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Phenolic Antioxidants from Green Tea Produced from *Camellia taliensis*

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The chemical constituents of green tea prepared from the leaves of *Camellia taliensis* (W. W. Smith) Melchior (Theaceae) were investigated for the first time. Of these, 19 phenolic compounds including 8 hydrolyzable tannins (**1**−**8**), 6 catechin derivatives **(9**−**14)**, 3 quinic acid aromatic esters (**15**−**17**), and 2 simple phenolics (**18**, **19**) were identified, along with caffeine (**20**). Their antioxidant activities were evaluated by DPPH radical scavenging and tyrosinase inhibitory assays. Moreover, the chemical composition was compared with that in the cultivated tea plant, *C. sinensis* var. *assamica*, by HPLC analysis. It was noted that *C. taliensis* has similar chemical features with the cultivated tea plant; that is, both of them contain rich flavan-3-ols and caffeine. In addition, there are abundant hydrolyzable tannins as specific characteristic constituents contained in the leaves of *C. taliensis*. Therein, 1,2-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucopyranose (**8**), as a major compound in *C. taliensis*, showed remarkable antioxidant activity. The results suggested that *C. taliensis* could be a valuable plant resource for the production of tea.

KEYWORDS: ***Camellia taliensis*; green tea; hydrolyzable tannins; 1,2-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucopyranose; phenolic antioxidants; HPLC analysis**

INTRODUCTION

Tea, one of the most popular beverages consumed in the world, is normally produced from the leaves of two cultivated tea plants, *Camellia sinensis* (L.) O. Kuntze var. *sinensis* and *C. sinensis* var. *assamica* (Masters) Kitamura (Theaceae). On the basis of the processing procedures, it can be generally divided as green tea (nonfermented), oolong tea (semifermented), black tea (fully fermented by polyphenol oxidase), and Pu-er tea (postfermented by microbe).

Camellia taliensis (W. W. Smith) Melchior, an evergreen tree about 10−20 m high belonging to *Camellia* sect. *Thea*, is endemic from western and southwestern Yunnan province of China to northern Myanmar and native in the subtropical mountain evergreen forest at altitudes of 1500−2400 m. This species, named "Da-Li-Cha" in Chinese, is commonly referred to be as "wild" tea plant by the local people of its growing areas. It is closely related to *C. sinensis* var. *assamica*, a widely cultivated tea plant, but differing in a leaf absent pubescence, a five-rip style, and five-locular ovary with tomenta (*1, 2*). The leaves of *C. taliensis* are often used to make tea by the local people living in Yunnan province. In the distribution area of *C. taliensis*, many different variations and relative species of *C. sinensis* var. *assamica* have been commonly and widely cultivated for producing tea from long ago. So far, there have been several studies reported on the tea leaves of *C. sinenesis* var. *assamica* (*3–5*), from which flavanoids and hydrolyzable tannins were suggested to be the major active constituents due to their stronger antioxidative properties (*6–8*). However, the chemical constituents of *C. taliensis* are so far not known. As a part of our continuing research on tea and its original and related plants, the present paper describes the isolation and identification of phenolic constituents of green tea produced from the leaves of *C. taliensis*. Moreover, to evaluate the quality of this wild tea plant, the isolated compounds were tested for their antioxidant activities by DPPH radical and tyrosinase assays. Its chemical composition was compared with that in the cultivated tea plant, *C. sinensis* var. *assamica*, by HPLC analysis.

MATERIALS AND METHODS

General. ^{1}H NMR, ^{13}C NMR, and 2D NMR spectra were recorded in acetone-d_6 with Bruker AM-400 and DRX-500 spectrometers operating at 400 and 500 MHz for ^{1}H NMR and at 100 and 125 MHz for ^{13}C, respectively. Coupling constants were expressed in hertz, and chemical shifts were given on a parts per million scale with tetramethylsilane as internal standard. FAB-MS were recorded on a VG Auto

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Spec-300 spectrometer with glycerol as the matrix. DPPH radical scavenging and tyrosinase inhibitory assays were performed on an Emax precision microplate reader. Column chromatography was done on Sephadex LH-20, 25−100 μm (Pharmacia Fine Chemical Co., Ltd.), MCI-gel CHP20P, 75−100 μm (Mitsubishi Chemical Co., Ltd.), and Toyopearl HW-40F (Tosoh Co., Ltd.). Thin-layer chromatography (TLC) was performed on precoated slica gel H plates, 0.2−0.25 mm thick (Qingdao Haiyang Chemical Co.), with benzene/ethyl formate/formic acid [3:6:1 (a) or 2:7:1 (b), v/v/v], and spots were detected by spraying with 2% ethanolic $FeCl_3$ or anisaldehyde−H_2SO_4 reagent followed by heating.

