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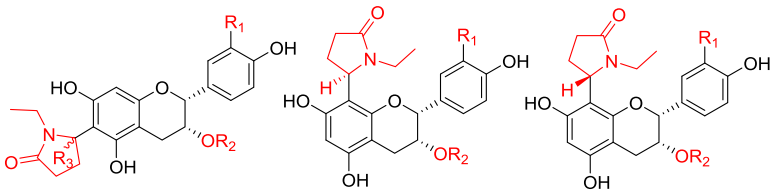
Black Tea 'Jin-Ya'



1 R_1=H, R_2=H, R_3=βH
5 R_1=OH, R_2=G, R_3=βH
6 R_1=OH, R_2=G, R_3=αH

2a R_1=H, R_2=G
4 R_1=OH, R_2=G
8 R_1=OH, R_2=H

2b R_1=H, R_2=G
3 R_1=OH, R_2=G
7 R_1=OH, R_2=H

New Flavoalkaloids with Potent *α*-Glucosidase and Acetylcholinesterase Inhibitory Activities from Yunnan Black Tea 'Jin-Ya'

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ABSTRACT

As the subgroup of flavoalkaloids, *N*-ethyl-2-pyrrolidinone substituted flavan-3-ols are reported to possess various biological activities that may play important roles in the beneficial healthcare functions of tea. The HPLC and LC-MS analyses showed the existence of *N*-ethyl-2-pyrrolidinone substituted flavan-3-ols in 'Jin-Ya', which is a Yunnan black tea produced only from buds of tea plant, *Camellia sinensis* var. *assamica*. Further phytochemical study on this precious black tea led to the identification of eight flavoalkaloids, **1**−**8**, along with 11 known flavan-3-ols (**9**-**14**) and flavonol glycosides (**15**-**19**). The new compounds, (−)-6-(5"*S*)-*N*-ethyl-2-pyrrolidinone-epiafzelechin (**1**), (−)-8-(5"*R*)-*N*-ethyl-2-pyrrolidinone-epiafzelechin-3-*O*-gallate (**2a**) and (−)-8-(5"*S*)-*N*-ethyl-2-pyrrolidinone-epiafzelechin-3-*O*-gallate (**2b**), were identified based on extensive spectroscopic analysis. Flavoalkaloids **2**-**6** showed inhibitory activity on α-glucosidase with IC_{50} values ranging from 2.09 to 8.47 μM, comparing to those of quercetin and acarbose (IC_{50} = 6.87 and 228.9 μM, resp.). Moreover, compounds **2**, **3** and **6** displayed inhibitory effect on acetyl-cholinesterase with IC_{50} values of 14.23, 33.79 and 34.82 μM, respectively, comparing to tacrine (IC_{50} = 0.223 μM).

KEYWORDS: *Camellia sinensis* var. *assamica*, Yunnan black tea, Jin-Ya, flavoalkaloids, *N*-ethyl-2-pyrrolidinone substituted flavan-3-ols, α-glucosidase inhibitory activity, AChE inhibitory activity.

Introduction

Black tea is a globally popular beverage that originated in China. It is welcomed by consumers from different ages, places, cultures, and societies, because of its attractive and strong flavor and health benefits. In the processing of black tea, the withered leaves or buds are rolled and crushed, which disrupts cellular compartmentation and brings phenolic compounds into contact with polyphenol oxidases to form oligomers such as theaflavins (TFs) and polymers known as thearubigins (TRs), before drying.[1-4] The chemical constituents of black tea have been investigated for a longer time, thus discovered a variety of compounds, e.g., flavonoids, theaflavins, theaflagallins, theadibenzotropolones, and theacitrins.[5-22] Despite the fact that TFs from black tea were first discovered in the early 1960s and explored extensively after then, not much progress has been made on other unknown polyphenols, which constitute a significant part of the black tea polyphenols.[23-28]

Flavoalkaloids are a class of natural products that possess a flavonoid framework in addition to one or more nitrogen atoms.[29] From tea and tea plants, a kind of flavoalkaloids, with a *N*-ethyl-2-pyrrolidinone moiety at the position C-6 or/and C-8 of the A ring of flavan-3-ol skeleton, were isolated, displaying diverse biological activities, e.g., inhibitory effect on acetylcholinesterase (AChE) and advanced glycation end products (AGE), protective effect on HMEC injured by H_2O_2, and antioxidant activity.[30-33] These compounds were first discovered in a blended black tea,[12] and Pu-er tea[33]. Their biosynthesis pathway was considered as, the richest amino acid in tea, i.e. theanine, was degraded to a Strecker aldehyde and combined with flavan-3-ol A-ring during tea fermentation process under the help of enzyme or microbes.[12, 33] In our previous work, eight flavoalkaloids together with the substitute unit, *N*-ethyl-5-hydroxy-2-pyrrolidinone were identified in the leaves of *C. sinensis* var. *pubilimba*.[30]

Meanwhile, these compounds were also found in Xi-Gui green tea (*C. sinensis* var. *assamica*) and white tea (*C. sinensis* var. *sinensis*).[31, 32]

Yunnan black tea, originated from Fengqing County of Yunnan Province, China, is made of the leaves and/or buds of *C. sinensis* var. *assamica*. Among which, 'Jin-Ya' is a precious Yunnan black tea produced with only buds of the tea plant growing in Fengqing area. It has attracting more and more consumers due to the significant pleasant flavor and mild taste. Our preliminary analysis by high-performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC-ESI-MS), indicated the existence of flavoalkaloids in 'Jin-Ya' black tea. Further phytochemical study led to the isolation and identification of eight flavoalkaloids (**1-8**), along with 11 flavan-3-ols (**9-14**) and flavonol glycosides (**15-19**). Most of the isolated compounds were also evaluated the inhibitory activity on α-glucosidase and AChE. Herein, we present the study.

MATERIALS AND METHODS

General Procedure. Optical rotations were measured with a Rudolph Research Analytical Autopol IV automatic polarimeter (Rudolph Research Analytical, Hackettstown, USA). The circular dichroism (CD) spectrum was detected with an Applied Photophysics Chirascan V100 spectrometer (Applied Photophysics Ltd., Leatherhead, Surrey, UK). IR and UV spectra were measured on a Bio-Rad FTS-135 series spectrometer (Bio-Rad, Hercules, USA) with KBr pellets and a Shimadzu UV2401A ultraviolet-visible spectrophotometer (Shimadzu Co., Kyoto, Japan), respectively. ESIMS and HRESIMS were run on a Shimadzu LCMS-IT-TOF spectrometer (Shimadzu Co., Kyoto, Japan), Agilent G6230 TOF MS (Agilent Technologies Inc., Santa Clara, USA) and API QSTAR Pular-1 spectrometers (Thermo Fisher Scientific Inc., Waltham, USA), respectively. 1D and 2D NMR spectra were

recorded in CD_3OD with Bruker DRX-600 and DRX-800 spectrometers (Bruker Co., Karlsruhe, Germany) operating at 600 and 800 MHz for ^1H and 150 and 200 MHz for ^{13}C, respectively. Chemical shifts were described in units of δ (ppm) with TMS as an internal standard, and coupling constants (J) were expressed in Hertz (Hz). Water was purified by Milli-Q system (Millipore Co., Billerica, USA).

