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Inhibition of the intestinal postprandial glucose transport by gallic acid and gallic acid derivatives

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Abbreviations

SGLT1, Sodium-dependent glucose transporter 1; **GLUT2,** Glucose transporter 2; **PZ,** Phloridzin; **PT,** Phloretin; **2DG,** 2-deoxyglucose; **ECG,** (-)-Epicatechin gallate; **EC,** (-)-Epicatechin ; **EGCG,** (-)-Epigallocatechin gallate; **EGC,** (-)-Epigallocatechin; **TFMG**, Theaflavin-3-gallate; **TF,** Theaflavin; **PE,** Petroleum ether; **DCM,** Dichloromethane; **EA,** Ethyl acetate; **1-But,** 1-butanol; **Tri-gallic-glu,** 3,4,6-Tri-O-galloyl-β-D-Glucose

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Abstract

Inhibition of glucose uptake in the intestine through sodium-dependent glucose transporter 1 (SGLT1) or glucose transporter 2 (GLUT2) may be beneficial in controlling postprandial blood glucose levels. Gallic acid and ten of its derivatives were identified in the active fractions of *Terminalia chebu*la Retz. fructus immaturus, a popular edible plant fruit which has previously been associated with the inhibition of glucose uptake. Gallic acid derivatives (methyl gallate, ethyl gallate, pentyl gallate, 3,4,6-tri-O-galloyl-β-D-Glucose, and corilagin) showed good glucose transport inhibition with inhibitory rates of 72.1 ± 1.6%, 71.5 ± 1.4%, 79.9 ± 1.2%, 44.7 ± 1.2%, and 75.0 ± 0.7% at 5 mM D-glucose and/or 56.3 ± 2.3, 52.1 ± 3.2%, 70.2 ± 1.7%, 15.6 ± 1.6%, and 37.1 ± 0.8% at 25 mM D-glucose. However, only 3,4,6-tri-O-galloyl-β-D-Glucose and corilagin were confirmed GLUT2-specific inhibitors. Whilst some tea flavonoids demonstrated minimal glucose transport inhibition, their gallic acid derivatives strongly inhibited transport effect with GLUT2 specificity. This suggests that gallic acid structures are crucial for glucose transport inhibition. Plants, such as *T. chebula*, which contain high levels of gallic acid and its derivatives, show promise as natural functional ingredients for inclusion in foods and drinks designed to control postprandial glucose levels.

Keywords: *Terminalia chebula*, glucose transport, gallic acid derivatives, tea flavonoids; Diabetes

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■ Introduction

Type 2 diabetes (T2D) has become an increasingly common disease, estimated to affect 380 million people worldwide by 2025[1]. The current T2D prevention strategies include lifestyle therapy, stimulating insulin secretion, α-glucosidase inhibition, increasing insulin sensitivity and/or inhibiting intestinal glucose transport. A possible strategy to reduce postprandial glucose and subsequent insulin spikes is to inhibit glucose absorption in the intestine[2]. Foods or meals with high carbohydrate content such as starch or sucrose, will increase postprandial blood glucose levels. According to Node *et al.*[3], people with repeated high postprandial plasma glucose ''spikes'' have increased risk of developing type 2 diabetes. It is important to control postprandial blood glucose levels in both normal healthy subjects and diabetic patients[4]. Unregulated blood glucose fluctuations are undesirable, and any reduction or ''blunting'' of the postprandial blood glucose levels is potentially beneficial. The molecular targets of intestinal glucose uptake inhibition include sodium-dependent glucose transporter-1 (SGLT1), a high-affinity, low-capacity, active transport protein, and glucose transporter 2 (GLUT2), a low-affinity, high-capacity, facilitated transport protein. Some herbal extracts and phytochemicals have reported glucose transport inhibitory effect in vitro and in vivo. For example, Guava (*Psidium guajava*) fruit extract prepared by supercritical-CO_2 extraction inhibited intestinal glucose resorption in a double-blind, randomized clinical study[2, 5]. Anthocyanin-rich berry-extract modulated postprandial glycaemia by decreasing glucose transporter expression in human intestinal Caco-2 cells[6]. Polyphenol-rich herbal extract attenuated glucose transport across Caco-2 cell monolayers through interactions with SGLT1 and GLUT2 transporters[7]. Extracts and polyphenols from apple reduced postprandial blood glucose levels through inhibition of the intestinal SGLT1 in mice and humans[8].

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Terminalia chebula Retz.(*T. chebula*), a member of the *Combretaceae* family, is a popular traditional medicine and edible food in India and East Asia[9]. It is widely used to treat diseases that include Alzheimer's disease, constipation, and diabetes[10], and has been reported to exhibit several pharmacological properties, such as, anti-ageing, anti-ulcer, anti-cancer, anti-HIV, anti-mutagenic, anti-diabetic, antioxidant, cardioprotection, cytoprotective effects, and wound healing[11, 12]. The extract of *T. chebula* contains geraniin, punicalagin, terflavin B, as well as gallic, chebulagic, chebulic, chebulinic, and tannic acids[12, 13]. Studies have shown that the extracts or components from the fruits of *T. chebula* had the effect of lowing glucose level through antioxidants, enhancing insulin secretion from the β-cells in the islets of Langerhans, and hypolipidemic properties[10, 12]. We have previously shown that the ethanol extract of *T. chebula* showed potent effective inhibition of intestinal glucose transport[14]. In this study, we investigated the chemical constituents of ethanol extracts of *T. chebula* and their role as potential glucose transport inhibitors. A series of gallic acid and its derivatives have been isolated from *T. chebula* using bioassay-directed fractionation and tested for glucose transport inhibitory activity. In addition, the glucose transport inhibitory effect and mechanism of gallic acid derivatives of flavonoids were investigated.