Kojic acid, tyrosinase mushroom, L-DOPA, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Chemicals (Steinheim, Germany), and ascorbic acid was obtained from Xinxing Chemical Industrial Reagent Institute (Shanghai, China). Compounds **8**−**12**, **14**−**16**, and **20** purified from this study were used as authentic samples for HPLC quantitative analysis.

The HPLC analysis was performed on a Waters 2695 separation module combined with the accessory of the Waters 2996 photodiode array detector, using a computer-controlled system with Millennium^{32} software, along with a 250 × 4.6 mm i.d., 5 μm, Zorbax SB-C_{18} reversed-phase column (Agilent). Water was purified in a Milli-Q (Millipore). Acetonitrile (chromatographic grade) and phosphoric acid (reagent grade) were purchased from Merck (Darmstadt, Germany).

Plant Materials. The green tea used in this study was produced from the leaves of *C. taliensis* (W. W. Smith) Melchior, collected at Dazongshan Mountain, Yunxian County, Lincang City, Yunnan Province, China. The cultivated green tea used for comparison study was produced from the leaves of "Yunkang No. 10" of *C. sinensis* (L.) O. Kuntze var. *assamica* (Masters) Kitamura, collected from Puwen, Xishuangbanna, Yunnan Province, China. Both plant materials were identified by Prof. Chong-Ren Yang, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation. Green tea produced from *C. taliensis* (5.0 kg) was extracted by soaking in aqueous acetone (60%, v/v, 10 L) three times (7 days each) at room temperature. After removal of the organic solvent under reduced pressure, the concentrated water solution was filtered to remove precipitate and then extracted with $CHCl_3$ to afford crude compound **20** (100 g). The water layer was further extracted with ethyl acetate to afford an organic extract (400 g), which was subjected to a Sephadex LH-20 column (35 × 8 cm), eluting with aqueous MeOH from 0 to 100% (v/v) in increments of 10% (800 mL each) followed by aqueous acetone (1:1, v/v, 1500 mL). The eluted parts were monitored by using TLC system a to give five fractions. Repeated column chromatography (CC) over MCI-gel CHP20P and Toyopearl HW40F, eluting with MeOH/H_2O (0:1−1:0), afforded compounds **1** (61 mg) and **5** (20 mg) from fraction 2 (7.0 g) and compounds **15** (76 mg), **16** (127 mg), **17** (61 mg), **18** (10 mg), and **19** (110 mg) from fraction 3 (9.0 g), respectively. Fraction 4 (108 g) was repeatedly chromatographed over Sephadex LH-20 and Chromatorex ODS, eluting with MeOH/H_2O (0:1−1:0), to yield compounds **2** (6 mg), **3** (245 mg), **4** (207 mg), **6** (46 mg), **7** (267 mg), and **8** (1.01 g). Fraction 5 (233 g) was applied to CC over MCI-gel CHP20P, Diaion HP20SS, and Sephadex LH-20 columns, eluting with MeOH/H_2O (0:1−1:0), to give compounds **9** (30 mg), **10** (0.1 g), **11** (10.15 g), **12** (2.37 g), **13** (9.0 mg), and **14** (0.55 g).

DPPH Radical Scavenging Activity. The DPPH assay was performed as previously described (*9*), and ascorbic acid was used as positive control. The starting concentration for the samples or positive control was prepared as 1 mg/mL, and then a 2-fold serial dilution was prepared to make a gradient with 10 concentrations. One hundred microliters of samples or positive control ethanol solution was added to 100 μL of DPPH ethanol solutions (200 μM). After incubation at room temperature for 30 min, the remaining DPPH radicals were determined by the absorbance at 517 nm. Scavenging activity was determined by the following equation: % scavenging activity = $100 \times (A_{control} - A_{sample})/A_{control}$. The SC_{50} value was obtained through extrapolation from linear regression analysis and denoted the concentration of sample required to scavenge 50% of DPPH radicals. The data presented are means ± SD of three determinations.