Chemicals and Reagents. Column chromatography (CC) was carried out over Sephadex LH-20 (25-100 μm, GE Healthcare Bio-Science AB, Uppsala, Sweden), RP-18 (40-60 μm, Merck, Darmstadt, Germany), MCI-gel CHP20P (75-100 μm, Mitsubishi Chemical Co. Ltd., Tokyo, Japan), and TSK gel Toyopearl HW-40F (37-70 μm, Tosoh Co. Ltd., Tokyo, Japan). Semi-preparative HPLC was carried out using a Hanbon series (Hanbon Sci. & Tech., Huai'an, China) HPLC system and a Agilent ZORBAX SB-C18 column (5 μm, 250 mm × 9.4 mm), and the flowing rate was 3 mL/min. An Agilent series 1200 (Agilent Technologies) liquid chromatography column equipped with a 1290 diode array detector (DAD), and a 6540 Ultra-High-Definition Accurate-Mass Q-TOF mass spectrometry system (Agilent Technologies) with electrospray ionization, were used for HPLC-DAD-MS analysis, operating in full-scan MS mode from 100 to 1500 *m/z*. Thin-layer chromatography (TLC) was carried on 0.2-0.25 mm silica gel H-precoated plates (Qingdao Haiyang Chemical Co., Ltd., Qingdao, China) with benzene/ethyl formate/formic acid (Shanghai Macklin Biochemical Co. Ltd., Shanghai, China) (3:6:1, 4:5:1, 5:4:1, v/v/v) as the mobile phase, and spots were detected by spraying with 2% ethanolic $FeCl_3$ or anisaldehyde-H_2SO_4 reagent (Shanghai Xilong Biochemical Technology Co. Ltd., Shanghai, China) followed by heating. 4-Nitrophenyl-α-D-glucopyranoside (PNPG), α-glucosidase, quercetin, *S*-acetylthiocholine iodide, *S*-butyrylthiocholine iodide, 5,5'-dithio-bis-(2-nitrobenzoic) acid (DTNB, Ellman's reagent), acetylcholinesterase derived from human erythrocytes

were purchased from Sigma Chemical (Merck KGaA, Darmstadt, Germany).

Materials. 'Jin-Ya' black tea were purchased from Fengshan town, Fengqing County, Yunnan Province, P. R. China, in 2017. A standard sample (KIB-Z-1705002) has been deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany (KIB), Chinese Academy of Sciences (CAS).

HPLC and LC-MS Analysis. The fine powder (1.500 g) of 'Jin-Ya' was soaked in 70% MeOH (100 mL) at room temperature for 12 h, during which ultrasonic baths were carried out three times, each time for 15 min. To eliminate the impact of caffeine, the crude extract was suspended into water (50 mL) and extracted with chloroform (30 mL) for five times, in order to remove most of the caffeine. After removal of the organic solvent under reduced pressure, the aqueous fraction was filtered through a 0.45 μm nylon membrane for HPLC and LC-DAD-MS analyses.

HPLC analysis was performed on an Agilent Zorbax SB-C18 column (4.6 × 250 mm, 5 μm), with a gradient elution of 4−40% (45 min) acetonitrile aqueous solution. The flow rate of 1.0 mL/min, and the column temperature was set at 30 °C. HPLC comparison of standards with 70% MeOH extract of 'Jin-Ya' (Figure S2) was did under the same condition except for the mobile phase was acetonitrile aqueous solution with 50 mM H_3PO_4. The injection volume was 2 μL, and UV wavelength was monitored between 210 and 400 nm.

MS analysis was performed with electrospray interface (ESI), operating in full-scan MS mode from 100 to 1500 *m/z*. Sample was analyzed using both negative and positive ionization modes. ESI-MS parameters were as follows: ion spray voltage was 4 kV, and capillary temperature was 300 °C.

Extraction and Isolation. The Yunnan black tea 'Jin-Ya' (4.0 kg) were extracted four times (each time 1 week) with 60% aqueous acetone at room temperature. After acetone

was removed under reduced pressure, the extract was fractionated with chloroform and EtOAc, successively. The EtOAc fraction (185 g) was applied to Sephadex LH-20 column chromatography (CC), eluting with MeOH-H_2O (0:1-1:0), to afford six fractions (Fr. 1-Fr. 6). Fr. 4 (50.0 g), the flavoalkaloid enriched fraction was further divided into six sub-fractions (Fr. 4-1-Fr. 4-6) by MCI-gel CHP20P CC using MeOH-H_2O (0:1-1:0) as the eluent. Fr. 4-4 (6.98 g) was applied to MCI-gel CHP20P CC, eluting with MeOH-H_2O (0:1-1:0), to afford seven fractions (Fr. 4-4-1-Fr. 4-4-7). Fr. 4-4-5 (2.46 g) was applied to repeated CC over Sephadex LH-20, Toyopearl HW-40F, and RP-18, eluting with MeOH-H_2O (0:1-1:0), followed with semi-preparative HPLC (MeCN-H_2O, 16:84) to yield **1** (1.8 mg), **2** (3.3 mg), **9** (3.0 mg), **14** (1.5 mg), **18** (3.8 mg), and **19** (3.7 mg). Fr. 4-4-4 (2.56 g) gave compounds **3** (41.2 mg), **4** (62.2 mg), **5** (11.6 mg), **6** (11.8 mg), **10** (13.3 mg), **12** (1.8 mg), **15** (33.2 mg), **16** (8.4 mg), and **17** (6.6 mg) by the same method (semi-preparative HPLC: MeCN-H_2O, 18:82). Fr. 4-3 (4.0 g) was subjected to MCI-gel CHP20P and Sephadex LH-20 CC, eluting with MeOH-H_2O (0:1-1:0), followed with semi-preparative HPLC (MeCN-H_2O, 13:87) to yield **7** (6.7 mg), **8** (4.4 mg), **11** (3.6 mg), and **13** (3.6 mg).

Compound **1**. Brown amorphous powder; ESI-MS: *m/z* 384 $[M-H]^-$. HRESI-MS: *m/z* 384.1455 $[M-H]^-$ (calcd for $C_{21}H_{22}NO_6^-$, 384.1453). $[\alpha]_D^{20}$−29.7 (*c* 0.16, MeOH). IR (KBr): ν_{max} 3411, 2969, 1617, 1517, 1452, 1384, 1231, 1126, 988, 819, 793 cm^{-1}. UV λ_{max} (methanol) (log ε): 276 (3.57), 211 (4.63) nm. ^1H NMR (800 MHz, CD_3OD): δ_H 4.85 (1H, s, H-2), 5.45 (1H, br s, H-3), 2.70 (1H, br s, H-4α), 2.88 (1H, br s, H-4β), 6.02 (1H, s, H-8), 7.30 (2H, d, *J* = 8.4 Hz, H-2',6'), 6.78 (2H, d, *J* = 8.4 Hz, H-3',5'), 2.46-2.40 (1H, m, H-3"α), 2.66 (1H, dd, *J* = 12.2, 5.5 Hz, H-3"β), 2.16 (1H, dq, *J* = 17.1, 5.6 Hz, H-4"α), 2.38-2.29 (1H, m, H-4"β), 5.45 (1H, s, H-5"), 2.65 (1H, dd, *J* = 12.2, 5.5 Hz, H-6"α), 3.48 (1H, br s, H-6"β), 1.01 (1H, t, *J* = 7.2 Hz, H-7"). ^{13}C NMR

(200 MHz, CD_3OD): δ_C 79.7 (C-2), 67.1 (C-3), 29.5 (C-4), 156.6 (C-5), 108.0 (C-6), 158.1 (C-7), 97.6 (C-8), 156.6 (C-9), 100.7 (C-10), 131.4 (C-1'), 129.1 (C-2', 6'), 115.8 (C-3', 5'), 158.1 (C-4'), 177.6 (C-2"), 32.6 (C-3"), 24.5 (C-4"), 54.2 (C-5"), 36.3 (C-6"), 12.6 (C-7").