■ Materials and methods

Materials and chemicals. Dried *Terminalia chebula* Retz., fructus immaturus, was purchased from Shanghai Kangqiao Pharmacy Co. Ltd. with the producing area (Anhui) and lot number (111108), authenticated by Prof. L.H. Wu at the Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai. The reagents of sodium pyruvate solution, D-(+)-Glucose, Lucifer Yellow, fetal bovine serum (FBS), phloridzin (PZ, ≥98.0%), and phloretin (PT, ≥99.0%), 2-deoxyglucose (2DG, ≥98.0%), gallic acid (≥98.0%), methyl gallate (≥90.0%), ethyl gallate (≥

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98.0%), pentyl gallate (⩾98.0%), 1,3,4-tri-O-galloyl-β-D-glucose (⩾98.0%), corilagin (⩾98.0%), (-)-epicatechin gallate (ECG, ⩾98.0%), (-)-epicatechin (EC, ⩾98.0%), (-)-epigallocatechin gallate (EGCG, ⩾95.0%), (-)-epigallocatechin (EGC, ⩾95.0%), theaflavin-3-gallate (TFMG, ⩾95.0%), theaflavin (TF, ⩾95.0%) were purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO), Non-Essential Amino Acids and DMEM+Glutamax-1 were obtained from Merck (Gibbstown, NJ). Human epithelial colorectal adenocarcinoma (Caco-2) cells were purchased from ATCC (Manassas, VA).

Extraction and isolation of the fruit of *T. chebula*. The dried fruit of *T. chebula* (9.0 kg) was extracted with 36 L of 95 % v/v ethanol for 2 hours at 80 °C using extraction and concentration equipment. The residue was then extracted with 27 L of 70 % v/v ethanol for another 2 hours at 80 °C. The two aqueous ethanol extracts were then combined and concentrated under reduced pressure, after vacuum dried to yield *T. chebula* aqueous ethanol extract (4.887 kg, yield rate 54.3 %) (Figure S1). Subsequently, the herbal residues were extracted three times with boiling water for 2 h each, at a solid-liquid ratio of 1: 6. The water extracts were concentrated and precipitated at 4 volumes of 95% ethanol, and the precipitate was freeze-dried to obtain a crude polysaccharide fraction (559.8g, yield rate 6.22%)[14].

The *Terminalia chebula* aqueous ethanol extract (5.0 g) was dissolved in 20 mL of water and extracted twice with 10 mL of petroleum ether (petroleum ether extract), twice with 10 mL of dichloromethane (dichloromethane extract), twice with 10 mL of ethyl acetate (ethyl acetate extract), and twice with 10 mL of 1-butanol (butanol extract) to obtain 4.7 mg in the petroleum ether phase (yield rate 0.94 %), 157.9 mg in the dichloromethane phase (yield rate 3.2 %), 876.5 mg in the ethyl acetate phase (yield rate 17.5 %), 1125.1 mg in the 1-butanol extract phase (yield rate 22.5 %), and 2018.2 mg

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in the water phase (yield rate 40.4 %) (Table S1, figure S1), the separation method was based to our previous report[3].

The 1-butanol extract (10 μL) was further purified using an Agilent 1200 series HPLC system equipped with an Agilent ZORBAX Eclipse XDB-C18 column (Agilent, UK; 4.6 mm × 250 mm, 5 μm particle size) and an Agilent ZORBAX Eclipse XDB guard column (1.0 mm × 17 mm). The injector temperature was room temperature. The mobile phase consists of a gradient of methanol/acetic acid (2% v/v) (15:85 to 25:75 over 20 min; to 50:50 over 10 min; to 100:0 over 10 min; to 15:85 over 10 min) at a flow rate of 0.5 mL/min and a column temperature of 35 °C. An Agilent 1200 DA photodiode recorded signals at 280 nm. The data was acquired with Agilent ChemStation software. 1-butanol extract was divided into 17 fractions after separation by HPLC and collected by Agilent 1260 infinity LC fraction collector (G1364B) (1 tube/3 minutes) (Figure S2).

The fractions (5 μL) with high glucose transport inhibitory effect were injected into an Agilent 6540 Ultra High Definition QTOF fitted with a Waters ACQUITY CSH™ C18 1.7 μm 2.1×150 mm column. The injector temperature was room temperature. The mobile phase consists of a gradient of methanol/acetic acid (2% v/v) gradient (15:85 to 25:75 over 0.5 min; to 50:50 over 5.5 min; to 100:0 over 2 min; to 15:85 over 2 min), with a flow rate of 0.3 mL/min and a column temperature of 35 °C. Other settings were: capillary voltage, −2.5 kV (ESI^-); sample cone, −25 V (ESI^-); extraction cone, −4.0 V (ESI^-); source temperature, 120 °C; desolvation temperature, 350°C. The ESI source was operated in negative (ESI-) ionization modes. The nebulizer pressure was 40 psi., the cone gas (nitrogen) flow was 50 L/h and desolvation gas (nitrogen) flow rate was 600 L/h. Argon was used as collision gas. The data were collected and analyzed using Agilent MassHunter Workstation Data Acquisition and Agilent MassHunter Qualitative Analysis B.4.00 software.

