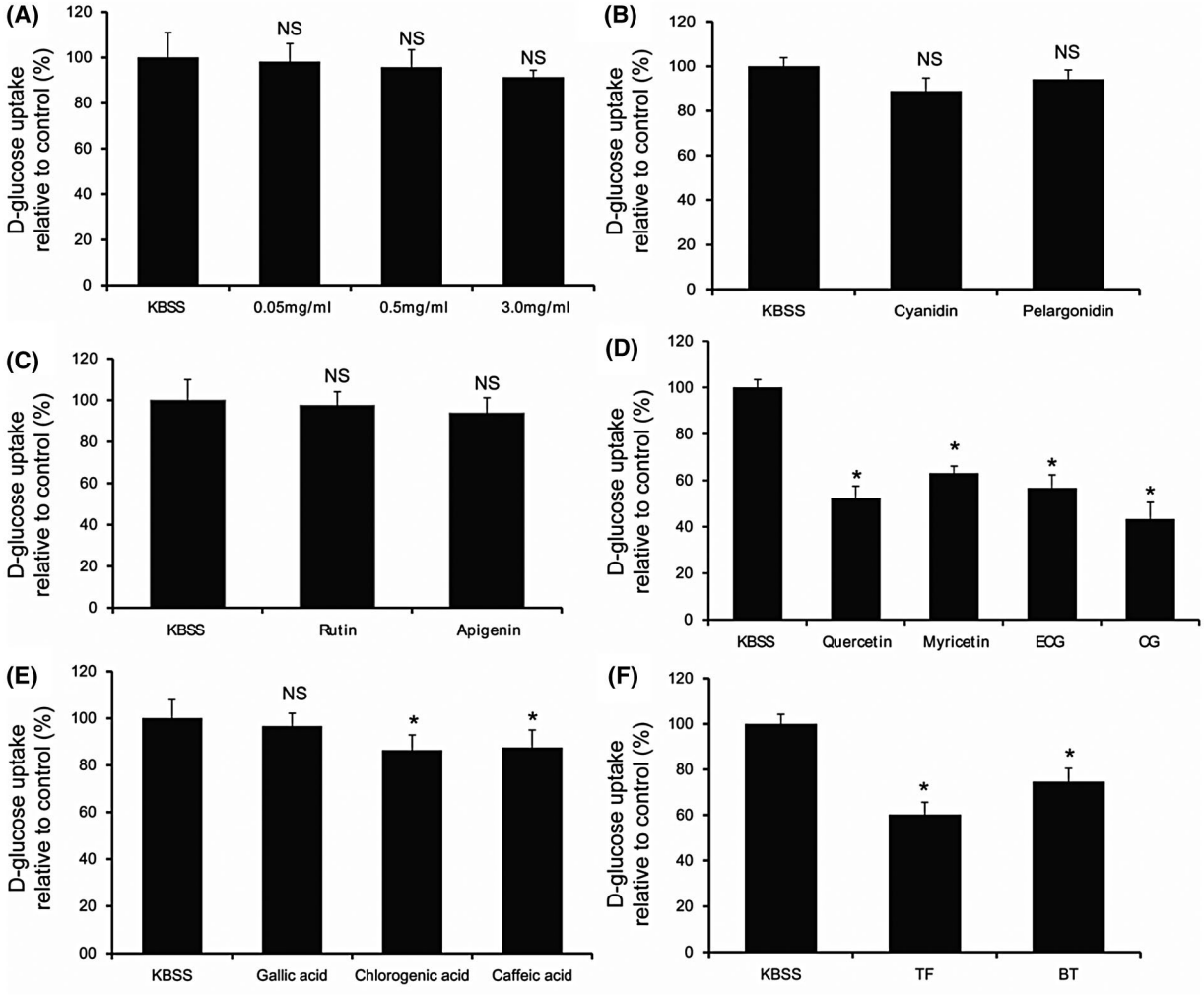

FIGURE 5 Effects of black tea polyphenolics and polysaccharides on D-[^{14}C] glucose uptake in Caco-2 cells. A, Effect of tea polysaccharides at a range of concentrations of 0 to 3.0 mg/mL on glucose uptake. B, Effect of cyanidin and pelargonidin at 100 μM on glucose uptake. C, Effect of rutin and apigenin at 100 μM on glucose uptake. D, Effect of quercetin, myricetin, ECG, and CG at 50 μM on glucose uptake. E, Effect of phenolic acids at 100 μM on glucose uptake. F, Effect of TF (0.077 mg/mL) and BT (0.35 mg/mL) at 100 μM on glucose uptake. Caco-2 cells were preincubated with each component, respectively, for 10 minutes, then, incubated for 2 minutes in an uptake medium containing 1 mM D-[^{14}C] glucose at room temperature in the presence of each component, respectively. KBSS was used as the control; other components were dissolved in DMSO (≤0.5%), and then, diluted with KBSS. Results are expressed as means ± SD (N = 4). *Indicates results are significantly different between the test samples and control at $P < .05$ level; NS indicates results are not significantly different between the test samples and control at $P > .05$