Compound **2**. Brown amorphous powder; ESI-MS: *m/z* 536 $[M - H]^-$. HRESI-MS: *m/z* 536.1560 $[M - H]^-$ (calcd for $C_{28}H_{26}NO_{10}^-$, 536.1557). $[\alpha]_D^{20}$–232.3 (*c* 0.14, MeOH). IR (KBr): ν_{max} 3400, 2976, 1694, 1615, 1518, 1455, 1369, 1232, 1114, 1040, 971, 837, 768 cm^{-1}. UV λ_{max} (methanol) (log ε): 276 (4.07), 212 (4.77) nm. ^1H NMR (600 MHz, CD_3OD): major isomer (**2a**): δ_H 6.80 (2H, s, H-2"', 6"'), 4.95 (1H, s, H-2), 5.26 (1H, s, H-3), 2.82 (1H, d, *J* = 17.5 Hz, H-4α), 2.92 (1H, d, *J* = 17.5 Hz, H-4β), 5.96 (1H, s, H-6), 7.15 (2H, d, *J* = 8.2 Hz, H-2', 6'), 6.62 (2H, d, *J* = 8.2 Hz, H-3', 5'), 2.11-2.02 (1H, m, H-3"α), 2.26-2.18 (1H, m, H-3"β), 2.08-1.99 (1H, m, H-4"α), 2.15 (1H, t, *J* = 10.8 Hz, H-4"β), 5.40-5.32 (1H, m, H-5"), 2.80-2.75 (1H, m, H-6"α), 3.58 (1H, dd, *J* = 13.4, 6.9 Hz, H-6"β), 0.99 (1H, t, *J*=6.6 Hz, H-7"); minor isomer (**2b**): δ_H 6.84 (2H, s, H-2"', 6"'), 4.95 (1H, s, H-2), 5.26 (1H, s, H-3), 2.82 (1H, d, *J* = 17.5 Hz, H-4α), 2.92 (1H, d, *J* = 17.5 Hz, H-4β), 5.96 (1H, s, H-6), 7.20 (2H, d, *J* = 8.2 Hz, H-2', 6'), 6.61 (2H, d, *J* = 8.2 Hz, H-3', 5'), 2.11-2.02 (1H, m, H-3"α), 2.26-2.18 (1H, m, H-3"β), 2.08-1.99 (1H, m, H-4"α), 2.15 (1H, t, *J* = 10.8 Hz, H-4"β), 5.40-5.32 (1H, m, H-5"), 2.80-2.75 (1H, m, H-6"α), 3.58 (1H, dd, *J* = 13.4, 6.9 Hz, H-6"β), 0.99 (1H, t, *J*=6.6 Hz, H-7"). ^{13}C NMR (150 MHz, CD_3OD): major isomer (**2a**): δ_C 121.2 (C-1"'), 110.3 (C-2"', 6"'), 146.7 (C-3"', 5"'), 140.6 (C-4"'), 167.8 (C-7"'), 79.2 (C-2), 70.3 (C-3), 27.4 (C-4), 156.4 (C-5), 96.3 (C-6), 158.4 (C-7), 105.6 (C-8), 156.8 (C-9), 100.3 (C-10), 130.6 (C-1'), 129.2 (C-2', 6'), 116.2 (C-3', 5'), 157.8 (C-4'), 177.3 (C-2"), 32.6 (C-3"), 24.3 (C-4"), 54.4 (C-5"), 36.9 (C-6"), 12.9 (C-7"); minor isomer (**2b**): δ_C 120.9 (C-1"'), 110.2 (C-2"', 6"'), 146.6 (C-3"', 5"'), 140.6 (C-4"'), 167.8 (C-7"'), 78.7 (C-2), 69.7

(C-3), 27.4 (C-4), 156.4 (C-5), 96.3 (C-6), 158.4 (C-7), 105.6 (C-8), 156.8 (C-9), 100.2 (C-10), 130.5 (C-1'), 129.3 (C-2', 6'), 116.1 (C-3', 5'), 157.8 (C-4'), 177.7 (C-2''), 32.6 (C-3''), 24.6 (C-4''), 54.4 (C-5''), 36.5 (C-6''), 12.7 (C-7'').

α-Glucosidase Inhibitory Activity. An enzyme-inhibitor screening model was chosen using 4-nitrophenol-α-D-glucopyranoside (PNPG) and slightly modified.[34] Briefly, test compound (50 μM), α-glucosidase solution (0.025 U/mL), phosphate buffer (pH 6.9) and PNPG (1 μM) were incubated in 96-well plates at 37 °C for 50 min. Absorbance at 405 nm was recorded on a micro-plate reader. Blank readings (no enzyme) were subtracted from each well and results were compared to the control. The quercetin was selected as the positive control. All the reactions were repeated three times. The inhibition percentage was calculated as follows: % inhibition = (E − S)/E × 100 (E is the activity of the enzyme without tested compound and S is the activity of enzyme with tested compound), and IC_{50} were calculated according to the Reed & Muench method.[35]

AChE Inhibitory Activity. As described in literature with slight modification, AChE inhibitory activity of C-6/8 *N*-ethyl-2-pyrrolidinone substituted flavan-3-ols was performed.[36] The compounds to be tested were dissolved in DMSO. A mixture (total 200 μL) containing phosphate buffer (pH 8.0), compound (50 μM) and acetyl cholinesterase (0.02 U/mL) was incubated for 20 min (37 °C). Then, the reaction was initiated by addition of 40 μL of a solution containing DTNB (0.625 mM) and acetylthiocholine iodide (0.625 mM) for AChE inhibitory activity assay. The hydrolysis of acetylthiocholine was monitored at 405 nm every 30 seconds for 1 hour. Tacrine was used as positive control with a final concentration of 0.333 μM. All the reactions were repeated three times. The percentage inhibition was calculated as follows: % inhibition = (E − S)/E × 100 (E is the activity of the enzyme without tested compound and S is

the activity of enzyme with tested compound).

RESULTS AND DISCUSSION

HPLC and LC-MS Analysis. The 70% aqueous MeOH extract of 'Jin-Ya' black tea was subjected to HPLC (Figure 1) and LC-DAD-MS analysis. Based on their retention times (t_R), absorbance spectrum, quasimoleculars and fragment ions, and HPLC analysis comparing with standards (Figure S2), and reported data, eighteen flavan-3-ol derivatives (including six catechins, six flavoalkaloids, four flavan-3-ol dimers and two theaflavins), five phenolic acid derivatives, together with three flavonol and its glycosides were identified, and the results are shown in Table 1. Most of these compounds were obtained from 'Jin-Ya' black tea during further isolation.