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Caco-2 cell monolayer glucose transport assay. The assay methods were constructed according to our previous report[4]. Briefly, 96-well transwell insert supports were collagen coated with 40 μL of type I rat tail collagen in acetic acid (0.02 M) for one hour at room temperature under sterile conditions. The inserts were washed twice in PBS and Caco-2 cells were seeded into the 96-well transwell inserts at 9.6×10^5 cell/mL (75 μL per insert) and incubated in growth medium, and 30 mL of Growth Medium added to the feeder plate below. The cells were left to attach to the collagen matrix and form monolayers over 48 hours at 37 °C, 5 % CO_2. Both inserts and feeder plate were washed in PBS and the cells incubated with BD Entero-STIM™ Enterocyte Differentiation Medium containing MITO+™ Serum Extender solution (both BD Biosciences), 75 μL per insert and 30 mL in the feeder plate, for a further 48 hours at 37 °C, 5 % CO_2. Differentiated cell monolayers were washed and incubated with fresh PBS (+) (containing Ca^{2+} and Mg^{2+}) for 60 min. Subsequently, 75 μL per insert of either 5 mM D-glucose ± test compound or 25 mM D-glucose ± test compound was replaced the PBS (+) in the apical side of the inserts, whereas, 225 μL per well of glucose-free PBS (+) were quickly added to each well of the basolateral side. After incubation at 37 °C, 5 % CO_2 for 15 min in 5 mM glucose wells and 30 min in 25 mM glucose wells, respectively, the liquid from basolateral compartments was collected for glucose quantification using Amplex Red Glucose/Glucose oxidase Assay Kit (Invitrogen, Carlsbad, CA). The kit was used as recommended by the manufacturer, the limit of detection of the kit used to quantify glucose was $<$ 1.0 μM in the test. Then, Lucifer Yellow was used to check the permeability of the Caco-2 membranes at 485 nm (excitation) and 530 nm (emission). The glucose transport inhibition (% inhibition) was calculated using the following formula:

$$\%\text{Inhibition} = \frac{\text{Glucose concentration (Control)} - \text{Glucose concentration (Test)}}{\text{Glucose concentration (Control)}} \times 100$$

Where, Glucose concentration (Control) is the glucose concentration measured in the basolateral side

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in control group and Glucose concentration (Test) is the glucose concentration measured in the basolateral side in the test group.

2-Deoxyglucose transport assay. 2DG transport assays were performed according to our previous report [4]. Briefly, Caco-2 cells were seeded in 24-wells plate at a density of 12.5×10^4 cell/well (500 µL per insert) in growth medium and growth medium (30 mL) was added to the feeder plate below. After 24 hours incubation, both inserts and feeder plate were incubated with Enterocyte Differentiation Medium containing MITO (BD Biosciences) and Serum Extender solution (BD Biosciences). After a further 48 hours at 37 °C, 5 % CO_2, differentiated cell monolayers were used for 2DG transport assay. 500 µL per insert of 25 mM 2DG ± test compound was replaced the PBS (+) in the apical side of the inserts, whereas, 1 mL per well of glucose-free PBS (+) were quickly added to each well of the basolateral side for 30 min incubation. Then, Lucifer Yellow was used to check the permeability of the Caco-2 membranes. GC-MS were used to quantify 2DG[4]. The 2DG transport inhibition (% inhibition) was calculated using the following formula:

$$\%\text{Inhibition} = \frac{\text{2DG concentration (Control)} - \text{2DG concentration (Test)}}{\text{2DG concentration (Control)}} \times 100$$

Where, 2DG concentration (Control) is the 2DG concentration measured in the basolateral side in control group and 2DG concentration (Test) is the 2DG concentration measured in the basolateral side in the test group.

Statistics. The data were analyzed using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA) and one-way ANOVA with Tukey's test. The data were expressed as mean ± standard error of the mean. Differences with $p < 0.05$ were considered statistically significant.

■ Results

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Isolation and identification of gallic acid and its derivatives from *T. chebula*. In our previous study, the aqueous ethanol extracts of the fruits of *T. chebula* showed significantly inhibitory effect of glucose transport across the cell monolayer at 5 mM and 25 mM glucose[14]. However, treatment with the crude polysaccharide extracts of the fruits of *T. chebula* did not have a potent inhibitory effect on glucose transport[14]. The *T. chebula* aqueous ethanol extract (5.0 g) was dissolved in water and partitioned in sequence with petroleum ether, dichloromethane, ethyl acetate, and 1-butanol to obtain petroleum ether fraction (PE), dichloromethane fraction (DCM), ethyl acetate fraction (EA), 1-butanol fraction (1-But), and water fraction (Water), respectively. The glucose transport inhibitory rates of PE, DCM, EA, 1-But, and water at 200 μg/mL were 29.7±1.5%, 48.4±1.0%, 82.0±0.7%, 88.0±0.5%, and 61.0±1.1% at 5 mM D-glucose (the simulated fasting state), and 7.9±3.4%, 15.9±2.3%, 43.4±2.8%, 57.6±2.1%, and 45.0±0.5% at 25 mM D-glucose (the simulated fed state), respectively. No Lucifer Yellow was detected in the feeder plate below after use, suggesting that the Caco-2 cells were differentiated. In order to find novel and potent glucose transport inhibitors, bioassay-directed fractionation of the 1-But fraction of the aqueous ethanol extract of a dried fruit part of *T. chebula* was carried out by semi-preparative HPLC system, and led to isolation of 17 fractions. Glucose transport inhibition assay showed that fractions 2, 5, 6, 8, 9, 10, and 11 exhibited good activities with inhibitory rate ≥ 50% at 5 mM D-glucose or/and 25 mM D-glucose (Table S1). Ultra-High Definition QTOF-MS analysis results showed that gallic acid and terflavin B were identified in 1-Bu-2; chebulanin and 3,4,6-Tri-O-galloyl-β-D-Glucose were identified in 1-Bu-5; corilagin was identified in 1-Bu-6; chebulagic acid, gallagic acid and 1,3,4,6-Tetra-O-galloyl-β-D-Glucose were identified in 1-Bu-8; neochebulagic acid was identified in 1-Bu-9; chebulinic acid and ellagic acid were identified in 1-Bu-11 (Table S2, figure S3). Clearly, all the identified active compounds were gallic acid or its derivatives.

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Therefore, it was hypothesized that gallic acid and its derivatives might inhibit glucose transport at 5 mM D-glucose or/and 25 mM D-glucose. In addition, gallic acid and 33 of its derivatives were reported in *T. chebula* Retz., fructus immaturus[15, 16].