in SGLT1 mRNA levels. In contrast, SGLT1 protein expression levels were largely unchanged by green tea catechins (Figure 6C,D), although a modest but statistically significant decrease was detected after 2 hours incubation with CG (27.0%), ECG (23%), and EGCG (20%).

Following a 2 hours incubation with catechins, GLUT2 gene and protein expression levels were downregulated by approximately 40%-50% (Figure 6E,G). In contrast, GLUT2 gene expression was upregulated 400%-1400% following 24 hours incubation with catechins (Figure 6F). GLUT2 protein expression levels were also significantly upregulated after 24 hours incubation with catechins but only for CG, ECG, GCG, and EGCG exposed cells (Figure 6H).

4 | DISCUSSION

In this report, we studied the effects of green, black, and oolong tea on intestinal sugar transport mediated by the Na^+ dependent glucose transporter (SGLT1) and the facilitative sugar transporters, (GLUT1, 2 and 5). We found that green tea, which contained the highest levels of catechins, was the most potent inhibitor of intestinal sugar transport via a partial non-competitive type of mechanism, that is, it did not lead to complete loss of transport activity even at high levels of tea extract. The most likely targets for the tea extracts were the GLUTs. We also showed that CG and GCG and their epiforms were the most potent inhibitors of glucose transport

