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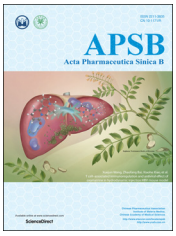

ORIGINAL ARTICLE

Flavanols from the *Camellia sinensis* var. *assamica* and their hypoglycemic and hypolipidemic activities

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KEY WORDS

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Abstract α-Glucosidase and lipase inhibitors play important roles in the treatment of hyperglycaemia and dyslipidemia. To identify novel naturally occurring inhibitors, a bioactivity-guided phytochemical research was performed on the pu-erh tea. One new flavanol, named (–)-epicatechin-3-*O*-(*Z*)-coumarate (**1**), and **16** known analogs (**2**–**17**) were isolated from the aqueous extract of the pu-erh tea. Their structures were determined by spectroscopic and chemical methods. Furthermore, the water extract of pu-erh tea and its fractions exhibited inhibitory activities against α-glucosidases and lipases *in vitro*; compound **15** showed moderate inhibitory effect against sucrase with an IC_{50} value of 32.5 μmol/L and significant inhibitory effect against maltase with an IC_{50} value of 1.3 μmol/L. Compounds **8**, **10**, **11** and **15** displayed moderate activity against a lipase with IC_{50} values of 16.0, 13.6, 19.8, and 13.3 μmol/L, respectively.

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1. Introduction

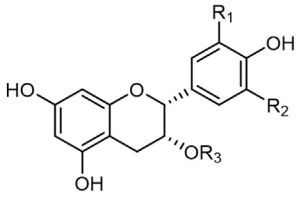
Type 2 diabetes mellitus (DM) is a metabolic disorder that is characterized by hyperglycemia caused by insulin resistance. It has become the third largest chronic noninfectious disease threatening the world. The classic symptoms include excess thirst, frequent urination, and constant hunger. Long-term complications from high blood sugar include heart disease, strokes, diabetic retinopathy and kidney failure. The use of herbal remedies to treat DM has been practiced since ancient times. Currently, more than 1000 plant species are being used as folk medicines to treat DM throughout the world[1].

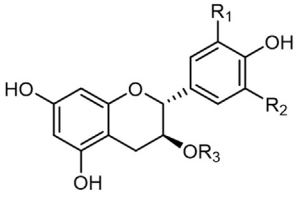
Tea is one of the most popular beverages consumed worldwide. According to the manufacturing process, Chinese commercial teas can be classified as green tea, yellow tea, white tea, oolong tea, black tea, and dark tea. Pu-erh tea is a kind of dark tea, which comes from "rough" *Camellia sinensis* (Linn) var. *assamica* (Masters) Kitamura (mao cha) in Yunnan province in China. The key production process of pu-erh tea is secondary fermentation, in which microorganisms play a very important role in producing the taste, color, fragrance, and functional components[2]. Pu-erh tea is not only a popular tea, but also a traditional Chinese medicine, which has many biological and biochemical effects, such as antiobesity[3], antiviral[4], antioxidative[5], hepatoprotective[6], hypoglycemic[7] and hypolipidemic[8]. In our research, we found that the water extract of pu-erh tea showed potential *in vitro* hypoglycemic and hypolipidemic effects. Bioassay-guided fractionation of these active extracts yielded one new flavanol (**1**) and 16 known compounds (**2**–**17**, Fig. 1), and the *in vitro* α-glucosidase inhibitory activity and lipase inhibitory activity were evaluated. Herein, the purification, structural determination and biological assays of these isolates were discussed.

2. Results and discussion

2.1. Compounds from pu-erh tea

The EtOAc section of the water extract from the pu-erh tea was subjected to multiple column chromatographic purification steps and further purified by preparative HPLC, affording one new flavanol (**1**) and 16 known compounds (Fig. 1). These compounds were identified as (–)-epicatechin-3-*O*-*(Z)*-coumarate (**1**), (–)-epicatechin-3-*O*-*(E)*-coumarate (**2**)[9], (–)-epicatechin-3-*O*-(*E*)-caffeate (**3**)[10], (+)-catechin (**4**)[11], ampelopsin (**5**)[12], (–)-epicatechin (**6**)[11], (–)-epiafzelechin (**7**)[13], (–)-epicatechin-3-*O*-gallate (**8**)[11], (–)-epiafzelechin-3-*O*-gallate (**9**)[11], (+)-catechin-3-*O*-gallate (**10**)[14], (+)-epiafzelechin-3-*O*-gallate (**11**)[15], epicatechin- 3-*O*-*p*-hydroxybenzoate (**12**)[16], (–)-epigallo-catechin (**13**)[11], (±)-gallocatechin (**14**)[11], (–)-epigallo-catechin-3-*O*-gallate (**15**)[11], (+)-gallocatechin-3-*O*-gallate (**16**)[17] and (–)-epicatechin-3-*O*-(3′′-*O*-methyl)-gallate (**17**)[18].