Inhibition of the intestinal glucose transport by gallic acid derivatives of alkyl. In this study, the commercially available gallic acid and its alkyl derivatives (methyl gallate, ethyl gallate, pentyl gallate, tri-gallic-glu, corilagin) were obtained to investigate their inhibitory effect on glucose transport (Figure 1). As hypothesized, the results showed that gallic acid and its derivatives showed glucose transport inhibitory effect at 5 mM D-glucose or/and 25 mM D-glucose (Table 1). Gallic acid at the concentration of 200 μM exhibited the glucose transport inhibition of 29.7 ± 3.9% at 5 mM D-glucose and 0.03 ± 3.3% at 25 mM D-glucose. This indicated that gallic acid may be a SGLT1 inhibitor, as Berry *et al.*[17] previously demonstrated that SGLT1 inhibitors have ≥ 20 % inhibition of glucose transport at 5 mM D-glucose and ≤20 % inhibition at 25 mM D-glucose (Table 1). However, methyl gallate, ethyl gallate, pentyl gallate, and corilagin may be GLUT2 inhibitors, because they have ≥20 % inhibition at both 5 mM and 25 mM D-glucose levels at the concentration of 200 μM. Tri-gallic-glu (200 μM) exhibited the glucose transport inhibitory effects with 44.7 ± 1.2% at 5 mM D-glucose and 15.3 ± 1.6% at 25 mM D-glucose, which indicated that gallic acid may a SGLT1 inhibitor (Table 1, figure S4).

Identification of SGLT1 or GLUT2 inhibitors of gallic acid derivatives of alkyl. Berry *et al.*[17] previously suggested that a compound that inhibits glucose transport by more than 20% at both low (5 mM) and high (25 mM) glucose concentrations might indicate a specific inhibitor of GLUT2. 2DG can be used to confirm GLUT2 specificity of compounds, since 2DG is only transported by GLUT2 and slightly transported by SGLT1[18]. A compound with an inhibitory effect on glucose transport ⩾

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20 % at low (5 mM) glucose concentration and ≤20 % at high (25 mM) glucose concentration are SGLT1-specific inhibitors. Our results show that gallic acid and tri-gallic-glu may be SGLT1 inhibitors, while methyl gallate, ethyl gallate, pentyl gallate, and corilagin may be GLUT2 inhibitors (Table 1). However, methyl gallate, ethyl gallate, and pentyl gallate did not inhibit 2DG transport (25 mM), suggesting that these compounds are not GLUT2-specific inhibitors or that they have different inhibiting sites of 2DG transport. Gallic acid did not inhibit 2DG transport (25 mM), however, tri-gallic-glu inhibited 2DG transport (25 mM) with the inhibition rate corresponding to the glucose transport inhibition rate, which indicated that tri-gallic-glu was not a SGLT1 inhibitor, but is a GLUT2 inhibitor with low inhibitory effect at 200 μM. Corilagin also inhibited 2DG transport (23.0 ± 3.3%), however, the inhibition rate did not correspond to the D-glucose transport inhibition rate (37.1 ± 0.8%) (Figure 2). This suggests that corilagin is not just a GLUT2-specific inhibitor but may also have non-specific activity.

Inhibitory effect of the intestinal glucose transport by gallic acid derivatives of flavonoids. Flavonoids are well-characterized glucose transport inhibitors[18, 19]. To investigate the effect of gallic acid derivatives of flavonoids, three green tea gallic acid derivatives (ECG, EGCG, and TFMG) were investigated for glucose transport inhibition (Figure 3). Table 1 showed that ECG, EGCG, and TFMG have ≥ 20 % glucose transport inhibition at 5 mM and 25 mM D-glucose levels, and may inhibit GLUT2 in intestinal cells. ECG, EGCG, and TFMG are the gallic acid derivatives from green tea, however, the non-gallated flavonoids EC and EGC only demonstrated minimal inhibition at 5 mM or/and 25 mM D-glucose levels. TF, another non-gallated flavonoid, at 200 μM inhibited glucose transport by 34.8 ± 4.2% at 5 mM D-glucose and by 11.6 ± 1.7% at 25 mM D-glucose, which indicated that TF may be a SGLT1 inhibitor (Table 1 and figure S5). Interestingly, ECG, EGCG, and TFMG are

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GLUT2 inhibitors, however, TF with >20% inhibition of glucose transport at high (25 mM) glucose concentrations and <20% inhibition of glucose transport at low (5 mM) glucose concentrations was indicative of a SGLT1-specific inhibitor, possibly due to the lack of a conjugated gallate group. TF may therefore have a different mechanism for inhibiting the glucose transport to ECG, EGCG, and TFMG. Our results indicated that gallic acid is a crucial group to exhibit the glucose transport inhibition effect in gallic acid derivatives of flavonoids, and the specific protein of inhibition may be GLUT2.

Identification of SGLT1 or GLUT2 inhibitors as gallic acid derivatives of flavonoids. We investigated whether gallic acid derivatives of flavonoids inhibited the transport of 2DG, a GLUT2-specific substrate, across a differentiated Caco-2 monolayer. The data showed that all three gallic acid derivatives of flavonoids, ECG, EGCG, and TFMG at the concentration of 200 μM inhibited the transport of 25 mM 2DG, with the inhibition rates corresponding to those of the D-glucose transport inhibition rates. Therefore, we propose that gallic acid derivatives of flavonoids, including ECG, EGCG, and TFMG specifically inhibit GLUT2, the same target as phloretin. In addition, the non-gallated flavonoids EC and EGC showed minimal 2DG inhibition confirming the results from the D-glucose transport studies (Figure 4). Interestingly, TF at the concentration of 200 μM exhibited a similar inhibition (no significantly difference) of the 25 mM of 2DG transport and glucose transport, which could indicate that TF is a weak GLUT2 inhibitor.

