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ScienceDirect
Food Chemistry 108 (2008) 369–373
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical Methods

Analysis of chlorogenic acids in beverages prepared from Chinese health foods and investigation, *in vitro*, of effects on glucose absorption in cultured Caco-2 cells

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Received 6 August 2007; accepted 20 October 2007

Abstract

The effects of aqueous extracts of Kuding tea (*Ilex latifolia* Thunb.) and the large-leaf form of *Camellia sinensis* var. *sinensis*), chrysanthemum (*Dendranthema morifolium* Ramat), honeysuckle flower (*Lonicera japonica* Thunb.), and purple sweet potato (*Ipomoea batatas*) stem on glucose absorption were investigated using Caco-2 cells. Glucose absorption by Caco-2 cells was significantly inhibited by aqueous extract of Kuding tea, chrysanthemum and purple sweet potato stem under both Na^+-dependent conditions and Na^+-free conditions indicating effects on SGLT1 and GLUT transporters. Analysis of the (poly)phenols in these aqueous extracts suggested that dicaffeoylquinic acids and flavanols may be particularly important in producing these effects. Kuding tea extract was the most effective, suggesting that this merits evaluation in a clinical study.
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Keywords: Caco-2 cells; *Camellia sinensis*; Chlorogenic acids; Chrysanthemum; *Dendranthema morifolium*; Flavanols; Glucose absorption; Glucose transport; GLUT; Honeysuckle; *Ilex latifolia*; *Ipomoea batatas*; Kuding; SGLT1; Sweet potato

1. Introduction

Kuding (*Ilex latifolia* Thunb.), chrysanthemum (*Dendranthema morifolium* Ramat), honeysuckle (*Lonicera japonica* Thunb.), and purple sweet potato (*Ipomoea batatas*) are consumed as beverages and vegetables in China where their consumption is believed to provide health benefits. For example, it has been reported that triterpenoids from Kuding have inhibitory effects on acyl CoA cholesterol acyl transferase, thus potentially protecting against arteriosclerosis and obesity (Negishi, Negishi, Yamaguchi, & Sugahara 2004), that chrysanthemum has been used to cure 'liver-fire' and treat eye disease (Anonymous, 1985), and that honeysuckle has antipyretic properties (Anonymous, 1985). In contrast, there have been few studies on the physiological and pharmacological effects of consuming sweet potato. The phytochemical composition of sweet potato has been recently reported (Wang & Clifford, 2008), revealing the presence of chlorogenic acids (Clifford, Wu, Kirkpatrick, & Kuhnert, 2007; Wang & Clifford, 2008), flavanols (Liang, Xu, Hu, & Liu, 1992) and other flavonoids (Liu, Xu, Liang, & Hu, 1992). The most widely occurring chlorogenic acid and one of the few that is available commercially, 5-caffeoylquinic acid, is a competitive inhibitor of hepatic glucose 6-phosphatase (Arion et al., 1997) but the extent to which 5-caffeoylquinic acid reaches the liver unmetabolised *in vivo* is unclear.

5-caffeoylquinic acid is found in abundance in coffee beans and commercial coffee products. Recently, several independent studies in Europe (Rosengren, Dotevall, Wilhelmsen, Thelle, & Johansson, 2004; Tuomilehto, Hu, Bidel, Lindstrom, & Jousilahti, 2004; van Dam & Feskens, 2002) have indicated that greater coffee consumption is

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doi:10.1016/j.foodchem.2007.10.083

associated epidemiologically with a reduced risk of developing type 2 diabetes mellitus. The substances and mechanism(s) responsible remain uncertain, though there is some evidence from *in vitro* studies that 5-caffeoylquinic acid might dissipate the Na^+ electrochemical gradient which provides the driving force for active absorption of glucose (Welsch, Lachance, & Wasserman, 1989).

More recently, Johnston, Clifford, and Morgan (2003) have demonstrated that coffee consumption by volunteers delayed the absorption of glucose. Indeed, many dietary (poly)phenols have been shown, at least *in vitro*, to modulate glucose uptake. Green tea polyphenols such as (–)-epigallocatechin gallate (EGCG) and (–)-epicatechin gallate (ECG) also inhibit the Na^+-dependent glucose transporter (Hossain et al., 2002; Kobayashi et al., 2000) and the Na^+-independent facilitative transporter (Johnston, Sharp, Clifford, & Morgan, 2005). Some quercetin glucosides inhibit glucose uptake into brush-border-membrane vesicles of porcine jejunum (Cermak, Landgraf, & Wolffram, 2004) and Gee, DuPont, Rhodes, and Johnson (1998) have shown that some quercetin glucosides are capable of interacting with the Na^+-dependent glucose transporters in the mucosal epithelium and, as a consequence, this may even be the route by which these flavonol glucosides are absorbed by the small intestine *in vivo* (Gee et al., 1998).

