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ARTICLE

Inhibition of Three Selected Beverage Extracts on α-Glucosidase and Rapid Identification of Their Active Compounds Using HPLC-DAD-MS/MS and Biochemical Detection

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Inhibition of α-glucosidase is a therapeutic approach for diabetes. In this study, a method based on online liquid chromatography−diode array detection−tandem mass spectrometry and biochemical detection (LC-DAD-MS/MS-BCD) was developed to screen and identify α-glucosidase inhibitors from selected beverage extracts, including pu-erh tea (*Camellia sinensis* var. *assamica*), eagle tea (*Litsea coreana* Levl.), and radix glycyrrhizae (*Glycyrrhiza uralensis* Fisch.). As a result, two components, (−)-epigallocatechingallate (EGCG) and (−)-epicatechingallate (ECG), as potent α-glucosidase inhibitors, were found in pu-erh tea. The IC_{50} values of EGCG and ECG on α-glucosidase (EC 3.2.1.20, from *Saccharomyces cerevisiae*) were 175.1 and 246.9 μM, respectively, and both were lower than that of acarbose (IC_{50} = 3553.0 μM), a commercial α-glucosidase inhibitor. Kinetic studies revealed that both EGCG and ECG inhibited α-glucosidase activity in a noncompetitive manner. The study suggests that the developed LC-DAD-MS/MS-BCD system is a powerful tool for rapid screening and identification of α-glucosidase inhibitors in complex samples and that EGCG and ECG may be good candidates as α-glucosidase inhibitors.

KEYWORDS: Pu-erh tea; eagle tea; radix glycyrrhizae; α-glucosidase; HPLC; biochemical detection

INTRODUCTION

Type II diabetes mellitus, characterized by high blood sugar and metabolic disorder, has been recognized as a serious global health problem, often resulting in substantial morbidity and mortality. α-Glucosidase plays an important role in the regulation of postprandial blood glucose levels in the human body (*1*), and its inhibitors, which can suppress postprandial hyperglycemia, are usually used to prevent or treat type II diabetes (*2*). To date, some inhibitors such as acarbose and voglibose are widely used in clinics to control blood glucose levels of patients, although they can cause negative gastrointestinal symptoms (*3*). Natural products, due to their unmatched chemical diversity and biological relevance, have been widely accepted as potential, high-quality chemical pools for screening of drug candidates. Therefore, discovery of α-glucosidase inhibitors from natural materials such as food matrices is very helpful in the development of new antidiabetic drugs (*4*−*6*).

Actually, the discovery of bioactive compounds from complex mixtures is difficult work. In recent years, a hyphenated technique, HPLC coupled with diode array detection and/or mass spectrometry and online biochemical detection (BCD), has been applied to screening bioactive compounds, which targets enzymes such as angiotensin-converting enzyme (*7*), acetylcholinesterase (*8*), or receptor-like estrogen receptor (*9*, *10*) from natural plants or food products.

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In the present study, the effects of three selected beverages, including pu-erh tea (*Camellia sinensis* var. *assamica*), eagle tea (*Litsea coreana* Levl.), and radix glycyrrhizae (*Glycyrrhiza uralensis* Fisch.), on α-glucosidase were investigated and an HPLC-DAD-MS/MS-BCD method was first developed for screening of α-glucosidase inhibitors in the extracts. As a result, two compounds with α-glucosidase inhibitory effects in pu-erh tea were found and identified. Their inhibition potency and kinetics were also determined.

MATERIALS AND METHODS

Chemicals and Materials. α-Glucosidase type I (EC 3.2.1.20) from *Saccharomyces cerevisiae*, *p*-nitrophenyl α-D-glucopyranoside (PNPG), as the substrate of α-glucosidase, and acarbose were purchased from Sigma (St. Louis, MO). The compounds (−)-epigallocatechingallate (EGCG) and (−)-epicatechingallate (ECG) were purchased from Shanghai Ronghe Pharmaceutical Corp. (Shanghai, China). Methanol and formic acid for liquid chromatography were purchased from Merck (Darmstadt, Germany). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Billerica, MA).