■ Discussion

Known treatments of diabetes and main classes of anti-diabetic agents are to stimulate insulin secretion, delay digestion and absorption of intestinal carbohydrates, suppress hepatic glucose

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production, improve insulin sensitivity, and peripheral glucose uptake[14, 20]. In our previous study, 10 recommended edible traditional Chinese medicine herb candidates were identified from 12 highly cited herbs, the ethanol extracts of the fruit of *T. chebula* showed a potent glucose transport inhibitory effect, when the initial glucose concentration on the apical side was either 5 mM or 25 mM[14]. Many phenolic compounds were identified in the fruit of *T. chebula*, such as gallic acid, 4-O-methyl gallic acid, methyl (S)-flavogallonate, 1,3,6-tri-O-galloyl-β-D-glucopyranoside, chebulagic acid, chebulinic acid, chebulic ellagitannins, methyl neochebulinate, methyl neochebulagate, chebulanin, methyl neochebulanin, chebulic acid, isoterchebulin, and punicalagin [14, 15, 21]. In this study, gallic acid and its derivates tri-gallic-glu, chebulanin, chebulagic acid, chebulinic acid, corilagin, terflavin B, ellagic acid, gallagic acid, neochebulagic acid, tetra-gallic-glu were identified in the 1-butanol fraction of *T. chebula,* (Figure S3). Based on the principle of active tracking and bioassay directed fractionation, only the fraction with the highest inhibition rate (1-butanol) was selected for further testing and UPLC-ESI-Q-TOF/MS analysis in our study. Future studies could however investigate other fractions with moderately high activity, such as the EA fraction, which may reveal other classes of active compounds. The various extraction solvents used in the fractionation, such as petroleum ether, dichloromethane, ethyl acetate, and 1-butanol, are not applicable for the preparation of food-grade extracts, but serve as good model systems to determine optimal conditions for extracting bioactive compounds. Additionally, the effects of the digestion system on the extracts could be investigated to determine the stability of these compounds after consumption.

Previously studies showed the fruits from *T. chebula* contained abundant corilagin, chebulagic acid, chebulanin, tetra-gallic-glu, ellagic acid, chebulinic acid, gallic acid, and tri-gallic-glu[15, 16]. The fruit of *T. chebula* has an abundance of gallic acid and its derivates, and the concentration of compounds

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obtained as pure substances were applied at high concentrations (200 μM). This suggested that corilagin, a GLUT2-nonspecific potent inhibitor and the most abundant active gallic acid derivate in *T. chebula* Retz, may be the main component of glucose transport inhibition.

Flavonoids are well-characterized glucose transport inhibitors. Gallic acid and some derivatives were demonstrated as potent glucose transport inhibitors in our study, which leads us to believe that a combination of flavonoids and gallic acid derivatives may exhibit good or better glucose transport inhibitory effects. Many catechins, the major polyphenol constituents of green tea (*Camellia sinensis*), are gallic acid derivatives of flavonoids, such as (−)-epicatechin-gallate (ECG), theaflavin-3-gallate (TFMG) and (−)-epigallocatechin-gallate (EGCG)[22]. It was reported that EGCG dose-dependently inhibited GLUT2, and decrease the uptake of both D-[^{14}C(U)]-glucose and of D-[^{14}C(U)]-fructose[23]. EC and EGC promote the translocation of GLUT4 through activation of the phosphatidylinositol 3' -kinase (PI3K), and (-)-catechin 3-gallate (CG) and EGCG inhibit insulin-induced translocation of GLUT4 by the insulin signaling pathway in 3T3-L1 mice adipocyte cells[24]. ECG inhibited SGLT1 in a competitive manner, although ECG itself was not transported via the glucose transporters[25]. Hossain and Johnston *et al.*[26-28] found that the effects of EGCG, ECG and EGC are likely to be the result of steric hindrance caused by incorporation into the membrane with subsequent disruption of the surrounding lipid-bilayer as shown previously by using transfected Xenopus oocytes as an expression vector. In the present study, ECG, EGCG, and TFMG, the gallic acid derivatives of flavonoids, demonstrated significant glucose transport inhibition with 5 mM and 25 mM D-glucose and 25 mM 2DG, confirming their GLUT2-specificity, which is different from that reported in Xenopus oocytes. However, the non-gallated flavonoids EC and EGC showed minimal D-glucose or 2DG inhibition. TF (200 μM) exhibited similar inhibition (no significant difference) of the 25 mM of 2DG transport and

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D-glucose transport, which could indicate that TF is a weak GLUT2-specific inhibitor. Our results indicated that gallic acid is a crucial group to exhibit the glucose transport effect in gallic acid derivatives of flavonoids.

In conclusion, eleven compounds, including gallic acid and ten of its derivatives, were identified from the active fractions of *T. chebula* by UPLC-QTOF-MS. Gallic acid and its alkyl derivatives (methyl gallate, ethyl gallate, pentyl gallate, tri-gallic-glu, corilagin; 200 μM) showed glucose transport inhibitory effect at 5 mM D-glucose or/and 25 mM D-glucose. Our study showed that methyl gallate, ethyl gallate, pentyl gallate, and corilagin were not GLUT2-specific inhibitors, but tri-gallic-glu was a GLUT2 inhibitor. Three gallic acid derivatives of flavonoids (ECG, EGCG, and TFMG; 200 μM) have a significant effect on glucose transport with >20% inhibition at 5 mM and 25 mM D-glucose, while the non-gallated flavonoids EC, EGC and TF did not. ECG, EGCG, and TFMG were therefore identified as GLUT2 inhibitors. Our results indicate that gallic acid is a crucial group to inhibit glucose transport, while the specific inhibition of GLUT2 was not affected by the addition of gallic acid moieties to flavonoids.

Conflict of interest

The authors declare that they have no competing interests.