Tyrosinase Inhibitory Assay. The tyrosinase inhibitory activity was measured as previously described, using L-dopa as the substrate (*10*). Kojic acid was used as positive control. Mushroom tyrosinase aqueous solution (40 μL, 25 IU/mL), phosphate buffer (pH 6.8) (120 μL) (*A*), or phosphate buffer (pH 6.8) (80 μL) along with testing samples (40 μL) (*B*) were mixed. A modified volume of 40 μL of sample (1 mg/mL) added to 120 μL of phosphate buffer (pH 6.8) (*C*) was made to eliminate the absorbance brought with the colored samples. The mixture was preincubated at 37 °C for 10 min, and then 40 μL of 10 μM/mL L-dopa was added. The reaction mixture was then further incubated at 37 °C for 15 min. The amount of dopachrome was measured at 475 nm in a microplate reader. The inhibiting activity was determined by the following equation: % inhibition rate = $[A - (B - C)]/A$. The IC_{50} value was obtained through extrapolation from linear regression analysis and denoted the concentration of sample required to inhibit 50% of tyrosinase activity. The data presented are means ± SD of three determinations.

Preparation of Tea Extracts for HPLC Analysis. Green teas from *C. taliensis* and the cultivated "Yunkang No. 10" of *C. sinensis* var. *assamica* were ground into powder, respectively. Then, each of the samples (2.500 g) was saturated in 70% aqueous methanol (100 mL) for 12 h at room temperature, during which an ultrasonic bath was twice carried out for 15 min. The extract was filtered through a 0.45 μm membrane filter for HPLC analysis.

HPLC Analysis for Identification of the Main Constituents. The optimal mobile phase for the analysis of green tea extracts from *C. taliensis* and *C. sinensis* var. *assamica* was a binary gradient elution system consisting of solvent A (acetonitrile) and solvent B (water containing 0.34% phosphoric acid). The gradient program used started from 4 to 40% of solvent A in solvent B in 40 min at a flow rate of 0.8 mL/min. Column temperature was set at 30 °C. The injection volume was 10 μL. The UV detection wavelength was monitored at 280 nm. The peaks were confirmed by the UV absorptions and retention times of authentic samples, which were 25.8 (**8**), 24.0 (**9**), 18.1 (**10**), 32.2 (**11**), 24.8 (**12**), 11.9 (**14**), 8.3 (**15**), 18.6 (**16**), and 20.0 (**20**) min, respectively.

Method Validation for the Quantification of the Main Constituents. Good linearity was established for all of the tested compounds **8**−**12**, **14**−**16**, and **20**. Gradient amounts of 1−20 μL of authentic sample solutions (0.5 mg/mL) were injected to examine the linear relations. Then calibration curves were made accordingly to yield the following regression equations and ranges for quantificative analysis: $y = 5E + 05x + 190811$, $r = 0.9991$, 0.56−11.27 μg (**8**); $y = 5E + 05x + 190811$, $r = 0.9992$, 0.43−8.67 μg (**9**); $y = 8E + 05x + 6870$, $r = 0.9993$, 0.49−9.83 μg (**10**); $y = 2E + 06x - 121282$, $r = 0.9999$, 0.42−8.33 μg (**11**); $y = 2E + 06x + 175992$, $r = 0.9996$, 0.50−10.03 μg (**12**); $y = 8E + 05x + 458$, $r = 0.9994$, 0.44−8.73 μg (**14**); $y = 3E + 06x + 253736$, $r = 0.9990$, 0.52−10.33 μg (**15**); $y = 1E + 06x + 238145$, $r = 0.9998$, 0.51−10.27 μg (**16**); $y = 3E + 06x + 638436$, $r = 0.9996$, 0.48−9.67 μg (**20**). The precision of the method was studied as the following procedure: Ten microliters of the prepared tea extract solution was injected six times, in which the intervals were 2 h. The calculation results showed that the RSD values of the peak areas for all nine tested compounds were below 3%. The stabilization of the method was confirmed by intraday injections of the prepared tea extract solution, and the RSD values of the peak areas for all the compounds in the intraday injections were below 3% as well. Finally, 1 and 10 μL of the prepared tea extract solution were injected to accommodate the calibration curves of compounds **8**, **11**, **12**, and **20** and compounds **9**, **10**, **14**, **15**, and **16**, respectively.