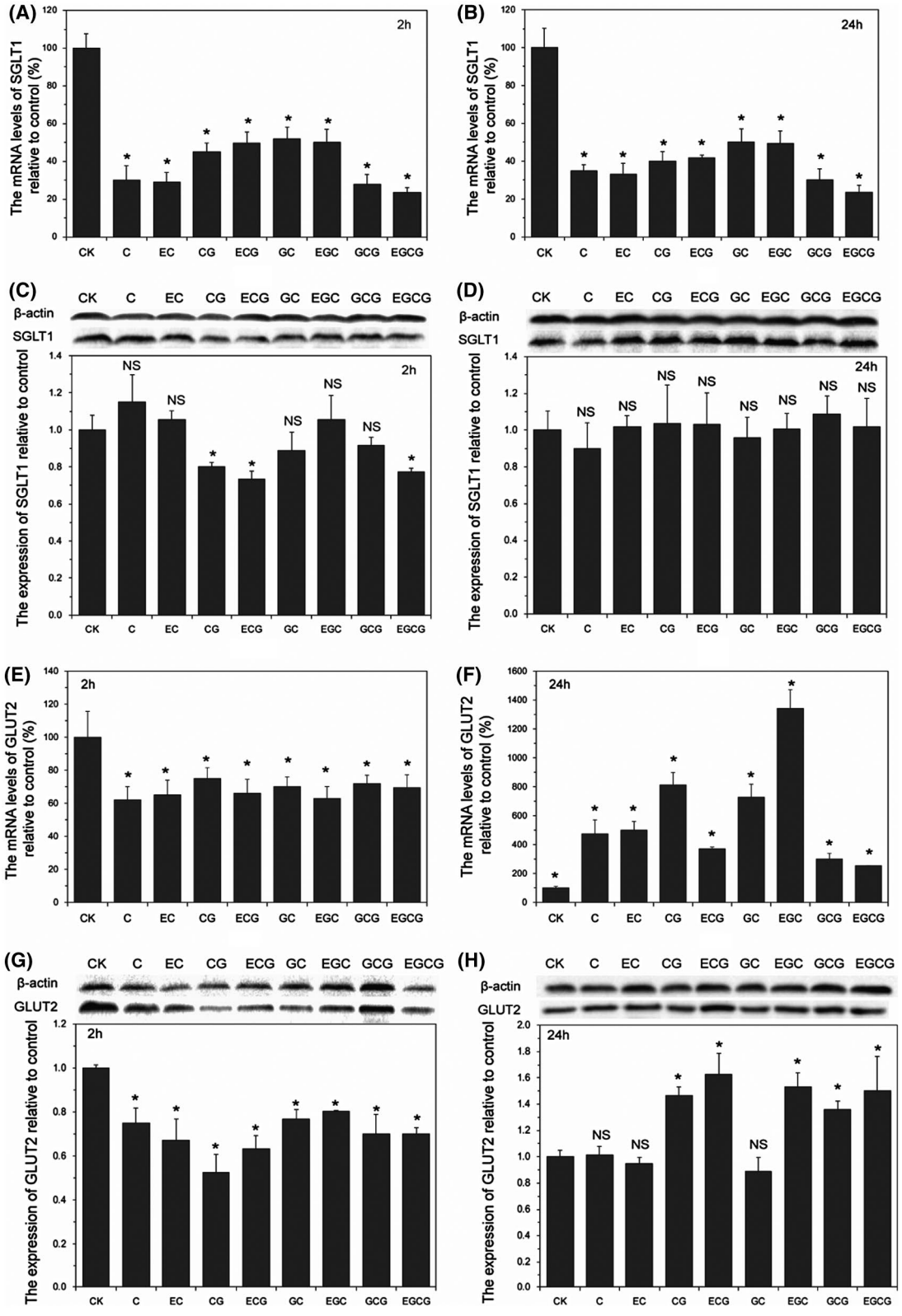

FIGURE 6 Effect of green tea catechins on mRNA and protein levels of SGLT1 and GLUT2 in Caco-2 cells. A-D, The protein and mRNA levels of SGLT1 after 2 or 24 hours of incubation with tea catechins. E-H, The protein and mRNA levels of GLUT2 after 2 or 24 hours of incubation with tea catechins. CK: Caco-2 cells were incubated with full culture medium at 37°C; C, EC, CG, ECG, GC, EGC, GCG, and EGCG: Caco-2 cells were incubated with 200 μM C, EC, CG, ECG, GC, EGC, GCG, and EGCG. Results are expressed as means ± SD (N = 3). C, EC, CG, ECG, GC, EGC, GCG, and EGCG were dissolved in DMSO (≤0.5%), and then, diluted with full culture medium. *Indicates results are significantly different between the test samples and control at $P < .05$ level; NS indicates results are not significantly different between the test samples and control at $P > .05$

activity. Selected catechins were also able to reduce SGLT1 and GLUT2 gene and protein expression levels.

Green tea is a non-fermented tea, and in its manufacture the primary goal is to preserve leaf catechins. Indeed, in this report, we showed the catechin content of GT was the highest of all the teas sampled, at 21.9% of total dry matter. During black tea manufacture, there is a high degree of enzyme-catalyzed aerobic oxidation (fermentation) of the leaf polyphenols followed by a series of chemical condensation reactions, which result in a significant decrease of catechins (3%-10% of dry weight) and production of theaflavins and thearubigins (3%-6% and 12%-18%, respectively). Oolong tea is partially fermented, and so catechin content is intermediate between green and black teas (8%-20% of the total dry matter).[46] In Caco-2 cells, inhibition of glucose transport by the tea extracts was in the following order: GT > OT > BT, and our inhibitory data (IC_{50} values) for D-[^{14}C] glucose uptake showed a significant correlation with the catechin content of teas, thus, providing strong evidence that tea catechins inhibit the small intestinal sugar transporters.

Previous studies have shown strawberries and blackberries rich in polyphenolics such as anthocyanins can inhibit intestinal glucose transport in isolated cells and in human dietary intervention studies.[47,48] Our in vitro data suggest that teas have the potential to inhibit the facilitative glucose transporters (eg, GLUTs) in the small intestine, which may attenuate postprandial glycemic responses in humans. These findings may also have clinical utility because it is known that in obesity and diabetes, GLUT1 and GLUT2 are over expressed in the apical membrane of enterocyte contributing toward enhanced glucose absorptive capacity.[25,49,50] In addition, intestinal GLUT1 expression is enhanced in inflammatory bowel disease (IBD)[51] and in cancers,[52] and high intakes of dietary fructose is associated with increased risk of non-alcoholic fatty liver disease (NAFLD).[53] Our tea extracts, and in particular our green tea extract, could be used as a concentrated source of polyphenolics (catechins) that in beverage form may be used in the dietary management of T2DM to lower the rise in postprandial blood glucose concentrations, improve insulin sensitivity and glycemic control in the long-term via inhibition of small intestinal GLUTs.