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CRediT authorship contribution statement

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Huijun Wang: Methodology, Software, Validation, Formal analysis, Investigation, Writing - original draft. **Mark I. Fowler and David J. Messenger:** Methodology, Investigation, Resources, Writing - review & editing. **Jose Juan Ordaz-Ortiz:** Methodology, Investigation, Writing - review & editing. **Xuelan Gu:** Investigation, Conceptualization. **Songshan Shi:** Methodology, Analysis and interpretation of data. **Leon A. Terry:** Resources, Supervision. **Mark J. Berry:** Funding acquisition, Conceptualization, Supervision. **Guoping Lian and Shunchun Wang:** Funding acquisition, Conceptualization, Supervision, Writing - review & editing.

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Figure Captions

Figure 1. Chemical structures of the gallic acid derivatives, among of them 1: gallic acid, 2: methyl gallate, 3: ethyl gallate, 4: pentyl gallate, 5: tri-gallic-glu, 6: corilagin.

Figure 2. Inhibition of glucose and 2DG transport by gallic acid and its derivatives. Inhibitory rate of gallic acid, methyl gallate, ethyl gallate, pentyl gallate, tri-gallic-glu, and corilagin on 25 mM glucose (white bars) and 25 mM 2DG (black bars) transport at the concentration of 200 μM in human intestinal Caco-2 cells (n = 3; the significant differences between groups are indicated by different letters a-f at $p<0.05$).

Figure 3. Chemical structures of the gallic acid derivatives of flavonoids, among of them 7: (-)-Epicatechin (EC), 8: (-)-Epigallocatechin (EGC), 9: Theaflavin (TF), 10: (-)-Epicatechin gallate (ECG), 11: (-)-Epigallocatechin gallate (EGCG), 12: Theaflavin-3-gallate (TFMG).

Figure 4. Inhibition of glucose and 2DG transport by gallic acid derivatives of flavonoids and their non-gallated flavonoids. Inhibitory rate of flavonoids (EC, EGC, and TF) and their gallic acid derivatives (ECG, EGCG, and TFMG) on 25 mM glucose (white bars) and 25 mM 2DG (black bars) transport at the concentration of 200 μM in human intestinal Caco-2 cells (n = 3; the significant differences between groups are indicated by different letters a-e at $p<0.05$).

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Table 1. Compounds tested for SGLT1 and GLUT2 inhibition activity in Caco-2 cells using 5 mM D-glucose for 15 minutes and 25 mM D-glucose for 30 minutes, respectively. The assigned class of transporter inhibited by each compound is based on SGLT1 inhibitors having ≥20% inhibition of glucose transport at 5 mM D-glucose and ≤20% inhibition at 25 mM D-glucose, and GLUT2 inhibitors having ≥20% inhibition at both 5 mM and 25 mM D-glucose levels. Triplicate values for % glucose transport inhibition.

No	Test compound*	Solvent	% Glucose transport inhibition		Class
			5mM	25mM	
1	Gallic acid	DMSO	29.7 ± 3.9	0.03 ± 3.3	SGLT1
2	Methyl gallate	DMSO	72.1 ± 1.6	56.3 ± 2.3	GLUT2
3	Ethyl gallate	DMSO	71.5 ± 1.4	52.1 ± 3.2	GLUT2
4	Pentyl gallate	DMSO	79.9 ± 1.2	70.2 ± 1.7	GLUT2
5	Tri-Gallic-Glu	DMSO	44.7 ± 1.2	15.3 ± 1.6	SGLT1
6	Corilagin	DMSO	75.0 ± 0.7	37.1 ± 0.8	GLUT2
7	EC	DMSO	12.0 ± 5.3	-0.6 ±3.4	None
8	EGC	DMSO	11.9 ± 0.8	2.1 ± 3.3	None
9	ECG	DMSO	93.8 ± 0.8	55.8 ± 3.9	GLUT2
10	EGCG	DMSO	85.6 ± 1.1	36.6 ± 3.2	GLUT2
11	TF	DMSO	34.8 ± 4.2	11.6 ± 1.7	SGLT1
12	TFMG	DMSO	94.2 ± 1.8	58.0 ± 1.7	GLUT2
13	Phloridzin	DMSO	37.0 ± 3.8	14.3 ± 0.8	SGLT1
14	Phloretin	DMSO	79.6 ± 3.7	58.3 ± 2.0	GLUT2

* Phloridzin was tested at 300 µM and Phloretin was tested at 150 µM. The other samples were tested at 200 µM.