Pu-erh tea (leaf, *Camellia sinensis* var. *assamica*) was supplied by Macao International Food Safety Association. Eagle tea (leaf, *Litsea coreana* Levl.) and radix glycyrrhizae (root, *Glycyrrhiza uralensis* Fisch.) were collected from Ninguo, Anhui, and Longxi, Gansu, respectively, China. Their species were identified by the corresponding author, Professor S.-P. Li, and voucher specimens were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao, China.

Sample Preparation. Sample materials (1 g) were extracted with 50% methanol (50 mL) for 30 min at room temperature under ultrasonication (43 kHz, 320 W). Then the extract was concentrated at 50 °C using a rotary

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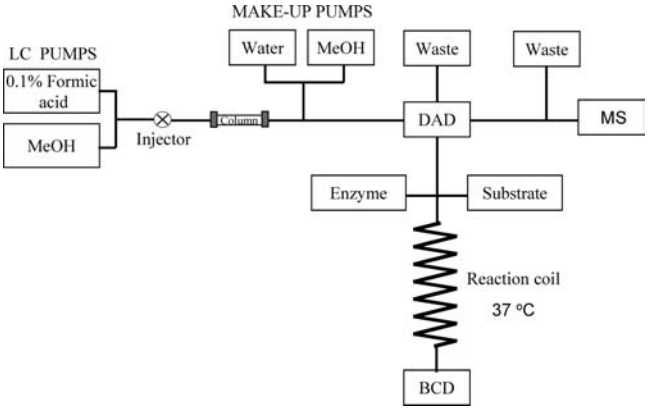

Figure 1. Diagrammatic scheme of HPLC-DAD-MS-BCD for rapid screening and identification of α-glucosidase inhibitors from beverage extracts.

evaporator (Büchi, Flawil, Switzerland), and the residue was transferred into a 5 mL volumetric flask and diluted to the mark with 50% methanol. The supernatant was centrifuged (13000 rpm for 5 min) and filtered through a 0.45 μm filter (Agilent Technologies) before injection into an HPLC system for analysis or was diluted to a series of suitable concentrations with 50% methanol for α-glucosidase inhibition activity assay.

α-Glucosidase Inhibition Assay of the Selected Beverage Extracts. The inhibitory activity of three selected beverage extracts on α-glucosidase was determined spectrophotometrically on 96-well microplate reader (*11*). In brief, to a total of 60 μL of reaction mixture containing 20 μL of 100 mM phosphate buffer (pH 6.8), 20 μL of 2.5 mM PNPG in the buffer, and 20 μL of investigated beverage extract in the wells was added 20 μL of 0.2 U/mL α-glucosidase in phosphate buffer and mixed well. After incubation for 15 min at 37 °C, the reaction was stopped by adding 80 μL of 0.2 M sodium carbonate solution. Then the absorbance (A_S) at 405 nm was recorded (Wallac Victor 1420 multilable counter, PerkinElmer, The Netherlands). The control sample was the mixture of the test sample with solvent instead. The sample and control blanks were the mixtures of sample and control, respectively, except α-glucosidase was instead with buffer, respectively. The inhibition (%) of test sample on α-glucosidase could be calculated as

$$\text{inhibition } (\%) = 100 \times (A_S - A_{SB})/(A_C - A_{CB})]$$

where A_S, A_{SB}, A_C, and A_{CB} are the absorbance of sample, sample blank, control, and control blank, respectively. The measurement was performed in triplicates.