Peaks 5, 7, 11, 12, 19, and 26 in Figure 1 were supposed to be catechins. Peak 5 was identified as (-)-epigallocatechin by the $[M - H]^-$ quasi-molecular ion of *m/z* 305 and $[M + H]^+$ quasi-molecular ion of *m/z* 306, and this could be further verified by comparing the t_R (12.97 min) and UV absorption (λ_{max} 214, 269 nm) with those of the standard sample. Similarly, peaks 7 (t_R 14.57 min; λ_{max} 212, 274 nm), 11 (t_R 17.75 min; λ_{max} 211, 274 nm), 12 (t_R 18.45 min; λ_{max} 209, 271 nm), and 19 (t_R 24.08 min; λ_{max} 209, 273 nm) were identified as (+)-catechin, EC (**9**), EGCG (**13**), and ECG (**11**), respectively. Peak 26 (t_R 28.11 min; *m/z* 425 $[M - H]^-$, 273 $[M - H -$ galloyl$]^-$, 255 $[273 - H_2O]^-$, 169 [gallic acid]$^-$) was identified as (-)-epiafzelechin 3-*O*-gallate (EAG, **10**), and this was confirmed by comparing to the isolated standard compound.

Peaks 16, 17, and 22-25 in Figure 1 were proposed to be flavoalkaloids, since their molecular weights are odd numbers. Due to the ions (*m/z* 400 $[M - H -$ galloyl$]^-$, 248,[33] 169, 112 [1-ethyl-5-hydroxy-2-pyrrolidinone]$^-$) and their t_R (Table 1), peaks 22 and 25 were identified as (−)-8-(5"*S*)-*N*-ethyl-2-pyrrolidinone-epicatechin-3-*O*-gallate (**3**) and (−)-6-(5"*R*)-*N*-ethyl-2-pyrrolidinone-epicatechin-3-*O*-gallate (**6**). Peaks 16 and

17 (*m/z* 400 $[M-H]^-$; t_R 21.64 and 22.20 min, resp.) were identified as (−)-8-(5"*S*)-*N*-ethyl-2-pyrrolidinone-epicatechin (**7**) and (−)-8-(5"*R*)-*N*-ethyl-2-pyrrolidinone-epicatechin (**8**). Similarly, peaks 23 and 24 (*m/z* 552 $[M-H]^-$; t_R 27.40 and 27.79 min, resp.) were identified to be (−)-6-(5"*S*)-*N*-ethyl-2-pyrrolidinone-epicatechin-3-*O*-gallate (**5**) and (−)-8-(5"*R*)-*N*-ethyl-2-pyrrolidinone-epicatechin-3-*O*-gallate (**4**), respectively. These compounds were acquired from further isolation and purification.

Peaks 4, 10, 14 and 15 in Figure 1 were inferred to be flavan-3-ol dimers. According to comparison with standards and reference data, peaks 4 (t_R 11.59 min; λ_{max} 269 nm; *m/z* 761 $[M-H]^-$) and 10 (t_R 16.67 min; λ_{max} 271 nm; *m/z* 913 $[M-H]^-$, 743 $[M-H-galloyl-H_2O]^-$,[37] 591 [743 – galloyl] $^-$) were identified as theasinensins B and A, respectively. Due to the molecular weight and quasi-molecular ions, peak 14 (t_R 19.69 min; *m/z* 885 $[M-H]^-$, 733 $[M-H-galloyl]^-$, 169), was thought to be an EGCG dimer. Peak 15 (t_R 20.35 min; *m/z* 729 $[M-H]^-$, 577 $[M-H-galloyl]^-$, 441 [ECG/CG] $^-$, 407 $[577-galloyl-H_2O]^-$, 289 [EC/C] $^-$, 169) was supposed to be a procyanidin dimer monogallate. Peaks 28 and 29 (*m/z* 715 $[M-H]^-$ and 867 $[M-H]^-$) were supposed to be theaflavin-3-gallate and theaflavin-3,3'-digallate, by comparing UV absorption (λ_{max} 273, 370 and 271, 370 nm, resp.) and t_R (34.99 and 35.95 min) with those of the standard samples.

Peaks 1, 2, 6, 8 and 13 in Figure 1 were supposed to be phenolic acid derivatives. On the basis of the ions (*m/z* 169 $[M-H]^-$, 125 $[169-COO]^-$) and t_R (2.20 min), peak 1 was identified as gallic acid, by combining with the comparison with standard. In the same way, peak 8 (*m/z* 633 $[M-H]^-$; t_R 14.80 min; λ_{max} 211, 274 nm), was identified as strictinin. Peak 2 (t_R 4.82 min; *m/z* 331 $[M-H]^-$, 169 $[M-H-Glu]^-$) was identified to be glucogallin. The molecular mass of peak 6 (t_R 13.35 min; *m/z* 483 $[M-H]^-$) was 152 Da more than that of peak 2. This 152 Da rise typically referring to a galloyl group,

and thus peak 6 was proposed to be digalloyl glucopyranose. Similarly, peak 13 (t_R 19.43 min; *m/z* 635 $[M - H]^-$, 483 $[M - H - 152]^-$, 169 $[galloyl]^-$), whose molecular mass was 152 Da more than that of peak 6, was supposed to be trigalloyl glucopyranose.

Peaks 18, 20 and 21 in Figure 1 were thought to be flavonol and its glycosides. Peak 18 (*m/z* 301 $[M - H]^-$) was supposed to be quercetin, due to its molecular weight and UV absorption (λ_{max} 212, 278 nm). Similarly, Peaks 20 (t_R 25.33 min; *m/z* 593 $[M - H]^-$, 595 $[M + H]^+$) and 21 (t_R 25.74 min; *m/z* 447 $[M - H]^-$) were identified as nicotiflorin (**15**) and kaempherol 3-*O*-*β*-D-galacopyranoside (**19**), and were further isolated from 'Jin-Ya' black tea (Figure S1).

Identification of Compounds 1-19. The 60% aqueous acetone extract of 'Jin-Ya' black tea was fractionated successively with chloroform and EtOAc. The EtOAc fraction was subjected to repeated CC and semi-preparative HPLC to provide eight flavoalkaloids (**1**-**8**), including two new ones, **1** and **2**, together with 11 known flavan-3-ols (**9**-**14**) and flavonol glycosides (**15**-**19**). The known compounds were identified as (−)-8-(5"*S*)-*N*-ethyl-2-pyrrolidinone-epicatechin-3-*O*-gallate (**3**),[31] (−)-8-(5"*R*)-*N*-ethyl-2-pyrrolidinone-epicatechin-3-*O*-gallate (**4**),[31] (−)-6-(5"*S*)-*N*-ethyl-2-pyrrolidinone-epicatechin-3-*O*-gallate (**5**),[31] (−)-6-(5"*R*)-*N*-ethyl-2-pyrrolidinone-epicatechin-3-*O*-gallate (**6**),[31] (−)-8-(5"*S*)-*N*-ethyl-2-pyrrolidinone-epicatechin (**7**),[33] (−)-8-(5"*R*)-*N*-ethyl-2-pyrrolidinone-epicatechin (**8**),[33] (-)-epicatechin (**9**),[38] (-)-epiafzelechin 3-*O*-gallate (**10**),[39] (-)-epicatechin 3-*O*-gallate (**11**),[38] (-)-epiafzelechin (**12**),[40] (-)-epigallocatechin 3-*O*-gallate (**13**),[41] bis(8-epicatechinyl)methane (**14**),[42] nicotiflorin (**15**),[43] rutin (**16**),[44] kaempferol 3-*O*-robinoside (**17**),[45] kaempherol 3-*O*-*β*-D-glucopyranoside (**18**),[44] and kaempherol 3-*O*-*β*-D-galacopyranoside (**19**),[44] respectively, by comparing their physical and spectroscopic data with reported values in literature and by authentic samples (Figures 2 and S1). In addition to **1**-**8**, **10** and **12**,

compound **14** was the first flavan-3-ol dimer with C-8/C-8 linkage through a methylene bridge reported from black tea.