In this study we examined aqueous extracts of plants rich in various (poly)phenols for their ability to inhibit glucose uptake by Caco-2 cells as a screening procedure to identify beverages which might merit further investigation in clinical studies.

2. Materials and methods

2.1. Materials

Kuding tea bags were purchased from the tea product factory in Hunan Agricultural University. These are a specially-developed product containing a mixture of Kuding (*I. latifolia* (Thunb.)) and the large-leaf form of *Camellia sinensis* var. *sinensis* grown characteristically in Yunnan Province, China, an unusual form of green tea where the dominant flavanol is (–)-epicatechin gallate (Shao, Clifford, & Powell, 1995; Shao, Powell, & Clifford, 1995). Purple sweet potato stem (*I. batatas*) was collected from Hunan Agricultural University; chrysanthemum (*Dendranthema morifolium* cv. *Gonju*) produced in Hanhui province, China, was purchased from a tea store in Changsha, China, and honeysuckle flower (*L. japonica* Thunb.) was collected from Zhang jiajie, Hunan province, China. All samples were freeze-dried and the dry material stored at 4 °C.

5-Caffeoylquinic acid and heat-inactivated fetal bovine serum were purchased from Sigma Chemical Company (Poole, UK). Cynarin (1,3-dicaffeoylquinic acid) was obtained from LGC Promochem (Hatfield, UK). D-[6-^{3}H] glucose was supplied by Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, UK. Cell culture medium and plasticware were purchased from Life Science Technologies (Paisley, UK) unless stated otherwise. Methanol and acetonitrile for HPLC were purchased from Fisher (UK). All other chemicals were of the highest grade available and bought from reputable commercial sources.

2.2. Preparation of plant extracts

To imitate the method commonly used to prepare beverages for human consumption, 1.5 g of freeze-dried plant material were soaked in 250 ml of boiling water and this suspension incubated for 60 min in a capped thermos flask. The insoluble material was removed from the extract by filtration with a Whatman No. 1 filter paper and 0.5 ml Carrez A reagent added to the filtrate, the mixture vortexed for 20 s and allowed to stand for 1 min. This procedure was repeated with Carrez B reagent and the mixture centrifuged at 2000*g* for 20 min. The aqueous supernatants were tested for effects upon glucose uptake by cultured Caco-2 cells.

2.3. Cell culture

Stock cultures of Caco-2 TC7 cells were maintained in 25 cm^2 plastic flasks and cultured in a 95% air/5% CO_2 atmosphere in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 20% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, 1% non-essential amino acids and 1% L-glutamine. All experiments were carried out on cells between passage numbers 30 and 40. For experiments, cells were seeded at a density of 1×10^4 cells/cm^2 into six-well plate inserts (Costar UK, Buckinghamshire, UK) and were grown for 19–21 days.

2.4. Measurement of glucose uptake by Caco-2 TC7 cell monolayers

Glucose uptake assays were performed using HEPES-buffered salt solution (HBSS, pH 7.5: 140 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES) containing 1 mM glucose and a 1 in 10 dilution of the plant extracts. D-[6-^{3}H] Glucose was used as the tracer and the final amount of radioactivity in the control or test solutions was 125 kBq/ml. When a sodium-free buffer was required for investigating facilitative transport, NaCl and Na_2HPO_4 in HBSS were replaced with equimolar amounts of KCl and K_2HPO_4, respectively.

Caco-2 cells were placed in serum-free media for 24 h prior to uptake studies and were incubated for 15 min at room temperature in HBSS prior to commencing experiments. Uptake was initiated by the addition of either the control or test solutions and the reaction was terminated after 2 min by aspiration of the uptake buffer, followed by the addition of ice-cold PBS. Cells were washed twice more in ice-cold PBS and solubilised overnight in 200 mM NaOH prior to scintillation counting.

2.5. Extraction of plant material for LC–MS analysis

Freeze-dried plant material (500 mg) was extracted (3 × 40 ml, 20 min each) with 70% v/v aqueous methanol, using an HT-1043 solid–liquid continuous extraction system (Tecator, Bristol, UK). The solvent cups containing the extract were allowed to cool for a few minutes and filtered through Whatman No.1 filter paper into a 100 ml volumetric flask and made up to volume with 70% v/v aqueous methanol. An aliquot (10 ml) was treated with Carrez reagents (0.5 ml reagent A plus 0.5 ml reagent B), mixed by inversion and vortexing at least five times for 20 s at 1 min intervals, and centrifuged (2000*g*, 20 min). An aliquot of supernatant (7 ml) was transferred to a glass-tube and evaporated to dryness under nitrogen at 60 °C (N-Evap-111, Organomation Associates Inc., Berlin, MA).