RESULTS AND DISCUSSION

Repeated column chromatography over Diaion HP-20SS, Sephadex LH-20, MCI-gel CHP20P, and Toyopearl HW-40F led to the isolation of 19 known phenolic compounds (**1**−**19**) together with caffeine (**20**) from the aqueous acetone extract of green tea produced from the leaves of *C. taliensis*. These compounds were identified as eight hydrolyzable tannins, 1-*O*-galloyl-β-D-glucopyranose (**1**) (*11*), 1,2-di-*O*-galloyl-β-D-glu-

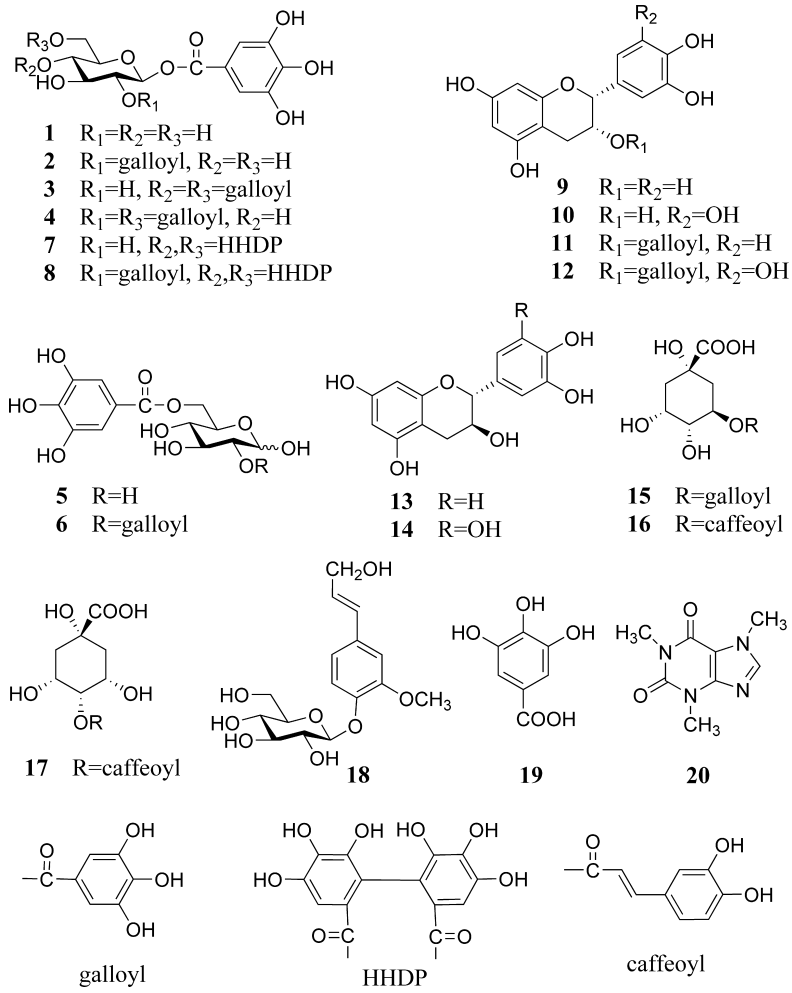

Figure 1. Compounds **1**−**20** isolated from *C. taliensis*.

copyranose (**2**) (*12*), 1,4,6-tri-*O*-galloyl-*β*-D-glucopyranose (**3**) (*13*), 1,2,6-tri-*O*-galloyl-*β*-D-glucopyranose (**4**) (*14*), 6-*O*-galloyl-D-glucopyranose (**5**) (*15*), 2,6-di-*O*-galloyl-D-glucopyranose (**6**) (*13*), 1-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-*β*-D-glucopyranose (**7**) (*16*), and 1,2-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-*β*-D-glucopyranose (**8**) (*17*); six catechin derivatives, (−)-epicatechin (**9**), (−)-epigallocatechin (**10**), (−)-epicatechin-3-*O*-gallate (**11**), (−)-epigallocatechin-3-*O*-gallate (**12**), (+)-catechin (**13**), and (+)-gallocatechin (**14**) (*18*); three quinic acid aromatic esters, theogallin (**15**) (*19*), chlorogenic acid (**16**) (*20*), and 3α,5α-dihydroxycaffeoylquinic acid (**17**) (*3*); and two simple phenolics, coniferin (**18**) (*21*) and gallic acid (**19**), by comparison with authentic samples and their spectroscopic and physical data with those previously reported (**Figure 1**). Among them, the hydrolyzable tannins **1**, **2**, **4**−**6**, and **8** were isolated from tea for the first time.