Compound **1**, $[\alpha]_D^{20}$−29.7 (*c* 0.16, MeOH), was obtained as brownish amorphous powder. Its molecular formula, $C_{21}H_{23}NO_6$, was deduced, according to the negative HR-ESI-MS (*m/z* 384.1455 $[M - H]^-$, calcd for $C_{21}H_{22}NO_6$: 384.1453). The IR spectrum indicated the presence of hydroxyl (3411 cm^{-1}), benzene ring (1617 and 1517 cm^{-1}), and carbonyl (1691 cm^{-1}) groups in the compound. The existence of a flavan-3-ol skeleton was indicated obviously from the ^1H and ^{13}C NMR spectrum of **1**. The typical proton signals for rings A, B, and C at δ_H 4.85 (1H, s, H-2), 5.45 (1H, br s, H-3), 2.70 (1H, br s, H-4α), 2.88 (1H, br s, H-4β) (ring C), 6.02 (1H, s) (ring A), 7.30, 6.78 (each 2H, d, *J* = 8.5 Hz, H-2'/6', 3'/5') (ring B) were quite similar to those of epiafzelechin (EA, **12**). However, unlike EA, the ^1H and ^{13}C NMR data of **1** displayed only one aromatic methine (δ_H 6.02, δ_C 97.6) and an additional quaternary aromatic carbon signals [δ_C 108.0], suggested a substituent at C-6 or C-8 of A ring. Besides signals arising from the EA unit, a set of signals assignable to one carbonyl (δ_C 177.6, C-2"), two methylenes (δ_C 32.6, 24.5, C-3" and C-4"), one methine (δ_C 54.2, C-5"), and an ethyl group (δ_C 36.3, 12.6, C-6" and C-7") were observed, whose corresponding protons were at δ_H 2.46-2.40 (1H, m, H-3"α), 2.66 (1H, dd, *J* = 12.2, 5.5 Hz, H-3"β), 2.16 (1H, dq, *J* = 17.1, 5.6 Hz, H-4"α), 2.38-2.29 (1H, m, H-4"β), 5.45 (1H, m, H-5"), 2.65 (1H, dd, *J* = 12.2, 5.5 Hz, H-6"α), 3.48 (1H, br s, H-6"β), and 1.01 (3H, t, *J* = 7.2 Hz, H-7") as assigned by the HSQC spectrum. A partial structure of -C(3")H_2-C(4")H_2-C(5")H- can be derived from the correlations of ^1H-^1H COSY. Furthermore, the HMBC spectrum showed that the methylene protons (H-6") of ethyl group at δ_H 3.48 and 2.65 were correlated with the methine carbon at δ_C 54.2 (C-5"), and the methylene protons at δ_H 2.46-2.40 and 2.66 (H-3") were correlated with the carbonyl carbon at δ_C 177.6

(C-2"). These correlations of HMBC and ^{1}H-^{1}H COSY proved the existence of a *N*-ethyl-2-pyrrolidinone ring, whose location on EA unit was further determined by comparison of the ^{13}C NMR data originating from the A ring of **1** with those of etc-pyrrolidinone E and G,[30] with a *N*-ethyl-2-pyrrolidinone ring at C-6 (C-6: δ_C 107.3, C-8: 96.0), and C-8 (C-6: δ_C 95.9, C-8: 104.3), respectively. The chemical shift of C-6 and C-8 were 108.0 and 97.6, which were more related to etc-pyrrolidinone E. Therefore, the structure of **1** was decided as C-6 *N*-ethyl-2-pyrrolidinone-substituted (−)-epiafzelechin.

The absolute configurations at C-2,3,5" of **1** were confirmed by comparing NMR data and CD curves with those of **5** (Figure 3). Previous studies have shown that the CD contribution of C-5" can be clarified by subtracting one CD spectrum from its stereoisomer with the same configurations at C-2/C-3.[30, 31, 46] As it presented a positive Cotton effect at 210 nm after subtracted the CD curves of **6** from that of **5** (Figure 4), compound **5** was determined to be the 5"*S*-configuration. Similarly, compound **6** was determined to be the 5"*R*-configuration (Figure 4). On the basis of the above evidence, compound **1** was determined to be (−)-6-(5"*S*)-*N*-ethyl-2-pyrrolidinone-epiafzelechin.

Compounds **2a** and **2b**, $[\alpha]_D^{20}$−232.3 (*c* 0.14, MeOH), obtained as brownish amorphous powder, are an inseparable mixture (Figure S13). The molecular formula, $C_{28}H_{27}NO_{10}$, was deduced, according to the negative HR-ESI-MS (*m/z* 536.1560 [M - H]$^-$, calcd for $C_{28}H_{26}NO_{10}$: 536.1557). The IR spectrum indicated the presence of hydroxyl (3400 cm^{-1}), benzene ring (1615 and 1518 cm^{-1}), and carbonyl (1694 cm^{-1}) groups in the compound. The existence of an additional galloyl group [δ_H 6.80 (2H, s, H-2"', 6"'), δ_C 121.2 (C-1"'), 110.3 (C-2"', 6"'), 146.7 (C-3"', 5"'), 140.6 (C-4"'), 167.8 (C-7"')] in **2** could be easily concluded from the ^{1}H, ^{13}C NMR and MS data (384 [M – H – galloyl]$^-$), as compared with those of **1**. The typical signals for C-2 and C-3 [δ_H

5.01-4.93 (1H, m, H-2), 5.26 (1H, s, H-3), 2.82 (1H, d, J = 17.5 Hz, H-4α), 2.92 (1H, d, J = 17.5 Hz, H-4β), δ_C 79.2 (C-2), 70.3 (C-3), 27.4 (C-4)] allowed the assignment of the galloyl group at C-3 position. The location of the *N*-ethyl-2-pyrrolidinone ring at C-8 in **2** was also established by comparison of the ^{13}C NMR data originating from A ring (C-6: δ_C 96.3, C-8: 105.6) with those of etc-pyrrolidinone E and G.[31] Thus, the structure of **2** was deduced as C-8 *N*-ethyl-2-pyrrolidinone-epiafzelechin-3-*O*-gallate.