LC-DAD-MS/MS and BCD Analysis. An Agilent Technologies series 1200 liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an autosampler, a diode array detector, and an ion-trap mass spectrometer with electrospray ionization interface, controlled by Agilent LC/MSD Trap software, was used for the analysis. A Zorbax SB C18 column (250 mm × 4.6 mm i.d., 5 μm) with a Zorbax SB C18 guard column (12.5 mm × 4.6 mm i.d., 5 μm) was used. Separation was achieved by a gradient elution of 0.1% formic acid aqueous (A) and methanol (B) at a flow rate of 1 mL/min: 0−30 min, 15−25% B; 30−45 min, 25−60% B. The column temperature was set at 37 °C, and the injection volume was 40 μL. To maintain a constant concentration of organic modifier in LC-BCD system, an additional postcolumn makeup was used, and methanol elution was adjusted to 30% with a constant flow rate of 2 mL/min. Subsequently, a splitter was inserted to obtain continuous flow rates of 100 μL/min to BCD and 1000 μL/min to DAD and then 1:1 split to MS, as well as 900 μL/min to waste (**Figure 1**). The analytes were detected at 270 nm by DAD, and mass spectrometry was operated in positive scan mode from m/z 50 to 1400. ESI-MS conditions were as follows: drying gas (N_2) flow rate, 7 L/min; drying gas temperature, 350 °C; nebulizer pressure, 35 psi. ESI-MS/MS conditions were as follows: compound stability, 10%; isolation width, 3; fragment amplification, 1.5.

For biochemical detection, 100 μL/min eluent, 50 μL/min 0.4 U/mL α-glucosidase, and 50 μL/min 2.5 mM PNPG on ice continuously entered and mixed in the reaction coil (200 cm × 0.50 mm i.d. PEEK tubing), where the mixture was allowed to interact for about 2 min. The reaction product, *p*-nitrophenol, was measured at 405 nm at the end of the reaction coil.

Table 1. Inhibition of the Selected Three Beverage Extracts on α-Glucosidase ($n = 3$)

	inhibition (%, av ± SD)		
concn[a] (mg/mL)	pu-erh tea	eagle tea	radix glycyrrhizae
1.0	17.1 ± 1.3		
1.3		17.0 ± 2.2	
2.5	42.1 ± 2.8	33.8 ± 4.6	
5.0	76.6 ± 3.5	62.6 ± 0.8	12.4 ± 3.7
10.0	101.4 ± 0.5	81.6 ± 1.0	32.2 ± 4.0
20.0		94.8 ± 0.6	50.8 ± 4.3
40.0			67.4 ± 4.6
IC_{50}[b] (mg/mL)	2.6	3.8	20.1
95% confidence interval[b] (mg/mL)	2.8−3.5	3.0−4.8	18.3−22.3

[a] Concentration of raw material. [b] IC_{50} value and 95% confidence interval of each sample were calculated on the basis of linear regression of the inhibition capacities to the logarithm of samples' concentrations, which was performed by GraphPad prism 5.0 for Windows (GraphPad Software Inc.).

Enzyme Inhibitory Activity and Kinetics of Investigated Compounds. α-Glucosidase inhibition activity and kinetics of investigated compounds were determined with microplate as mentioned above. For kinetic analysis of α-glucosidase inhibition, a certain concentration of 0.2 U/mL α-glucosidase and different contents of test compounds were incubated with a series of concentrations of substrate. The inhibitory kinetics of the investigated compounds on α-glucosidase was analyzed using the Lineweaver−Burk plot (*12*), double-reciprocal plot of the substrate concentration, and velocity (expressed as absorbance). Besides, the inhibitory activity and kinetic of acarbose, a positive drug, were also determined.

RESULTS AND DISCUSSION

Inhibition of Selected Beverage Extracts on α-glucosidase. Pu-erh tea, eagle tea, and radix glycyrrhizae have a long history as folk beverages due to their beneficial effects in the human body. Modern pharmacological studies have demonstrated that pu-erh tea ethanol extract might have the function of lowering blood glucose with $PPAR_\delta$ agonists using SMMC-7721 cells as model (*13*), total flavonoids of *L. coreana* had a hypoglycemic role on pathological mice and rat models (*14*), and the EtOAc extracts of licorice could decrease the blood glucose level of genetically diabetic KK-A^y mice (*15*). However, so far there have been few studies on the hypoglycemic active compounds responsible for their antidiabetic activities (*16*). In this study, their effects on α-glucosidase were investigated. The results (**Table 1**) showed that all of the extracts of the three selected beverages had α-glucosidase inhibitory activity.