	R_1	R_2	R_3
1	OH	H	(*Z*)-Coumaroyl
2	OH	H	(*E*)-Coumaroyl
3	OH	H	(*E*)-Caffeoyl
6	OH	H	H
7	H	H	H
8	OH	H	Galloyl
9	H	H	Galloyl
12	OH	H	*p*-Hydroxybenzoyl
13	OH	OH	H
15	OH	OH	Galloyl
17	H	OH	(3′′-*O*-methyl)-galloyl

	R_1	R_2	R_3
4	OH	H	H
5	OH	OH	H
10	OH	H	Galloyl
11	H	H	Galloyl
14	OH	OH	H
16	OH	OH	Galloyl

Figure 1 Chemical structure of compounds **1**–**17**.

Compound **1** was obtained as a pale amorphous powder and showed absorption bands for hydroxy group (3276 cm^{-1}), aromatic double bond (1623, 1604 and 1514 cm^{-1}), and carbonyl group (1709 cm^{-1}) in the IR spectrum. Its molecular formula, $C_{24}H_{20}O_8$, was deduced from HR-ESI-MS (*m/z* 437.1242 $[M+H]^+$). In the ^1H NMR spectrum (Table 1), an ABX system of protons were observed at δ_H: 6.88 (1H, d, J=1.8 Hz), 6.66 (1H, d, J=8.0 Hz) and 6.69 (1H, dd, J=1.8, 8.0 Hz); 2 methine proton signals at δ_H 5.79 (1H, d, J=1.8 Hz), 5.93 (1H, d, J=1.8 Hz) were assigned to the H-8 and H-6 in the A ring, respectively; 2 oxygenated methine proton signals at δ_H 4.99 (1H, br, s) and 5.32 (1H, br s), and 1 methylene proton signal at δ_H 2.91 (1H, dd, J=4.0, 17.5 Hz) and 2.64 (1H, br d, J=17.5 Hz) were ascribable to the C ring in flavanol; 2 *cis*-olefinic protons at δ_H 6.25 (1H, d, J=12.5 Hz) and 7.40 (1H, d, J=12.5 Hz) indicated the presence of *cis*-double bond. Four aromatic proton signals were assigned to a *p*-hydroxy benzene ring at δ_H 7.49 (2H, d, J=8.5 Hz, H-5′′, -9′′) and 6.75 (2H, d, J=8.5 Hz, H-6′′, -8′′). In the ^{13}C NMR spectrum (Table 1), 24 carbon signals, including 1 ketone carbon, 1 methylene carbon, 2 oxygenated methine carbons, 2 *cis*-olefinic carbons and 18 aromatic carbons, were also observed. In the HMBC spectrum (Fig. 2), correlations to a ketone carbonyl carbon at δ_C 165.2 (C-1′′) from 2 *cis*-olefinic protons of H-2′′ and H-3′′ indicated the presence of α,β-unsaturated ketone group. Correlations from H-3′′ to C-1′′, C-5′′ and C-9′′ and from H-2′′ to C-1′′ indicated the presence of a (*Z*)-coumaroyl moiety. The oxygenated methine proton H-3 was correlated with the carbonyl carbon signal at δ_C 165.2 (C-1′′), confirming that the (*Z*)-coumaroyl moiety was connected to the 3-OH. Based on these data, the planar structure of compound **1** can be deduced.

Compound **1** has 2 chiral centers at C-2 and C-3. The 2,3-*cis* configuration of **1** was determined on the basis of a broad singlet at δ_H 4.99 for H-2 and an upfield shift of C-2 to δ_C 76.2[19,20]. The optical rotation {$[\alpha]_D^{20}$20 D–66.8 (*c* 0.025, MeOH)} and CD data [217 ($\Delta\varepsilon$ –9.96), 267 ($\Delta\varepsilon$ +1.24) and 314 ($\Delta\varepsilon$ –4.05) nm, MeOH] of **1** were found to be similar to those of (–)-epicatechin-3-*O*-(*E*)-caffeate {$[\alpha]_D^{20}$–175.5 (*c* 0.21, MeOH); 233 ($\Delta\varepsilon$ –4.13), 273 ($\Delta\varepsilon$ +1.21) and 321 ($\Delta\varepsilon$ –4.37) nm, MeOH}[10]. Thus, the absolute configuration of compound **1** was determined to be 2*R* and 3*R*. The structure of compound **1** was determined as (–)-epicatechin-3-*O*-*(Z)*-coumarate.