It was confirmed that the absolute configurations of C-2 and C-3 in **2** were 2*R* and 3*R* by comparing NMR data, optical rotation value and CD curves with those of EAG (**10**) (Figure 3, S34 and S35). Compound **2** was a mixture of epimers **2a** and **2b** at C-5" position. Due to the negative optical rotation value of compound **2** ($[\alpha]_D^{20}$−232.3), the major isomer **2a** was defined as 5"*R*, and the minor isomer **2b** was 5"*S* configurations.[30] In addition, the negative Cotton effect at 211 nm ($\Delta\varepsilon$ −33.36) of compound **2** (major is **2a**) is obviously derived from the chirality of 5"*R*,[31] which was further proven by comparing CD curve with **4** and **10** (Figure 3). Therefore, compound **2a** was deduced as (−)-8-(5"*R*)-*N*-ethyl-2-pyrrolidinone-epiafzelechin-3-*O*-gallate, and compound **2b** was deduced as (−)-8-(5"*S*)-*N*-ethyl-2-pyrrolidinone-epiafzelechin-3-*O*-gallate.

α-Glucosidase Inhibitory Activity. All of the flavan-3-ols (**1**-**14**), including eight *N*-ethyl-2-pyrrolidinone-substituted ones, were evaluated for their α-glucosidase inhibitory activity. As shown in Table 2, most of the tested compounds exhibited significant α-glucosidase inhibitory activity, and more than half showed stronger activity than the positive control (quercetin). The activity order was of **11 > 13 > 14 > 10 > 2 > 6 > 5 > 13 >** quercetin. At a concentration of 50 μM, compounds **1** and **7**-**9** showed weak inhibitory effects on α-glucosidase. The results showed that the 3-*O*-galloyl group plays the most important role in the α-glucosidase inhibitory activity,

while the *N*-ethyl-2-pyrrolidinone moiety has no obvious effect.

AChE Inhibitory Activity. With tacrine as positive control, the AChE inhibitory activity of **1**-**8**, **10**, **12** and **14** was evaluated, and the results are shown in Table 3. At a concentration of 50 μM, compounds **2**, **3**, **6** and **10** showed strong inhibitory activity (**>** 60%) against AChE, with IC_{50} values of 14.23, 33.79, 34.82, and 10.81 μM, respectively. Compounds **7** and **8** without C-3 galloyl substitution showed weak inhibitory activity against AChE. The results suggested that the existence of galloyl group attaching to the C-3 position might enhance the AChE inhibitory activity of flavan-3-ols. The AChE inhibitory activity of **8** was not consistent with the previous report,[30] in which, the 5"*R* epimer (**8**) isolated from the leaves of *C. sinensis* var. *pubilimba* showed obvious, while 5"*S* epimer (**7**) showed weak AChE inhibitory activity. Flavoalkaloids with a *N*-ethyl-2-pyrrolidinone moiety at C-8 position of flavan-3-ol tend to be existed in and isolated as a mixture of C-5" *R* and *S* epimers from tea and tea plants.[30-32] After being kept in solution, both compounds **8** with 5"*R* and **7** with 5"*S* configurations became gradually a mixture of 5"*R* and 5"*S* epimers, causing the reduction of AChE inhibitory activity.

In conclusion, eight flavoalkaloids including two new ones, were isolated and identified from the high-end Yunnan black tea 'Jin-Ya', together with 11 known flavan-3-ols (**9**-**14**) and flavonol glycosides (**15**-**19**). The flavoalkaloids, **1**-**8**, flavan-3-ols, **10** and **12**, and flavan-3-ol dimer, **14**, were obtained from black tea for the first time. The absolute configuration at C-5" of the pyrrolidinone ring of the new compounds **1** and **2** was established by CD analyses. Five-eighth of the isolated flavoalkaloids (**2**-**6**) showed strong inhibitory activities on α-glucosidase with IC_{50} values ranging from 2.09 to 8.47 μM. And half of them (**2**, **5** and **6**) showed stronger inhibitory activities than positive control (quercetin). In addition, flavoalkaloids (**2**, **3** and **6**) showed an inhibitory effect

on AChE, with the IC_{50} values at 14.23, 33.79, and 34.82 μM, respectively. The flavoalkaloids, **1**-**8**, are supposed to be formed from theanine and catechins during the producing process of black tea, during which the enzyme may be play an important role. Different catechin skeletons lead to different flavoalkaloids. Epiafzelechin (**12**) and epiafzelechin 3-*O*-gallate (**10**), the precursor core structures of the new flavoalkaloids **1** and **2**, are not the commonly existed flavan-3-ols in tea, and were obtained from black tea for the first time. The isolations of **12** and **10**, though with very low yields, might explain the existence of **1**-**2** in 'Jin-Ya' black tea, rather than other teas. However, further comprehensive investigation on the fresh material and the processing process of black tea 'Jin-Ya' are necessary and on progress now.

ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C NMR, HSQC, HMBC, UV spectra of compounds **1**, **2**, **5**, and **6**, HRESIMS, IR, COSY, ROESY spectra of compounds **1** and **2**, OR, CD spectra of compounds **1**, **2**, **5**, **6**, and **10**, the structures of compounds **14-19**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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Figure captions

1. **Figure 1.** HPLC chromatogram of MeOH extract of the fresh buds (a), 70% MeOH extract (b) and the de-caffeine fraction (c) of Yunnan black tea 'Jin-Ya'.
2. **Figure 2.** Compounds **1-14** isolated from Yunnan black tea 'Jin-Ya'.
3. **Figure 3.** CD spectra of **1** and **5** (A), and **2a** and **2b**, **4**, and **10** (B).
4. **Figure 4.** Configurations of compounds **5** and **6** were arithmetically determined by subtracting CD curves from each other for a couple of stereoisomers.