Effects of Organic Solvent on α-Glucosidase Activity. For HPLC separation of complex mixtures, a gradient organic solvent such as methanol or acetonitrile is usually necessary. However, high concentrations of organic modifiers could lower enzyme activity and even denature the enzyme. In addition, volatile acid is commonly added to the mobile phase to improve separation, the shape of peaks, and/or MS response. Therefore, the influence of methanol or acetonitrile together with formic acid on the enzymatic activity of α-glucosidase at different ratios was investigated. It was found that the enzymatic activity was not significantly lowered by methanol below the concentrations of 30% but significantly decreased by acetonitrile (about 90% activity decreased at 30% ACN). Thus, methanol was selected as an organic modifier for LC-DAD-MS/MS-BCD analysis of beverage extracts, and its concentration in the final reaction mixture was controlled at 30%.

Inhibition of Acarbose on α-Glucosidase. For definite identification of peaks with inhibitory activities, a positive drug with definite α-glucosidase inhibition, acarbose, was used for testing system availability and the time delay between UV and biochemical

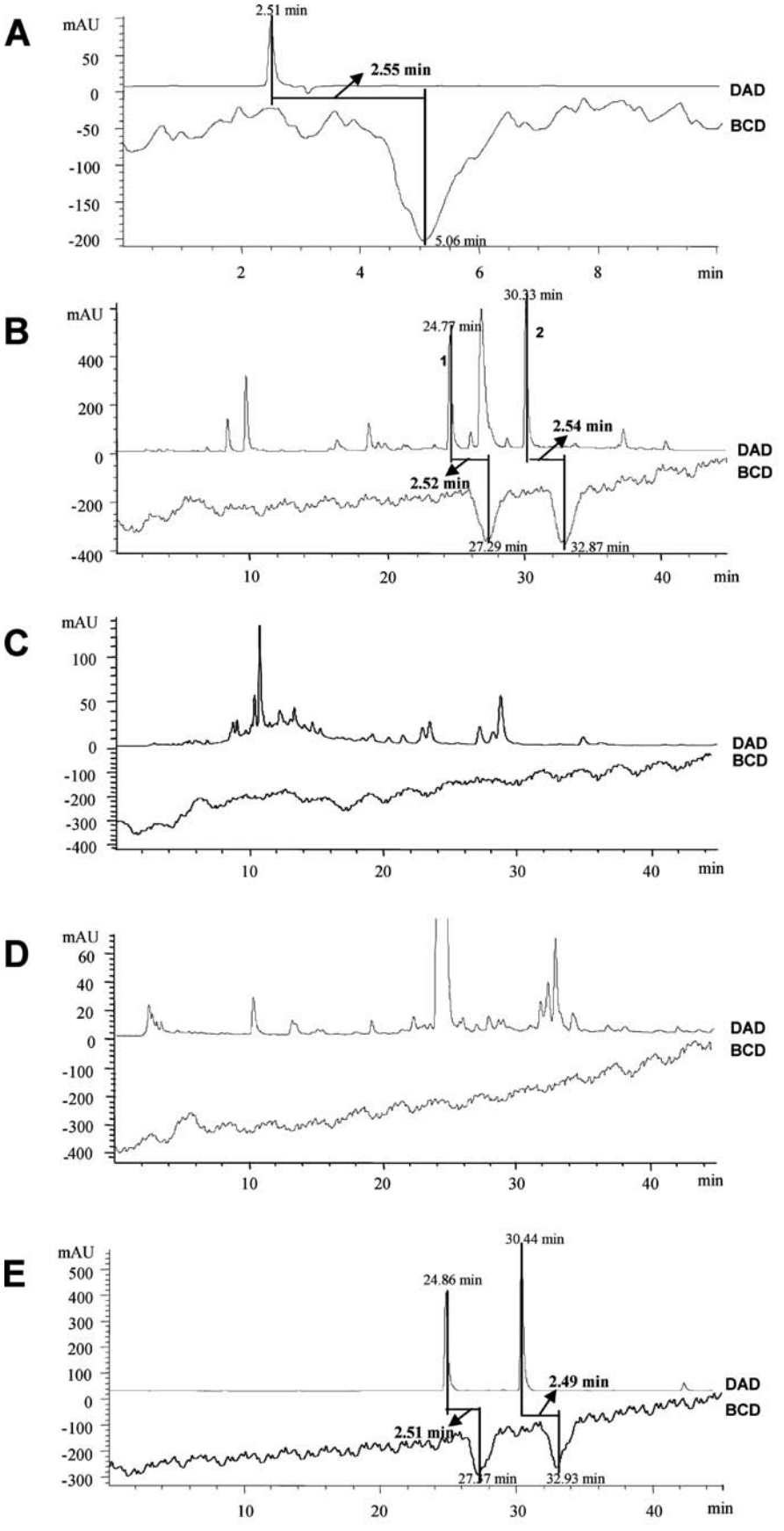

Figure 2. Typical UV chromatograms before (DAD) and after (BCD) online biochemical assay of acarbose (**A**), pu-erh tea (**B**), eagle tea (**C**), and radix glycyrrhizae (**D**) extracts and of mixed solution of EGCG and ECG (**E**).

detection. On the same system, 40 μL of acarbose (1 mg/mL) was injected and eluted with isocratic mobile phase of aqueous MeOH (70:30, v/v) containing 0.1% formic acid and detected at 210 nm. **Figure 2A** shows that acarbose had a definite negative peak to