The isolated phenolic compounds **1**−**19** could be divided into three groups, hydrolyzable tannins (**1**−**8**), flavan-3-ols (**9**−**14**), and simple phenolic compounds (**15**−**19**). Their yields from the leaves of *C. taliensis* in this study are listed in **Table 1**. Of them, ellagitannin **8** (1,2-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-*β*-D-glucopyranose) and flavan-3-ols **11** and **12** were the major phenolic constituents in the title plant.

The antioxidant activities of **1**−**19** were evaluated by DPPH radical scavenging and mushroom tyrosinase inhibitory assays, and the results are shown in **Table 1**. It is noted that most of the isolated phenolic compounds displayed significant DPPH radical scavenging activities, which were comparable to that of the positive control (ascorbic acid), but mild tyrosinase inhibitory activity. Among them, both hydrolyzable tannins and flavan-3-ols (**1**−**14**) with catechol and/or pyrogallol groups in the molecule showed stronger DPPH radical scavenging activities than the other kinds of compounds. The results were consistent with previous studies (*22*–*24*). Three major compounds, 1,2-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-*β*-D-glucoranose (**8**), (−)-epicatechin-3-*O*-gallate (**11**), and (−)-epigallocatechin-3-*O*-gallate (**12**), of this plant were proved to possess higher activity.

The contents of the main compounds, **8**−**12**, **14**−**16**, and **20**, in the green tea produced from *C. taliensis* were compared with those produced from *C. sinensis* var. *assamica*, a widely cultivated tea plant, by HPLC analysis (**Figure 2**), and the results are shown in **Table 2**. It is noted that the wild tea plant, *C. taliensis*, has chemical features similar to those of the cultivated tea plant. Both tea plants contain rich flavan-3-ols (**9**−**12**, **14**), theogallin (**15**), chlorogenic acid (**16**), and caffeine (**20**), and the contents of all of these compounds in the cultivated tea plant are higher than those in the wild tea plant, except for (−)-epicatechin-3-*O*-gallate (**11**), the contents of which in the wild and cultivated tea plants were 3.69 and 0.40%, respectively. (−)-Epicatechin-3-*O*-gallate (**11**) is the highest content phenolic compound in *C. taliensis*, whereas the compound with highest content in the cultivated tea plant, *C. sinensis* var. *assamica,* is (−)-epigallocatechin-3-*O*-gallate (7.35%). Both (−)-epicatechin-3-*O*-gallate (**11**) and (−)-epigallocatechin-3-*O*-gallate (**12**) are the main constituents in tea, with various bioactivities, for example, antioxidative (*25*), antimicrobial (*26*), and antimutagenic activities (*27, 28*).

Table 1. Yields of Compounds **1**−**19** from Green Tea Produced from *C. taliensis* and Their DPPH Radical Scavenging (SC_{50}) and Tyrosinase Inhibitory (IC_{50}) Activities

compound	yield (%)	SC_{50} [a,b]	IC_{50} [b,c]
hydrolyzable tannins			
1	0.0012	33.7 ± 0.7	−[d]
2	0.0001	32.3 ± 0.9	−
3	0.0050	19.4 ± 0.4	−
4	0.0041	20.2 ± 0.6	−
5	0.0004	18.9 ± 0.2	726 ± 6
6	0.0009	17.0 ± 0.7	731 ± 14
7	0.0053	14.0 ± 0.8	731 ± 12
8	0.0202	8.2 ± 0.9	616 ± 8
flavan-3-ols			
9	0.0006	39.2 ± 1.0	861 ± 7
10	0.002	33.5 ± 0.5	744 ± 12
11	0.203	24.6 ± 0.6	626 ± 19
12	0.047	20.3 ± 0.3	518 ± 15
13	0.0002	38.2 ± 1.1	852 ± 12
14	0.011	22.0 ± 0.7	754 ± 7
simple phenolics			
15	0.0015	51.6 ± 0.5	1007 ± 14
16	0.0025	203 ± 1	550 ± 13
17	0.0012	198 ± 3	561 ± 5
18	0.0002	−	−
19	0.0022	13.0 ± 0.7	821 ± 11
ascorbic acid		29.6 ± 0.8	
kojic acid			273 ± 5

[a] SC_{50}, DPPH radical scavenging activity (concentration in μM necessary for 50% reduction of DPPH radical). [b] Values represent means ± SD ($n = 3$). [c] IC_{50}, tyrosinase inhibitory activity (concentration in μM necessary for 50% inhibition of tyrosinase activity). [d] −, no activity at a concentration of 1 mg/mL.