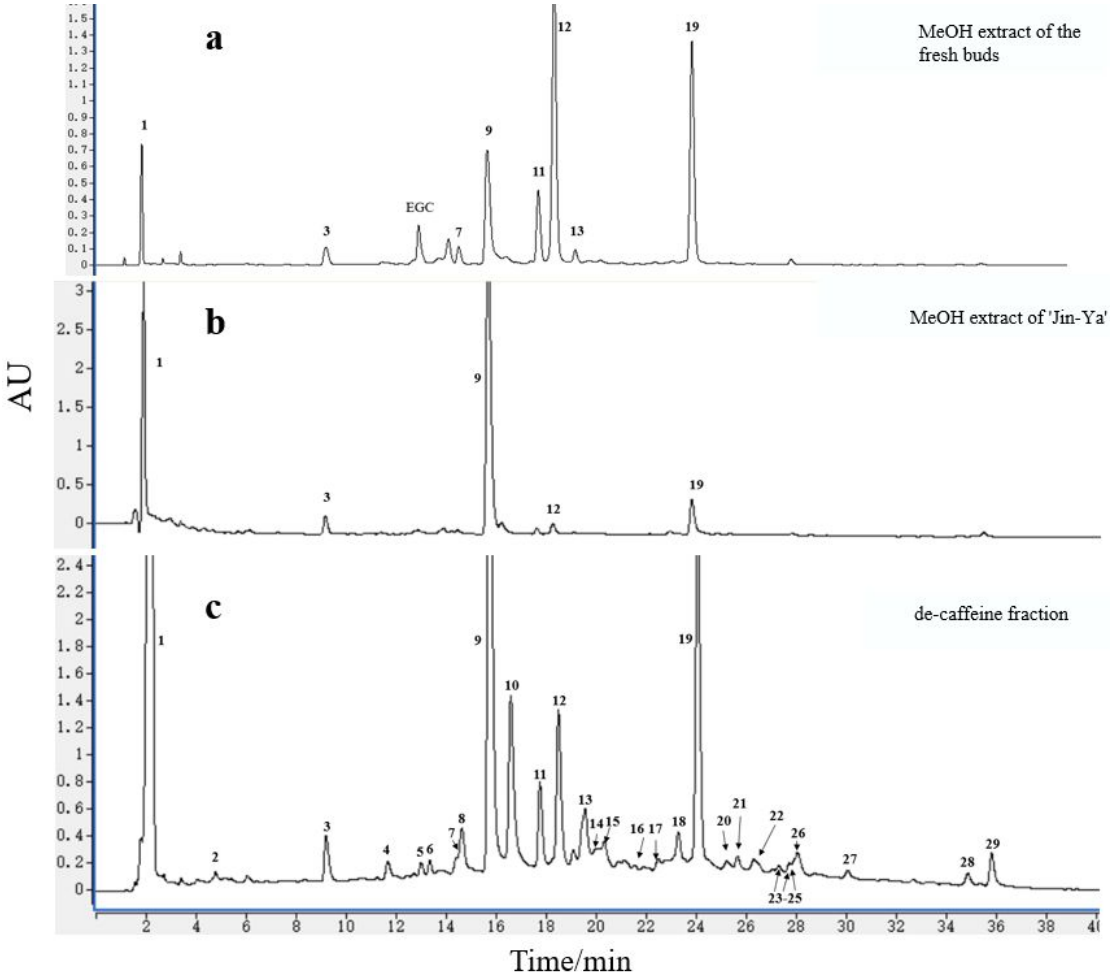

Figure 1. HPLC chromatogram of MeOH extract of the fresh buds (a), 70% MeOH extract (b) and the de-caffeine fraction (c) of Yunnan black tea 'Jin-Ya'.

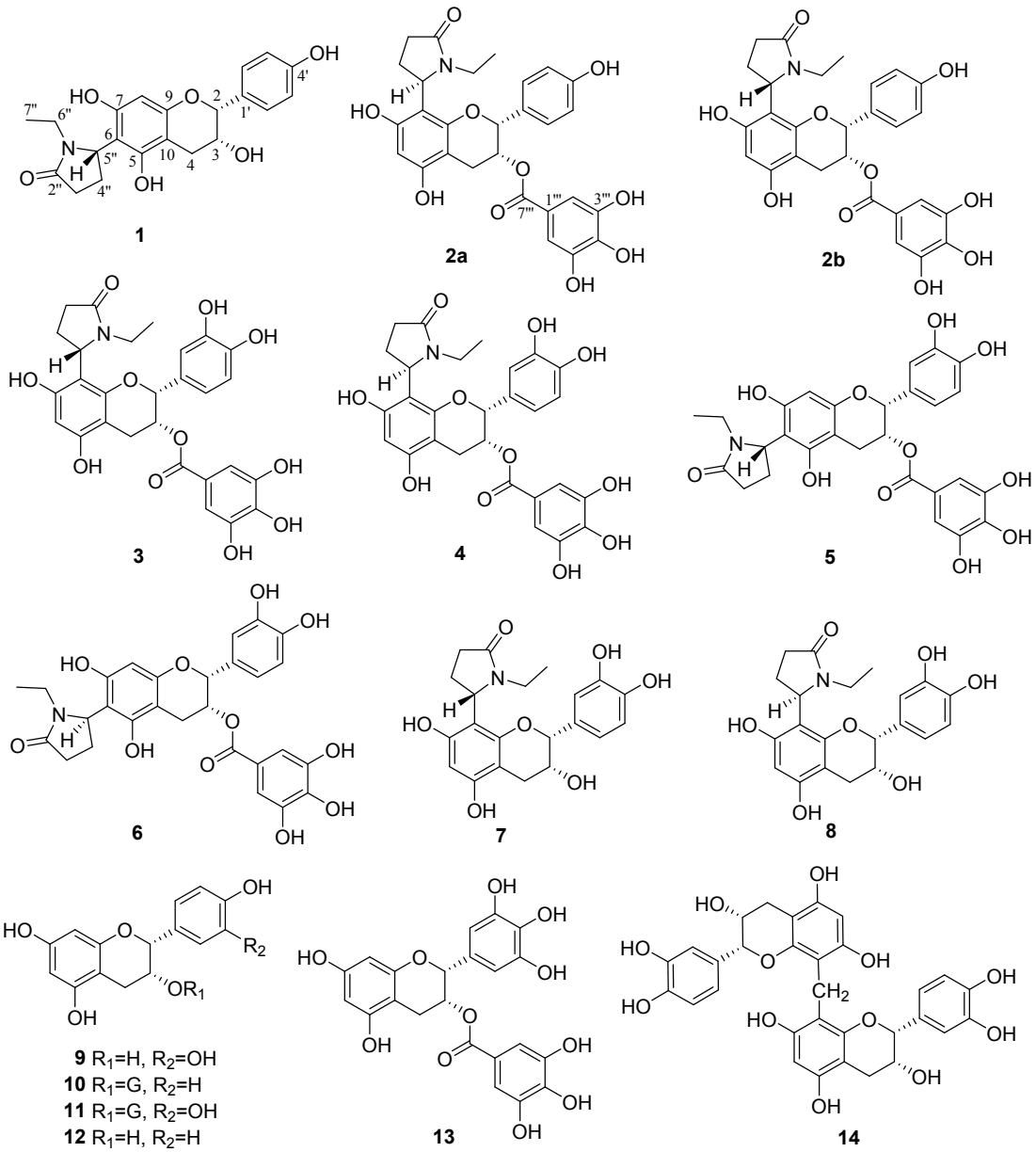

Figure 2. Compounds **1-14** isolated from Yunnan black tea 'Jin-Ya'

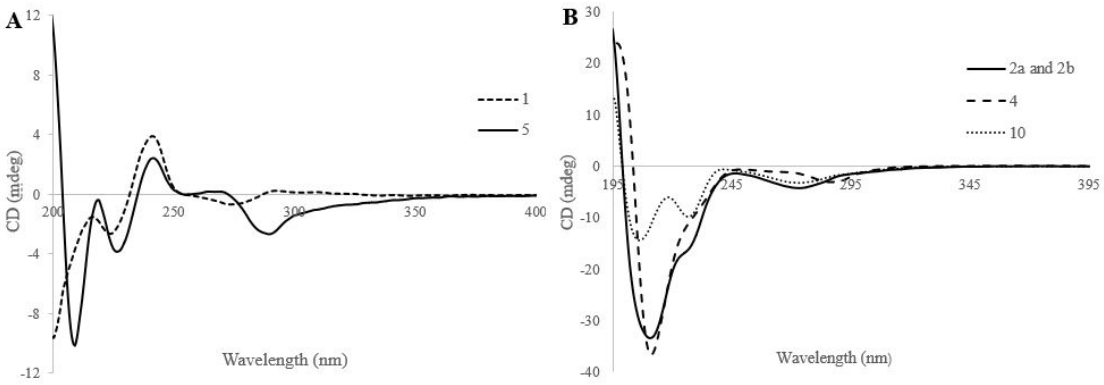

Figure 3. CD spectra of **1** and **5** (A), and **2a** and **2b**, **4**, and **10** (B).