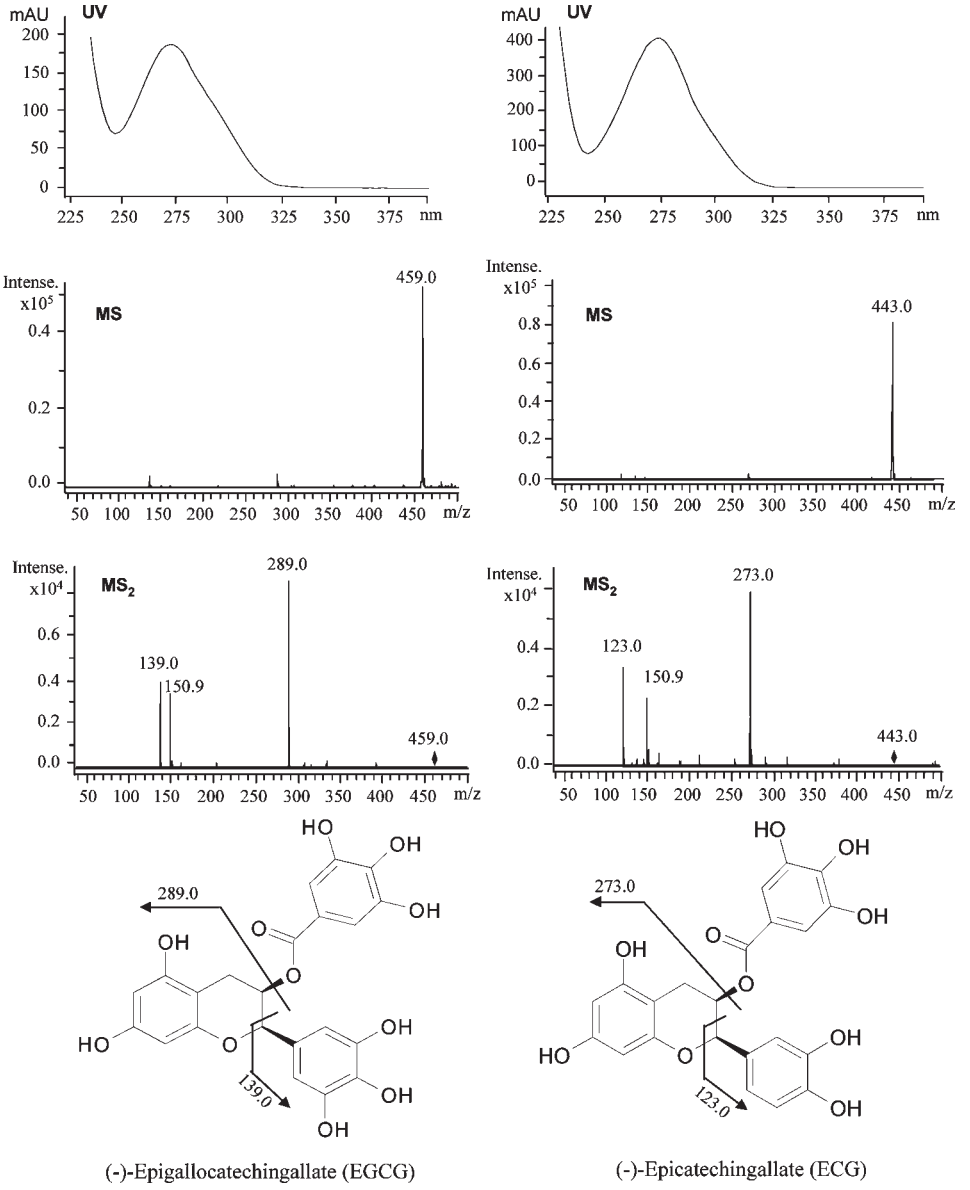

Figure 3. UV, MS, and MS_2 spectra and fragment illustration of peaks 1 (left) and 2 (right).

biochemical detection with the retention time of 5.06 min, but 2.51 min to UV detection. Finally, the system delay time between two detection modes was about 2.5 ± 0.1 min (n = 3).

Identification of α-Glucosidase Inhibitors in Beverage Extracts. Using the developed HPLC-DAD-MS/MS-BCD method, 40 μL of each of the three beverage extracts, pu-erh tea, eagle tea, and radix glycyrrhizae, at the concentration of 200 mg/mL of raw material was injected into the system and analyzed as mentioned above. **Figure 2B−D**) shows the chromatograms of three extracts detected by DAD and BCD, respectively. As a result, two components in pu-erh tea extract had α-glucosidase inhibitory activity. However, no components with activity in eagle tea and radix glycyrrhizae were found, although their extracts had strong inhibitory activity on α-glucosidase, which might be attributed to the slow inhibition kinetics of their active components or the effects of eagle tea and radix glycyrrhizae derived from the integrating effect of multiple weak and/or low concentration active components. For the two peaks with inhibitory effect on α-glucosidase in pu-erh tea, their retention times on BCD were 27.3 and 32.9 min, respectively. After calibration of delayed time (2.5 ± 0.1 min) between DAD and BCD, their retention times were 24.8 and 30.4 min on DAD, respectively, which should be peaks 1 and 2 (**Figure 2B**).

The components of peaks 1 and 2 were tentatively identified as EGCG and ECG, respectively, by comparison of their MS data (**Figure 3**) with previous literature (*17*−*19*). Furthermore, EGCG and ECG were also analyzed using the same system, and the results (**Figure 2E**) suggest that peaks 1 and 2 were EGCG and ECG, unambiguously, on the basis of their retention time and α-glucosidase inhibitory activity.

It was interesting that theaflavins, considered to be potent α-glucosidase inhibitiors (*20*), were not detected in this study. Actually, theaflavins that are formed by the oxidative condensation of catechins are characteristic polyphenols in black tea (*21*). However, raw pu-erh tea without fermentation was used in the present study, so there were no or little theaflavins to be detected.

Inhibitory Activity and Kinetics of EGCG and ECG. The inhibitory effects of two compounds identified in pu-erh tea, EGCG and ECG, and acarbose were investigated, and their activities were in dose-dependent manners (**Figure 4A**). The IC_{50} values of EGCG and ECG were determined to be 175.1 and 246.9 μM, respectively, both of which were much lower than that of acarbose (3553.0 μM).