2.2. In vitro hypoglycemic and hypolipidemic effects

α-Glucosidase inhibitors could restrain the liberation of glucose from oligosaccharides, thereby reducing the postprandial glucose levels and enhancing insulin responses. Such inhibitors, including acarbose and voglibose, are currently used clinically in combination with either diet or other anti-diabetic agents to control blood glucose levels. Natural resource provided a huge and highly

Table 1 ^{1}H NMR and ^{13}C NMR spectroscopic data for compound **1**[a].

No.	δ_H	δ_C	No.	δ_H	δ_C
2	4.99 (s)	76.2	**4′**		144.9
3	5.32 (br s)	67.7	**5′**	6.66 (d, J=8.0 Hz)	115.1
4	2.91 (dd, J=4.0, 17.5 Hz, H-a), 2.64 (br d, J=17.5 Hz, H-b)	25.5	**6′**	6.69 (dd, J=1.8, 8.0 Hz)	117.4
5		156.5	**1″**		165.2
6	5.93 (d, J=1.8 Hz)	95.5	**2″**	6.25 (d, J=12.5 Hz)	115.0
7		156.6	**3″**	7.40 (d, J=12.5 Hz)	143.6
8	5.79 (d, J=1.8 Hz)	94.3	**4″**		125.2
9		155.4	**5″**	7.49 (d, J=8.5 Hz)	132.5
10		97.2	**6″**	6.75 (d, J=8.5 Hz)	114.9
1′		129.2	**7″**		158.9
2′	6.88 (d, J=1.8 Hz)	114.2	**8″**	6.75 (d, J=8.5 Hz)	114.9
3′		144.9	**9″**	7.49 (d, J=8.5 Hz)	132.5

[a]Data were measured in DMSO-d_6 for **1** (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR).

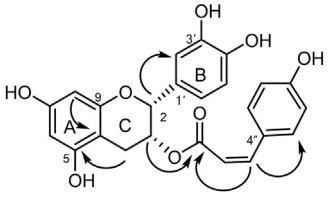


Figure 2 The key HMBC correlations of compound **1**.

Table 2 Inhibition effects of water extracts against α-glucosidase and lipase IC_{50} (μg/mL).

Sample	α-Glucosidase		Lipase
	Sucrase	Maltase	
1	49.3	4.72	9.69
2	14.4	11.4	7.52
3	50.1	22.4	12.42
4	>400	>400	>400

1: Water extract of pu-erh tea.
2: EtOAc fraction of water extract.
3: BuOH fraction of water extract.
4: Water fraction of water extract. Acarbose (IC_{50} value for sucrose: 0.262 μg/mL, for maltase: 0.084 μg/mL) and Orlistat (IC_{50} value for lipase: 0.006 μg/mL) were used as positive control.

diversified chemical bank from which we could explore for potential therapeutic agents by bioactivity-driven screenings. Thus, a bioassay-guided approach was tried to identify active ingredients from pu-erh tea. To that end, the inhibitory activity of the water extract of pu-erh tea and its fractions on sucrose and maltase was measured. The results showed that the EtOAc fraction had moderate inhibitory activity with the IC_{50} values of 14.4 μg/mL against surase and 11.4 μg/mL against maltase, respectively (Table 2). Then, the inhibitory effects of compounds **1**–**17** were also measured using the same methods. (–)-Epigallo-catechin-3-*O*-gallate (**15**) showed moderate activity with IC_{50} values of 32.5 μmol/L against sucrose and 1.3 μmol/L against maltase, respectively (Table 3). In addition, compounds **2**, **3**, **5**, **8**–**11**, and **13**–**16** displayed very weak inhibitory activities against sucrase with IC_{50} values ranging from 59.4 to 107.1 μmol/L (Table 3). In maltase inhibitory assay, compound **11** displayed moderate activity with an IC_{50} value of 15.8 μmol/L, and compounds **1**, **8**, **9**, **10**, **12**, and **17** showed weak activities with IC_{50} values ranging from 27.3 to 63.1 μmol/L (Table 3).