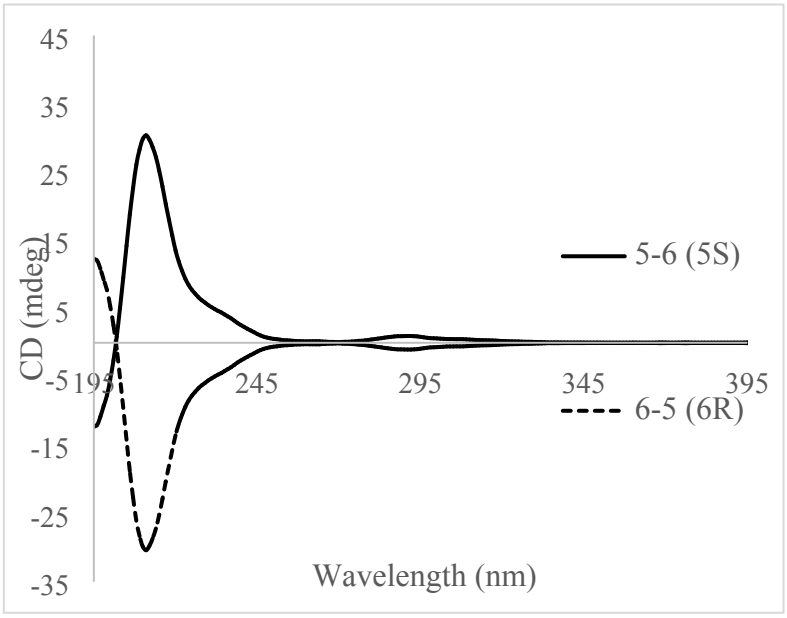

Figure 4. Configurations of compounds **5** and **6** were arithmetically determined by subtracting CD curves from each other for a couple of stereoisomers.

Table 1. Retention time (RT), molecular weight, as well as the *m/z* values of precursor ion and MS/MS fragments of the compounds in Yunnan black tea 'Jin-Ya'

Peak	RT (min)	Molecular weight	MS^+	MS^{2+}	MS^-	MS^{2-}	Compounds
1	2.20	170			169[M-H]$^-$	125[169-COO]$^-$	gallic acid[37]
2	4.82	332			331[M-H]$^-$	169[M-H-glucose]$^-$	*β*-glucogallin
3	9.24	218	219[M+H]$^+$				NI[a]
4	11.59	762			761[M-H]$^-$		theasinensin B
5	12.97	306			305[M-H]$^-$		(-)-epigallocatechin
6	13.35	484			483[M-H]$^-$		digalloyl glucopyranose
7	14.57	290			289[M-H]$^-$		catechin
8	14.80	634			633[M-H]$^-$		strictinin
9	15.73	194	195[M+H]$^+$				caffeine
10	16.67	914			913[M-H]$^-$	743[M-H-galloyl-H_2O]$^-$, 591[743-galloyl]$^-$	theasinensin A[37]
11	17.75	290			289[M-H]$^-$	245[M-COO]$^-$, 203, 123	**9**[37]
12	18.45	458	459[M+H]$^+$, 481[M+Na]$^+$	171[gallic acid]$^+$	457[M-H]$^-$	305, 169[gallic acid]$^-$	**13**
13	19.43	636			635[M-H]$^-$	483, 465, 441, 169[gallic acid]$^-$	trigalloyl glucopyranose[37]
14	19.69	886			885[M-H]$^-$	807, 733[M-H-galloyl]$^-$, 655, 427, 169[galloyl]$^-$	epigallocatechin gallate dimer
15	20.35	730			729[M-H]$^-$	577 [M-H-galloyl]$^-$, 441[ECG/CG]$^-$, 407[577-galloyl-H_2O]$^-$, 289[epicatechin/catechin]$^-$, 169[gallic acid]$^-$	procyanidin dimer monogallate
16	21.63	401			400[M-H]$^-$		**7**
17	22.20	401			400[M-H]$^-$		**8**
18	23.34	302			301[M-H]$^-$		quercetin
19	24.08	442			883[2M-H]$^-$,441[M-H]$^-$	289, 169[gallic acid]$^-$	**11**
20	25.33	594	595[M+H]$^+$	287[kaempferol]$^+$	593[M-H]$^-$	285[kaempferol]$^-$	**15**
21	25.74	448			447[M-H]$^-$		**19**
22	26.40	553			552[M-H]$^-$	400[M-H-galloyl]$^-$, 248, 169[gallic acid]$^-$, 112[1-ethyl-5-hydroxy-2-pyrrolidinone]$^-$	**3**[33]
23	27.40	553			552[M-H]$^-$		**5**
24	27.79	553			552[M-H]$^-$		**4**
25	27.99	553			552[M-H]$^-$	400[M-H-galloyl]$^-$, 248, 169[galloyl]$^-$	**6**[33]
26	28.11	426			425[M-H]$^-$	273[M-H-galloyl]$^-$, 255[273-H_2O]$^-$, 169[gallic acid]$^-$	**10**[37]
27	30.13	882			881[M-H]$^-$		NI
28	34.99	716			715[M-H]$^-$		theaflavin-3-gallate
29	35.95	868			867[M-H]$^-$	697[M-H-galloyl]$^-$, 545, 527[697-galloyl]$^-$	theaflavin-3,3'-digallate

[a] NI, not identified.

Table 2. α-Glucosidase inhibitory activities of compounds **1-14** from Yunnan black tea 'Jin-Ya'[a]

Sample	IC_{50} (μM)[b]	Sample	Inhibition ratio (%)[c]
quercetin[d]	6.870 ± 0.161	quercetin[d]	73.81 ± 0.60[e]
acarbose[d]	228.95 ± 0.38	**1**	14.59 ± 2.46
2	2.094 ± 0.173	**7**	26.61 ± 2.48
3	8.474 ± 0.402	**8**	31.31 ± 2.04
4	7.769 ± 0.013	**9**	17.00 ± 1.64
5	5.398 ± 0.273		
6	4.219 ± 0.281		
10	1.230 ± 0.115		
11	0.954 ± 0.013		
12	6.024 ± 0.008		
13	1.006 ± 0.003		
14	1.158 ± 0.033		

[a] Values represent means ± SD (n = 3). [b] IC_{50} = one-half maximal inhibitory concentration to α-glucosidase. [c] Inhibition ratio (%) at a concentration of 50 μM. [d] Positive control. [e] Concentration of quercetin was 10 μM.

Table 3. AChE Inhibitory activity of compounds **1**-**8**, **10**, **12** and **14** from Yunnan black tea 'Jin-Ya'[a]

Sample	IC_{50} (μM)[b]	Sample	Inhibition ratio (%)[c]
tacrine[d]	0.223 ± 0.007	tacrine[d]	64.88 ± 1.33[e]
2	14.22 ± 0.571	**1**	25.44 ± 0.85
3	33.79 ± 1.022	**4**	56.62 ± 1.81
6	34.82 ± 2.110	**5**	34.32 ± 2.41
10	10.81 ± 0.134	**7**	32.00 ± 2.42
		8	32.98 ± 0.46
		12	36.34 ± 2.49
		14	47.16 ± 0.22

[a] Values represent means ± SD (n = 3). [b] IC_{50} = one-half maximal inhibitory concentration to AChE. [c] At a concentration of 50 μM. [d] Positive control. [e] At a concentration of 0.333 μM.