The residue was dissolved in 200 μl methanol and transferred with washing (4 × 200 μl) into a volumetric flask (5 ml), made up to volume with water, centrifuged (13,400*g*, 10 min), syringe-filtered (0.45 μm), stored at −12 °C until required, thawed at room temperature, and used directly for LC–MS^n.

2.6. LC–MS^n

The LC equipment (ThermoFinnigan, San Jose, CA, USA) comprised a Surveyor MS Pump, autosampler with 50 μl loop, and a PDA detector with a light-pipe flow cell (recording at 320, 280 and 254 nm, and scanning from 200 to 600 nm). This was interfaced with an LCQ Deca XP Plus mass spectrometer fitted with an ESI source (ThermoFinnigan, San Jose, CA, USA) operating in data-dependent full scan mode for the determination of parent ion and fragment ion *m*/*z*.

2.7. Data analysis

Data for glucose uptake are presented as the means ± SEM. Statistical analysis was carried out using SPSS 10.0. A one-sample *t*-test was based on data from two control wells and four treatment wells. In order to correct for multiple comparisons, the level of significance (α-value) was $P < 0.05$.

3. Results

3.1. Effects of aqueous plant extracts on glucose uptake by cultured Caco-2 cells

Caco-2 cells are derived from a human colon adenocarcinoma and are used widely as a model of intestinal absorption by epithelial cells (Sambuy et al., 2005) and to study absorption of glucose *in vitro* (Johnston, Sharp, Clifford, & Morgan, 2005). In the presence of an aqueous extract of chrysanthemum flower, purple sweet potato stem and Kuding tea, Na^+-dependent (i.e. SGLT1-mediated) glucose uptake was significantly decreased to 82% ($p < 0.05$), 85% ($p < 0.05$) and 36% ($p < 0.0001$) of the control value, respectively (Fig. 1). In contrast, while the aqueous extract of honeysuckle flower reduced glucose uptake, the effect did not reach statistical significance.

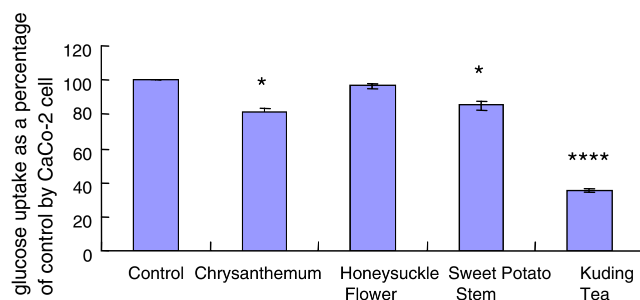

Fig. 1. Effects of various plant aqueous extracts on Na^+-dependent glucose uptake by CaCo-2 cells. Glucose concentration 1 mM, control mannitol 100 μM. Beverages prepared from 1.5 g of freeze-dried material in 250 ml of boiling water to simulate domestic preparation. Each data point is presented as the mean ± SEM ($n = 4$). $^{*}P < 0.05$, $^{****}P < 0.0001$.

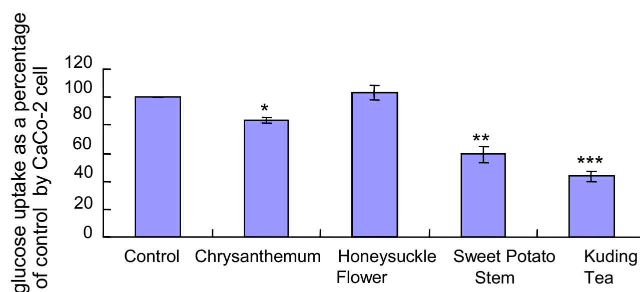

Fig. 2. Effects of various plant aqueous extracts on Na^+-independent glucose uptake by CaCo-2 cells. Glucose concentration 1 mM, control mannitol 100 μM. Beverages prepared from 1.5 g of freeze-dried material in 250 ml of boiling water to simulate domestic preparation. Each data point is presented as the mean ± SEM ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Under Na^+-independent conditions, i.e. GLUT-mediated, the same aqueous extracts reduced glucose absorption as follows: chrysanthemum flower to 82% ($p < 0.05$), purple sweet potato stem to 65% ($p < 0.001$) and Kuding tea to 50% ($p < 0.001$) whereas honeysuckle flower had no detectable effect (Fig. 2).