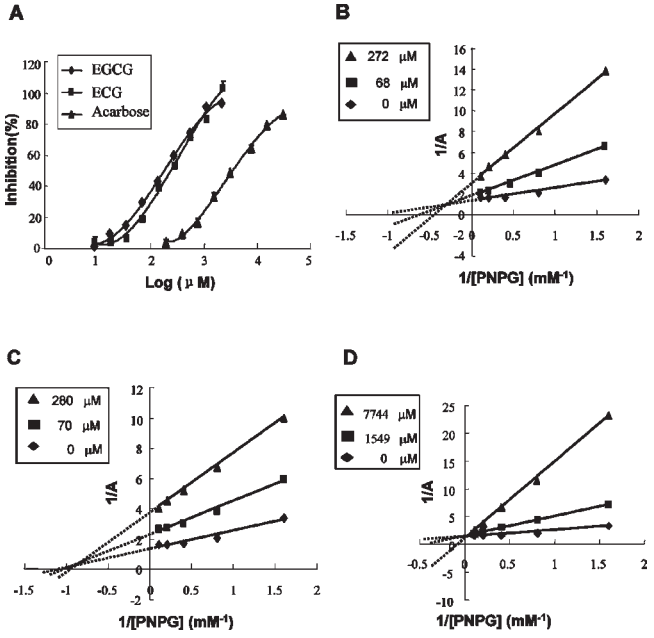

Figure 4. Inhibitory effects (**A**) and Lineweaver−Burk plots of EGCG (**B**), ECG (**C**), and acarbose (**D**) on α-glucosidase.

Table 2. Summarized Contents of EGCG and ECG in Different Teas

	content (%, w/w)		
tea	EGCG	ECG	ref
pu-erh	3.73−8.36[a]	2.06−8.38[a]	*17*
	0.07−0.24[b]	0.13−0.44[b]	*17*
	0.20	0.13	*30*
green	5.11−5.27[c]	1.13−2.18[c]	*30*
	6.11−7.74[d]	1.16−1.95[d]	*30*
	2.26	0.65	*19*
	4.91−8.71[d]	0.86−1.55[d]	*29*
oolong	2.22−2.82[c]	0.61−0.65[c]	*30*
	6.54	1.12	*29*
black	0.38	0.45	*30*
	0.43−6.13[d]	0.32−1.09[d]	*31*
	0.03	0.05	*19*
	5.73	1.80	*29*

[a] Four samples of raw tea. [b] Three samples of ripe tea. [c] Two samples. [d] Three samples.

The inhibition kinetics of EGCG, ECG, and acarbose were also determined and shown as Lineweaver−Burk plots (**Figure 4B−D**). The data indicated that EGCG and ECG inhibited the enzymatic activity in a noncompetitive manner, but acarbose was a competitive inhibitor. The findings are in accordance with the results previously reported for EGCG (*22*) and acarbose (*11*, *23*).

Contents of EGCG and ECG in Different Teas. Tea, a well-known traditional beverage, has attracted more attention in the world due to its wide range of beneficial health effects, special flavor and taste, and apparent lack of toxicity. The beneficial effects of tea, such as anticancer (*24*), antioxidant (*25*, *26*), and antiobesity (*27*, *28*), are largely attributed to EGCG. Similarly, the present study also indicated that EGCG and ECG responded to α-glucosidase inhibitory effect. **Table 2** summarizes the contents of EGCG and ECG in different teas on the basis of previous papers (*17*, *19*, *29*−*31*), which is helpful, at least partially, to understand the beneficial effects of tea.

Concluding Remarks. The developed LC-BCD-MS/MS-BCD system that generated both biological and chemical information of compounds is a valuable tool for effective screening and identification of α-glucosidase inhibitors in complex samples. EGCG and ECG mainly contribute to the inhibitory effect of pu-erh tea on α-glucosidase.

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