To identify the individual tea catechins that inhibit intestinal glucose transport, we studied eight catechins (C, EC, CG, ECG, GC, EGC, GCG, and EGCG) at concentrations ranging between 0.01 and 0.1 mg/mL (20-200 μM) and showed that the potency of glucose-uptake inhibition was in the order: CG > ECG > EGCG ≥ GCG > C > EC > GC > EGC. These data are consistent with previous findings showing ECG to be the most potent inhibitor of glucose transport.[23,33] Although the mechanisms by which tea catechins inhibit glucose uptake are currently unclear, their structural characteristics (as shown in Figure 7) may be important in three possible ways: (1) esterification affects glucose uptake, so that gallated catechin (ie, CG) is more inhibitory than the non-gallated structure (ie, C); (2) the –OH group on R2 of the B ring affects glucose uptake, with the non-hydroxylated structure (ie, ECG) being more inhibitory than hydroxylated catechin (ie, EGCG); (3) the trans-catechin isomer (ie, CG) is more inhibitory than the cis-catechin form (ie, ECG), but this difference was not observed in the geometric isomers EGCG and GCG. Pharmaceutical companies could use this information to develop drug treatments to alleviate postprandial hyperglycemia in the diabetic population. Individual catechins could also be used as nutraceuticals for inclusion in functional ingredients and foods/drinks to improve glycemic control.

The non-competitive inhibition of glucose uptake by catechins shown in our study suggests the aglycone catechins (eg, ECG and EGCG) do not interfere with the sugar binding

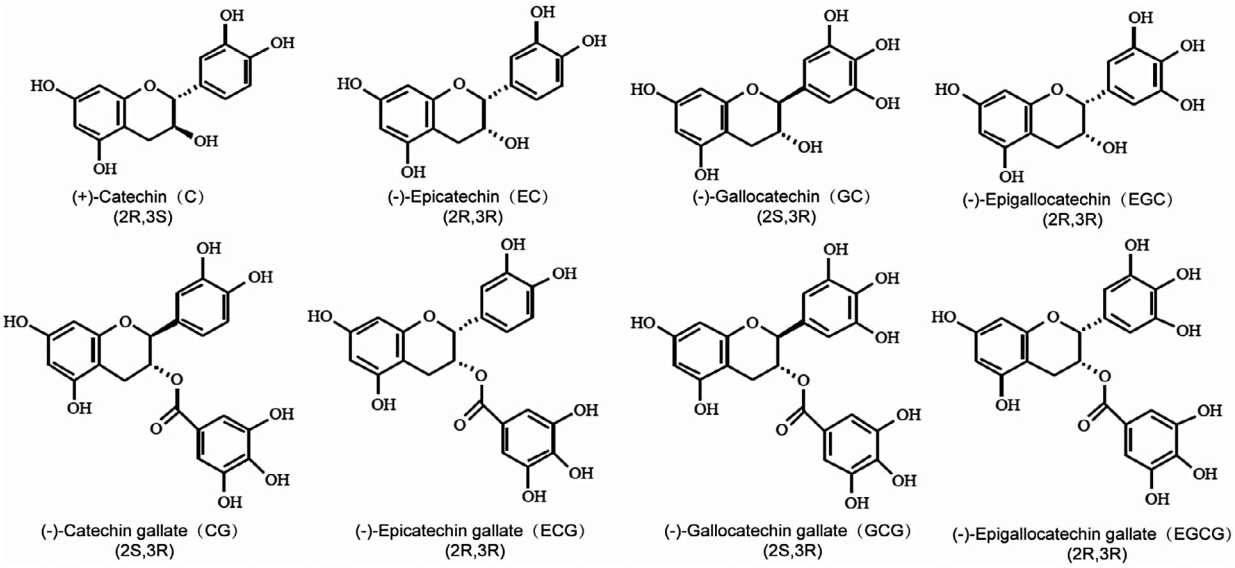

FIGURE 7 Structures of eight catechins

sites of the facilitative transporters. This is consistent with previous reports, which have shown that the aglycone phloretin non-competitively inhibits GLUTs, whereas the glycone phlorizin competitively inhibits SGLT1.[54] Indeed, ECG and EGCG are the major tea catechins and have a triphenol structure, which is reported to bind to the lipid bilayers with high affinity and to perturb the membrane structure.[55] Polyphenolics have also been shown to inhibit intracellular glucose metabolism.[56] Catechins could therefore inhibit internal glucose metabolism, leading to a build-up of intracellular glucose, which in turn could dampen facilitative glucose transport. We believe this to be an unlikely explanation of our data since uptake of the non-metabolizable glucose analog, 2-deoxy D-glucose, in GLUT2 expressing oocytes was inhibited by tea extracts. The mechanisms by which catechins and other polyphenolics enter the cell are also unclear. Previous work has shown that quercetin was not transported into oocytes injected to express GLUT2[38] and that ECG was not transported into rabbit intestinal BBMVs.[23]