3.2. Analysis of CQA and associated cinnamic acids by LC–MS^n

As summarised in Table 1, and consistent with previous reports, a range of chlorogenic acids was detected in each of the plant extracts used in this study. In the case of the Kuding, the chlorogenic acids derived from the *I. latifolia* were accompanied by significant amounts of flavonol glycosides and flavanols, dominated, respectively, by rutin and (–)-epicatechin gallate, and a significant but lesser amount of (–)-epigallocatechin gallate, derived from the

Table 1
The profiles in the beverages of chlorogenic acids and related compounds

	Chrysanthemum	Purple sweet potato stem	Kuding tea	Honeysuckle flower
CQA glycosides	+			
diCQA glycosides	+			
1-CQA	+			
3-CQA	+	+	+	+
4-CQA	+	+	+	+
5-CQA	+++	+++	+++	+++
3-*p*CoQA	tr		+	
4-*p*CoQA	tr		+	
5-*p*CoQA	tr			
3-FQA	tr	+	+	
4-FQA	tr	+	+	
5-FQA	tr	+	+	
1,3-diCQA	+			
1,4-diCQA	+			
1,5-diCQA	+++			
3,4-diCQA	+		+	
3,5-diCQA	+++	+++	+++	
4,5-diCQA	+	+	+	
3,4,5-triCQA	+			
CFQA		+[a]		

Key: tr = trace; + = present; +++ = dominant isomer; quantitative comparisons should only be made in the same column.
CQA = caffeoylquinic acids; *p*-CoQA = *p*-coumaroylquinic acids; FQA = feruloylquinic acids; triCQA = tricaffeoylquinic acids; CFQA = caffeoyl-feruloylquinic acids.
[a] At least four isomers of caffeoyl-feruloylquinic acids were detected but could not be fully characterised.

C. sinensis component. Chromatograms and their interpretation have been published elsewhere (Clifford et al., 2007; Shao, Clifford, et al., 1995; Shao, Powell, et al., 1995; Wang & Clifford, 2008).

4. Discussion

There is an increasing accumulation of data linking dietary (poly)phenols with effects on glucose absorption and carbohydrate metabolism. Thus there is the potential for these compounds to protect against the development of type 2 diabetes and the metabolic syndrome and the data underlying this assertion has been reviewed elsewhere (Clifford, 2004; Clifford & Brown, 2006). Of particular relevance to the present study are observations that, at normal dietary levels, chlorogenic acid-containing coffee beverage and apple juice acutely modify volunteers' gastrointestinal hormone secretion and glucose tolerance (Johnston, Clifford, & Morgan, 2002; Johnston, Sharp, Clifford, & Morgan, 2003). The biochemical mechanisms underlying such protection are unclear, but *in vitro* studies with various pure polyphenols (Johnston et al., 2005) suggest that slowing the intestinal absorption of glucose by inhibition of the active glucose transporter (SGLT1) and/or the facilitative glucose transporters (GLUT) may be one of these.

The studies reported here using aqueous extracts corresponding to the levels normally consumed in beverages suggest that the mono-caffeoylquinic acids are not particularly effective in inhibiting glucose uptake since the extract of honeysuckle flower did not demonstrate a statistically significant effect on either SGLT1 (Fig. 1) or GLUT (Fig. 2) transporters. This is consistent with our previous studies employing 5-caffeoylquinic acid (Johnston et al., 2005) and with the report from Welsch et al. (1989) where inhibition of SGLT1 was seen only at very high (millimolar) concentrations.

In contrast, aqueous extracts of chrysanthemum, purple sweet potato stem, and especially Kuding Tea, were much more effective in inhibiting both SGLT1 and GLUT transporters. In addition to the mono-caffeoylquinic acids, these beverages contain a range of other cinnamic acid derivatives, including various dicaffeoylquinic acids, plus flavanols and flavonol glycosides in the case of Kuding tea. We observed that 1,3-dicaffeoylquinic acid (100 μM), the only dicaffeoylquinic acid available to us in sufficient quantity and purity, reduced glucose uptake by 24% compared with the control under Na^+-dependent conditions, which was more potent than 5-caffeoylquinic acid (100 μM) against SGLT1 (data not shown).

The strongest effects on glucose absorption were observed with Kuding tea, suggesting that its dominant flavanols, (–)-epicatechin gallate, and (–)-epigallocatechin gallate, also caused a significant inhibition of both classes of transporters. This agrees with previous observations from our group and from others using pure flavanols (Johnston et al., 2005; Kobayashi et al., 2000). Our data suggest that Kuding tea merits further investigation in a clinical study to determine its effects on glucose absorption *in vivo*.

Acknowledgements

The authors thank the China Scholarship Council for funding a postdoctoral fellowship for Dr. Wang Zheng. Professor Luo Zemin and Professor Cao Yong kindly provided sweet potato and honeysuckle flower samples. Technical assistance from Mr. H. Roozendaal is gratefully acknowledged.

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