Lipase inhibitors could reduce the uptake of fat from food intake. The inhibitory activity of water extract and compounds **1**–**17** against a lipase was further evaluated. The results showed that the EtOAc fraction displayed strong activity with an IC_{50} value of 7.52 μg/mL (Table 2), and compounds **8**, **10**, and **15** displayed good activities with IC_{50} values of 16.0, 13.6, and 13.3 μmol/L, respectively (Table 3). And compounds **4**–**6**, **9**, **11**, **13**, and **16** showed weak activities with IC_{50} values ranging from 19.8 to 62.6 μmol/L (Table 3).

3. Conclusions

One new flavanol and 16 analogs were isolated from the water extract of pu-erh tea. The EtOAc fraction showed strong *in vitro* activity, which indicated that the functional materials were enriched in this fraction. The compounds isolated from the EtOAc fraction also showed inhibitory activity against α-glucosidases and a lipase. Compounds **11** and **15** displayed good activity, which may have the potential to be developed as therapeutic agents. The current data suggested that the activity of catechin-type compounds might be associated with the galloyl group.

4. Experimental section

4.1. General experimental procedures

The optical rotations were measured using a Jasco P2000 polarimeter, UV spectra were determined using a Jasco V650 spectrophotometer (JASCO, Corporation, Tokyo, Japan). IR spectra were carried out on a Nicolet 5700 spectrophotometer with KBr disks (Thermo Electron Scientific Instruments Corp.). ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), HSQC and HMBC spectra were run on a Mercury-500 with TMS as an internal standard (Varian, Palo Alto, CA, USA). HR-ESI-MS was performed on a 6520 Accurate-Mass Q-TOF LC/MS mass spectrometer. Sephadex LH-20 (Pharmacia, Uppsala, Sweden), silica gel (Qingdao Marine

Table 3 Inhibition effects of isolated compounds against α-glucosidase and lipase.

Compd.	Sucrase		Maltase		Lipase	
	Inhibition[a] (%)	IC_{50} (μmol/L)	Inhibition[a] (%)	IC_{50} (μmol/L)	Inhibition[a] (%)	IC_{50} (μmol/L)
1	45.4	–	71.8	49.7	42.6	–
2	71.3	62.1	–	–	41.6	–
3	64.5	107.1	–	–	36.3	–
4	21.5	–	16.1	–	64.9	39.7
5	90.4	74.6	92.0	199.9	92.1	20.4
6	39.2	–	48.5	–	70.4	32.2
7	31.5	–	25.5	–	44.9	–
8	61.9	104.6	84.7	27.3	91.8	16.0
9	83.5	84.2	91.4	63.1	97.0	43.9
10	79.2	71.9	89.7	27.5	96.4	13.6
11	88.1	59.4	91.8	15.8	95.9	19.8
12	41.2	–	67.6	61.8	55.8	–
13	84.6	71.3	–	–	89.5	51.7
14	93.8	78.1	78.5	–	56.7	–
15	92.7	32.5	98.7	1.3	93.8	13.3
16	77.5	78.7	–	–	75.8	62.6
17	41.9	–	68.7	60.3	47.5	–

Acarbose (IC_{50} value for sucrose: 0.97 μmol/L, for maltase: 0.13 μmol/L) and Orlistat (IC_{50} value for lipase: 0.012 μmol/L) were used as positive control.
– Not applicable.
[a]Inhibition at the concentration of 10^{-5} mol/L.

Chemical Factory, 200–300 mesh), and RP-18 (Merck, 40–60 μm) were used for CC and silica gel GF-254 (Qingdao Marine Chemical Factory, Qingdao, China) was used for TLC. The HPLC experiments were performed on a preparative YMC-Pack ODS-column (250 mm × 20 mm, 10 μm, YMC, Kyoto, Japan) equipped with a Shimadzu SPD-6A UV spectrophotometric detector and an SPD-6AD pumping system (Shimadzu, Japan).

4.2. *Plant material*

Dried pu-erh tea was purchased from "Da de sheng" tea-shop in Beijing, China, in August 2011 and authenticated by associate professor Lin Ma from Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College (IMM). A voucher specimen (S-2441) was deposited at the Herbarium of IMM.