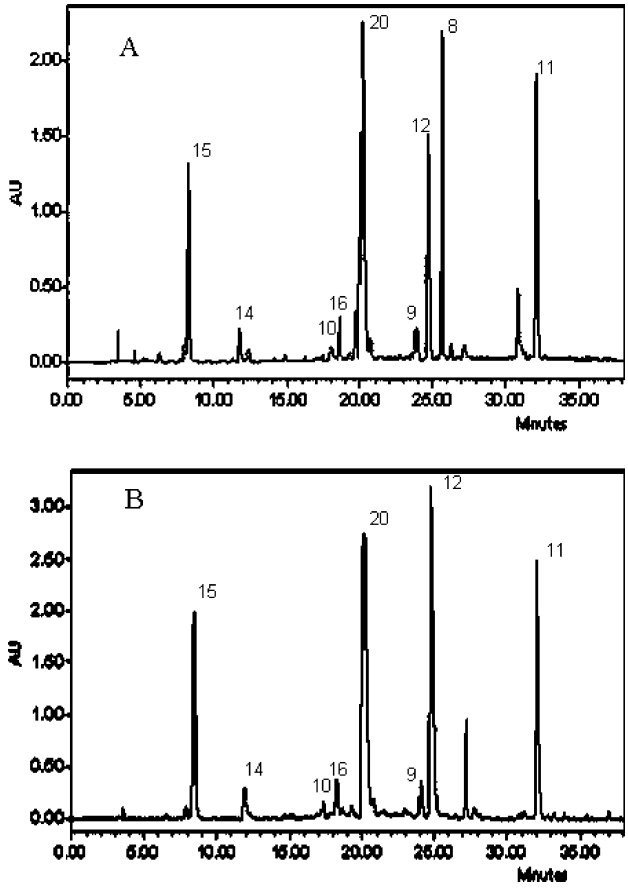

Figure 2. HPLC chromatogram of green tea extracts produced from *C. taliensis* (**A**) and *C. sinensis* var. *assamica* (**B**).

Moreover, ellagitannin 1,2-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucopyranose (**8**) was found to be one of the major and characteristic constituents in *C. taliensis*, the content of which reached 2.44%, comparable to those of (−)-epicatechin-3-*O*-gallate (3.69%) and (−)-epigallocatechin-3-*O*-gallate (2.47%). Compound **8** was first isolated from the leaves of *Camellia oleifera* (*29*) and then also reported from walnuts with superoxide dismutase (SOD)-like activity and remarkable radical scavenging effect against DPPH radical (*17*), the latter of which was consistent with this study. This is the first time that **8** was obtained from the *Thea* section of *Camellia*.

Table 2. Contents (Percent) of Compounds **8**−**12**, **14**−**16**, and **20** in Green Tea Produced from *C. taliensis* (A) and *C. sinensis* Var. *assamica* (B)

compound	green tea A[a]	green tea B[b]
8	2.44	
9	0.65	1.27
10	0.26	0.38
11	3.69	0.40
12	2.47	7.35
14	0.32	0.71
15	0.21	0.29
16	0.26	0.61
20	2.32	2.97

[a] Green tea A produced from the leaves of *C. taliensis*. [b] Green tea B produced from the leaves of *C. sinensis* var. *assamica*.

In the present work, eight hydrolyzable tannins (**1**−**8**) were identified from green tea produced from *C. taliensis*, in addition to flavan-3-ols (**9**−**14**), simple phenolic compounds (**15**−**19**), and caffeine (**20**). These hydrolyzable tannins have been mostly isolated from tea for the first time. The chemical features of *C. taliensis* are very similar to those of the widely cultivated tea plant, *C. sinensis* var. *assamica*. Both wild and cultivated tea plants contain rich flavan-3-ols and caffeine. Moreover, abundant hydrolyzable tannins, which showed remarkable antioxidant activities, are found to be the characteristic phenolic constituents in *C. taliensis*. They may play important roles in the health beneficial effects of tea produced from *C. taliensis*, together with the flavan-3-ols and the other phenolic compounds. The above evidence suggests that *C. taliensis* is a valuable plant resource for the production of tea.

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