It has been reported that ECG and EGCG, but not catechin, competitively inhibit SGLT1 mediated 100 nM D-glucose uptake into rabbit BBM vesicles[23] and also that ECG and EGCG non-competitively inhibited Na^+ currents in the presence of 0.5 mM D-glucose in oocytes expressing SGLT1.[57] In the current study there was no evidence of tea extracts inhibiting glucose uptake into oocytes expressing SGLT1, because the competitive inhibition of SGLT1-mediated glucose transport (K_m 0.1-0.6 mM)[39] by 1 mg/mL of teas containing ECG (2.5% of dry weight) and EGCG (10% of dry weight) would most likely be overwhelmed by the 10 mM of D-glucose concentration used in our transport assays. In addition, tea catechins are potent non-competitive inhibitors of Na^+/K^+-ATPase,[1,58] which might explain the reduction in Na^+ currents detected in an earlier report.

Thus far, we and others have shown that teas and tea catechins can inhibit intestinal sugar transporters in the short-term. It has also been reported that tea catechins can impact intestinal sugar transporter capacity in the long-term by altering gene expression levels.[59] In this study, rats treated with green tea for 6 weeks displayed significantly reduced SGLT1 and increased GLUT2 mRNA level in the jejunum mucosa.[59] We also found that SGLT1 gene expression was down-regulated in Caco-2 cells following 2- and 24-hours exposure to individual tea catechins; however, SGLT1 protein expression was only modestly reduced when exposed to CG, GCG, and EGCG for 2 hours. This discordance in gene and protein expression levels may reflect differing half-lives of the gene and protein and suggests that the blood glucose lowering properties of tea catechins over the postprandial period are not mediated via changes in small intestinal SGLT1 protein expression levels. The effects of individual tea catechins on GLUT2 gene and protein expression levels were also much more complicated with gene and protein expression levels being modestly reduced after 2 hours exposures to catechins but increased at 24 hours. Over the postprandial period tea catechins may inhibit glucose absorption via reductions in GLUT2 protein at the basolateral and apical membrane.[60] In the long-term, and perhaps because the cells are starved of fuel (glucose), GLUT2 gene and protein expression levels are upregulated. This however may not be physiologically relevant, because ingested polyphenolics are rapidly metabolized by the gut and liver.[61] Further studies in vitro and in animal models are necessary to determine the short- and long-term effects of teas on intestinal sugar transport capacity via alterations in sugar transporter gene and protein expression levels.

In conclusion, we report that tea extracts and individual tea catechins strongly inhibit small intestinal sugar transporters. This is likely to be a key mechanism along with other physiological effects of tea polyphenols, such as inhibition of starch digestion,[16] increased insulin sensitivity and/or insulin secretion[62] in explaining improvements in glucose tolerance in experimental animals[62] and humans.[41] Similar proof of principle studies in Caco-2 cells and in oocytes have been undertaken by other laboratories using berry extracts and have provided information on the mechanisms that underpin the effects of berries on postprandial glycemia in people.[45,46] Based on these findings, tea plants genetically engineered to enhance their CG or ECG content could be generated by plant breeders for the food industry and be used as nutraceuticals to reduce the glycemic index of foods and beverages, blunting postprandial blood glucose responses and providing the consumer with a healthier dietary choice.

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CONFLICT OF INTEREST

Authors state there is no conflict of interest.

AUTHOR CONTRIBUTIONS

D. Ni performed research, analyzed data and wrote the paper. Z. Ai performed research, analyzed data, and wrote the paper. D. Munoz-Sandoval performed research and analyzed data. R. Suresh performed research. P. R. Ellis designed research and wrote the paper. C. Yuqiong provided reagents, P. A. Sharp

provided reagents and edited the paper. P. J. Butterworth designed research, analyzed data and wrote the paper. Z. Yu provided reagents. C. P. Corpe designed research, analyzed data and wrote the paper.

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