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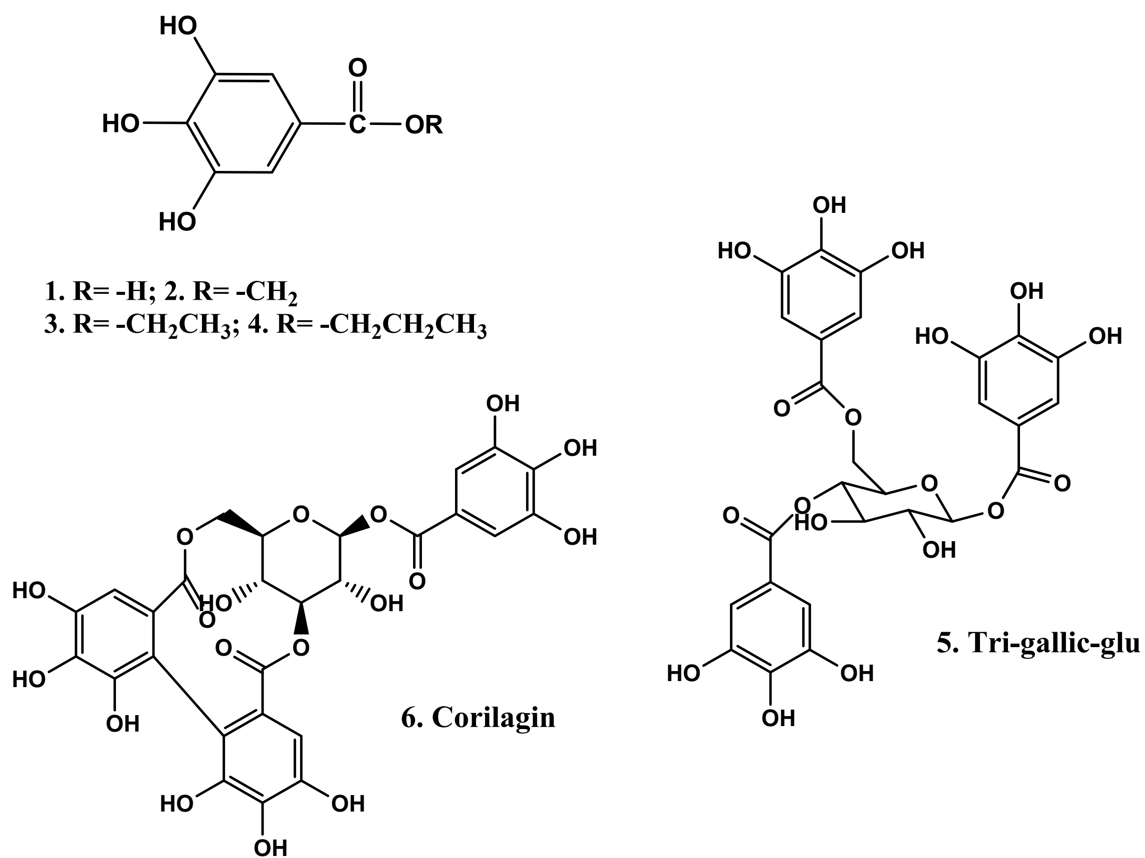

Figure 1

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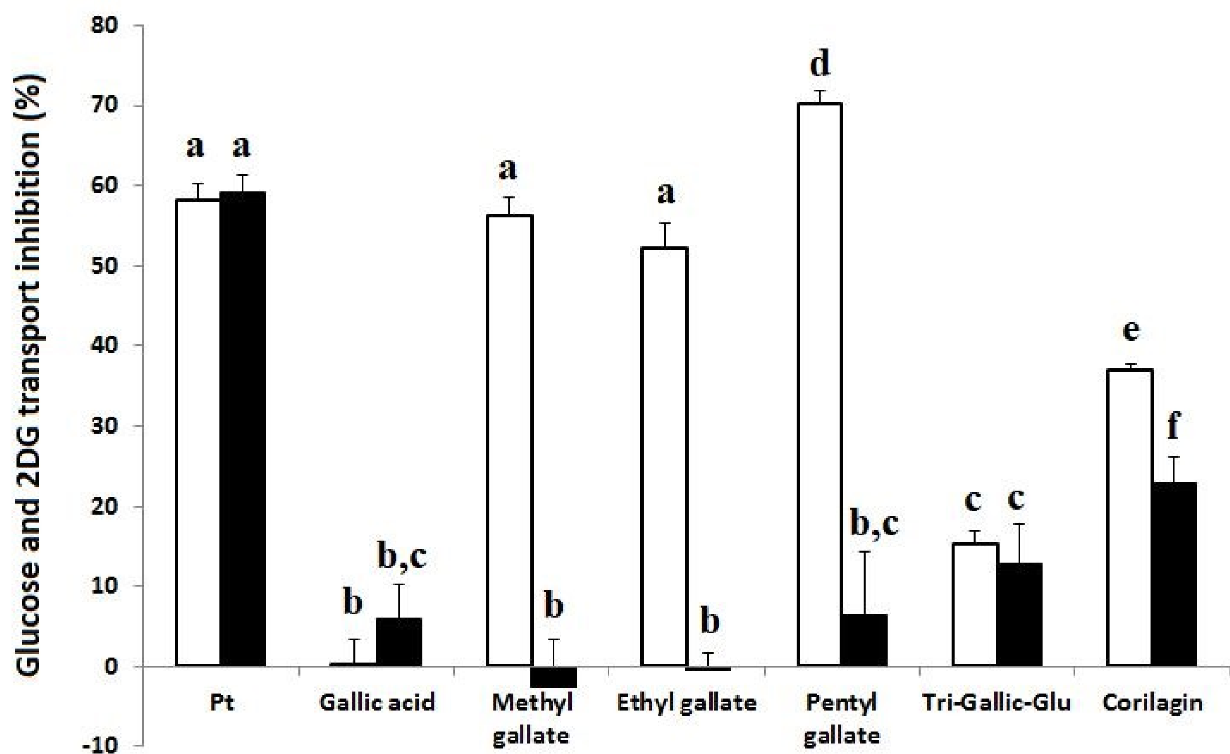