4.3. *Extraction and isolation*

4.3.1. *Isolation of* ***1***

Dried pu-erh tea (10.0 kg) was extracted three times with 20 L boiling water for 50 min. Then the solvent was evaporated and the resulting residue (3.4 kg) was suspended in H_2O and extracted with EtOAc for 3 times, yielding a concentrated extract (1.4 kg). The EtOAc fraction (1.4 kg) was subjected to CC (silica gel; $CHCl_3$–MeOH, 1:0–0:1) to obtain fractions A1–A6. Fr. A2 (127 kg) was submitted to repeated CC (silica gel, $CHCl_3$–MeOH, 9:1–0:1) to afford 4 fractions, Fr. B1–B4. Fr. B2 (32 g) was further purified using CC (RP-C18 silica gel, MeOH–H_2O, 5:95–1:0) to obtain fractions C1–C7. Fr. C3 (4.7 g) was separated by the RP-MPLC using an ODS column, and further purified by preparative HPLC to obtain **4** (85 mg), **5** (27 mg) and **6** (49 mg). Fr. C4 (3.2 g) was subjected to an NKA resin column (EtOH–H_2O, 0:1–1:0), and further purified by Sephadex LH-20 CC and preparative HPLC to afford **7** (47 mg). Fr. C5 (2.1 g) and Fr. C6 (1.4 g) were subjected to Sephadex LH-20 CC (MeOH–H_2O, 0:1–1:0) and further purified by preparative HPLC to obtain **8** (14 mg), **9** (46 mg), **10** (36 mg), **11** (21 mg), **12** (53 mg) and **17** (25 mg). Fr. B3 (22 g) was separated on a RP-C18 silica gel column eluting with MeOH–H_2O (5:95–1:0) to afford 7 fractions, Fr. D1–D7. Fr. D3 (1.1 g) and Fr. D7 (4.8 g) were subjected to repeated CC purifications (NKA resin, EtOH–H_2O 0:1–1:0; Sephadex LH-20, MeOH–H_2O 0:1–1:0) and further purified by preparative HPLC to obtain **15** (12 mg), **16** (8 mg), **1** (19 mg) and **2** (6 mg). Using analogous separation and purification procedures as for Fr. B4 (13 g), Fr. B6 (18 g) afforded **3** (5 mg), **13** (23 mg) and **14** (50 mg).

4.3.2. *Identification of* ***1***

White amorphous powder, $[\alpha]_D^{20}$–66.8 (*c* 0.025, MeOH); UV (MeOH) λ_{max} (logε) 208 (3.02), 314 (3.51) nm; IR (KBr) υ_{max} 3276, 1709, 1623, 1604, 1514 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz), and ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 1; positive ion mode HR-ESI-MS *m/z* 437.1242 $[M+H]^+$ (Calcd. for $C_{24}H_{21}O_8$ 437.1231).

4.4. *In vitro hypoglycemic and hypolipidemic activities*

4.4.1. *Assessment of α-glucosidase inhibitory activity*

Rat small intestinal brush border membrane vesicles were prepared and a suspension of this material in 0.1 mol/L phosphate buffer (pH 6.0) was used as the small intestinal α-glucosidases of maltase, sucrase, isomaltase and trehalase. The enzyme suspension was diluted to hydrolyze sucrose to produce D-glucose in the following reaction. Reaction was performed in a 96-well plate. The substrate (sucrose: 100 mg/dL), test compounds and the enzyme in 0.1 mol/L phosphate buffer (pH 6.0, 0.2 mL) were incubated together at 37 °C. After 30 min of incubation, the plate was immediately heated to 80–85 °C for 3 min to stop the reaction, then cooled. Glucose concentration was

determined by the glucose oxidase method. The assay was performed in triplicates with five different concentrations around the IC_{50} values. The IC_{50} values were calculated from the dose–response curves, thus being obtained in the experiments.

4.4.2. *Assessment of lipase inhibitory activity*

An enzyme buffer was prepared by the addition of 40 μL (5 mg/mL) of a solution of porcine pancreatic lipase in Tris buffer (pH 7.1) 20 μL of compounds or orlistat at the test concentration was mixed with the enzyme buffer, and incubated for 10 min at 37 °C. Then, 5 μL of the substrate solution (10 mg/mL triglyceride) was added and the enzymatic reaction was allowed to proceed for 60 min at 37 °C. Pancreatic lipase activity was determined by measuring the hydrolysis of triglyceride to glycerol, which was monitored at 500 nm using a plate reader. The inhibition of lipase activity was expressed as the percentage decreased in the optical density (OD) when porcine pancreatic lipase was incubated with the test compounds.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this paper can be found in the online version at http://dx.doi.org/10.1016/j.apsb.2016.12.007.

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