Figure 2

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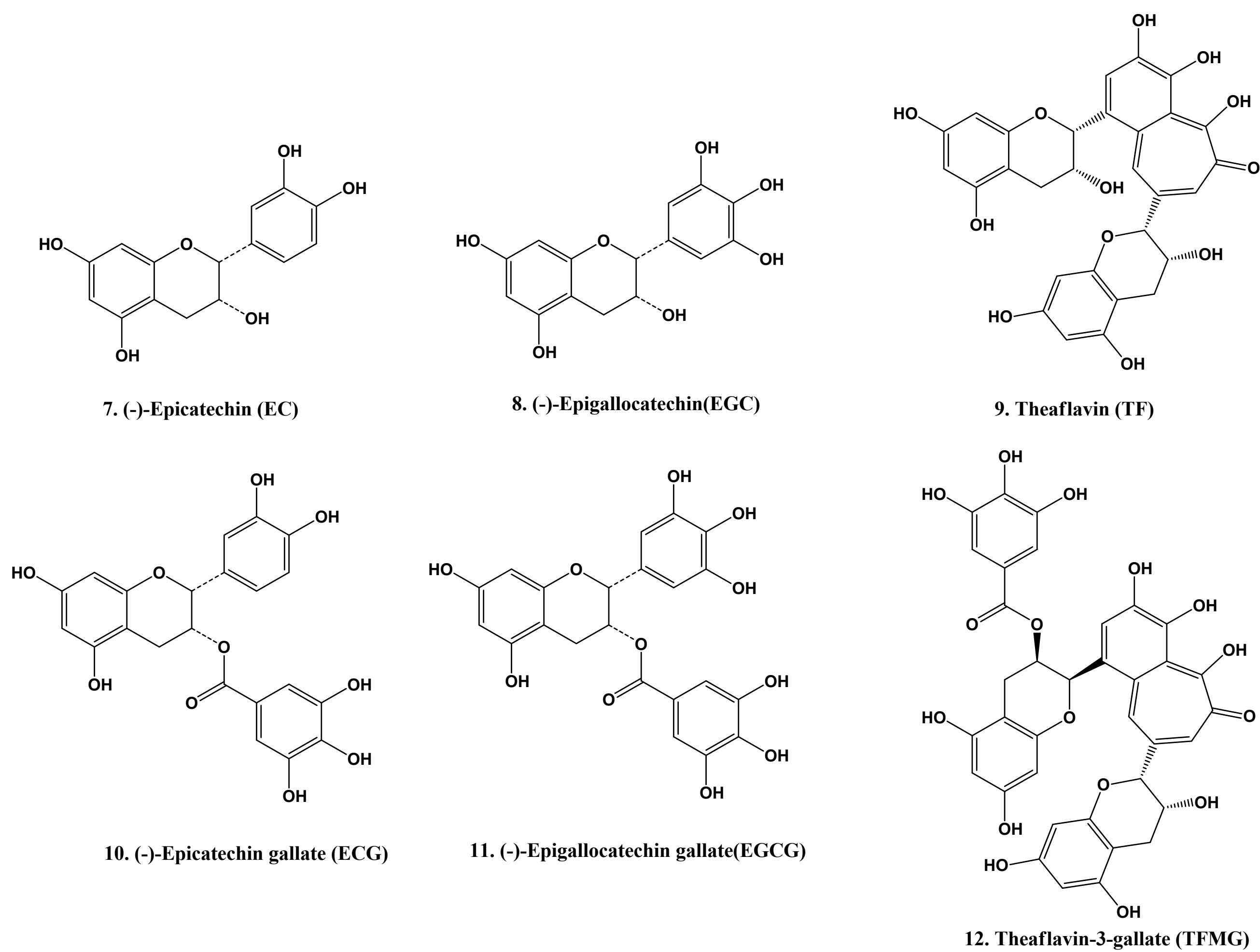

Figure 3

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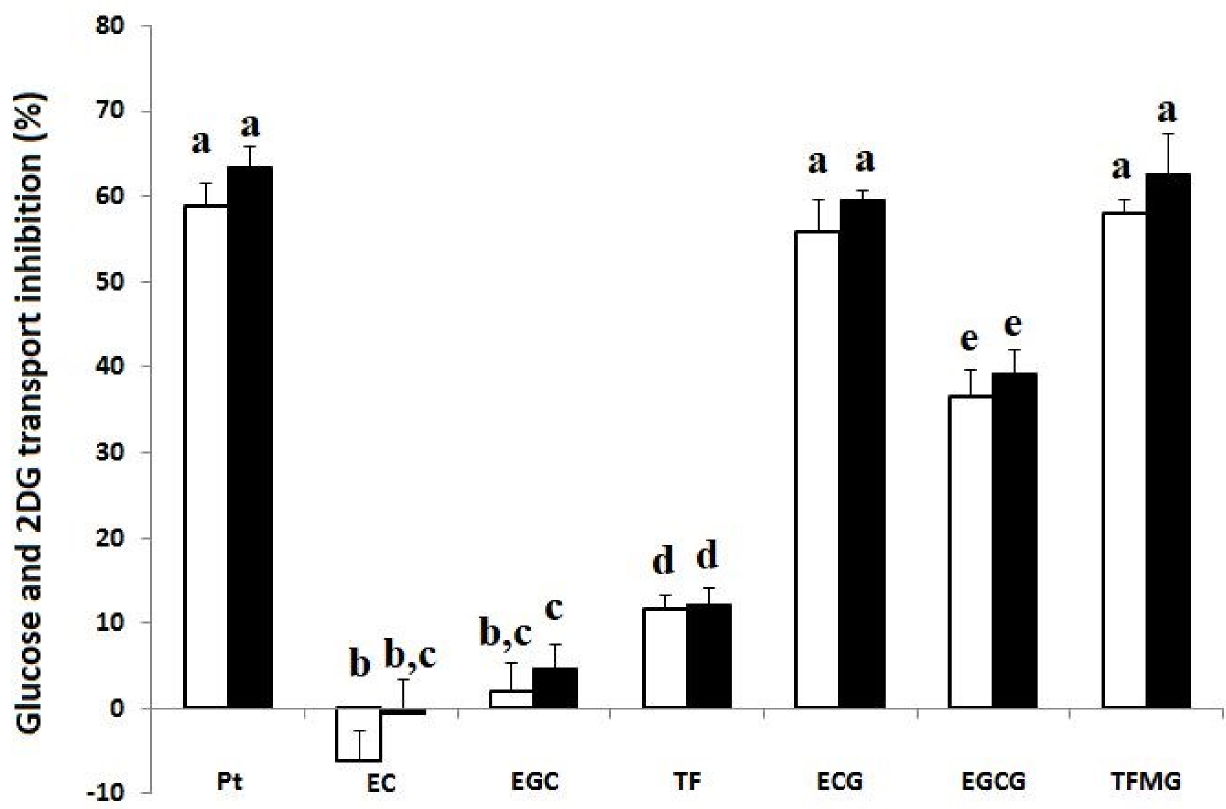

Figure 4

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