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Identification of a Major Polyphenol and Polyphenolic Composition in Leaves of *Camellia irrawadiensis*

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The polyphenolic composition of *Camellia irrawadiensis*, which is a closely related species of *Camellia sinensis* (cultivated tea), was investigated. The most predominant polyphenol, a kind of ellagitannin, was isolated from leaves of *C. irrawadiensis*. Its structure was established as 1,2-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-*β*-D-glucose (2) on the basis of spectral and chemical evidence. Moreover, the polyphenols [catechins, strictinin (1), compound 2, theogallin, and gallic acid] and two methylxanthines (theobromine and caffeine) in leaves of *C. irrawadiensis* were determined by HPLC-Photodiode array detector analysis, and were compared to those in *C. sinensis* and *Camellia taliensis*. Total catechin content in *C. irrawadiensis* was lower than that in *C. sinensis* and *C. taliensis*. Theobromine content in *C. irrawadiensis* was higher than that in *C. sinensis* and *C. taliensis*. The content of 2 in *C. irrawadiensis* was 8.4% of dry leaf weight and comprised approximately 60% of the total polyphenols detected, while the compound was not detected in *C. sinensis* and was reported to be 2.4% in *C. taliensis*.

Key words *Camellia irrawadiensis*; ellagitannin; polyphenol; *Camellia sinensis*; genus *Camellia*; section *Thea*

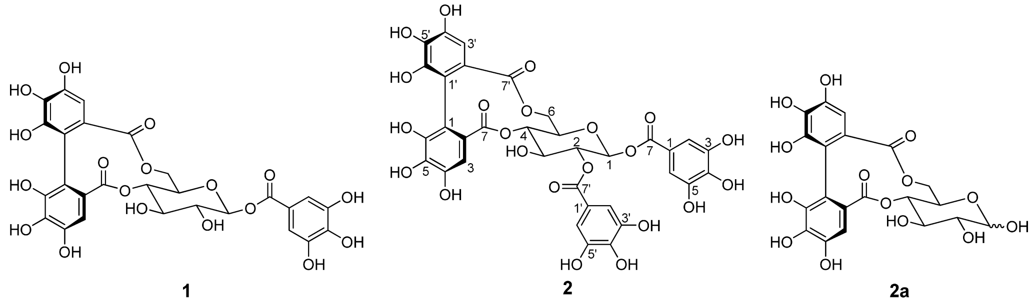
Green tea, the most popular beverage in East Asia, is made from leaves of *Camellia sinensis* belonging to the section *Thea* of genus *Camellia*.[1] It is well-known that the species *C. sinensis* contains catechins including epigallocatechin 3-*O*-gallate (EGCg), epigallocatechin (EGC), epicatechin 3-*O*-gallate (ECg), epicatechin (EC), gallocatechin 3-*O*-gallate (GCg), gallocatechin (GC), catechin 3-*O*-gallate (Cg), and catechin (C) as the major polyphenols, and the catechins show a variety of biological activities.[2] In the tea plant, hydrolyzable tannins [for example, strictinin (**1**) and theogallin], proanthocyanidins, flavonol glycosides and so on are also present as minor polyphenols.[3—9] In addition to these polyphenols, methylxanthines such as caffeine and theobromine exist in tea,[10] and are also well-known as pharmacologically active constituents.[11]

Camellia irrawadiensis and *Camellia taliensis* are well-known species in section *Thea*.[1] Nagata and Sakai[10,12] have reported the content of catechins and methylxanthines in the above species in addition to *C. sinensis*. According to their investigation, *C. sinensis* and *C. taliensis* contain a large amount of EGCg and ECg, while *C. irrawadiensis* has less amount of these catechins. It was also found that caffeine is the major methylxanthine in *C. sinensis* and *C. taliensis*, whereas theobromine is abundant in *C. irrawadiensis*. Moreover, Gao *et al.*[13] have recently reported the identification of polyphenols (hydrolyzable tannins and catechins) and determined the major polyphenolic composition in *C. taliensis*.

Contrastively, few studies have been carried out on the polyphenols other than catechins in *C. irrawadiensis*. Only one study published 50 years ago, has researched the presence of polyphenols other than catechins in *C. irrawadiensis*. Roberts *et al.*[14] reported that besides theogallin, unknown polyphenols were also detected as main components in *C. irrawadiensis* by two-dimensional paper chromatography. Although the unknown polyphenols were presumed to be derivatives of gallic acid, their structure and content in *C. irrawadiensis* are still undetermined. It is important to clarify the polyphenolic composition in *C. irrawadiensis* in order to utilize its potential application. This paper deals with the identification of strictinin (**1**) and 1,2-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-*β*-D-glucose (**2**) in leaves of *C. irrawadiensis*, and comparison of its polyphenolic composition with *C. sinensis* and *C. taliensis*. The major polyphenol in *C. irrawadiensis* was found to be **2**, which has already been reported to have 1,1-diphenyl-2-picrylhydrazyl radical scavenging-effect,[15] superoxide dismutase-like activity,[15] and tyrosinase inhibitory effect.[13] Compound **1** has also been found to show antiallergic effect[16] and hair growth promotion.[17] Thus, this study will open the door to potential utilization of *C. irrawadiensis* as a plant material with physiological functions.

Results and Discussion

As shown in Fig. 2A, an unknown major peak (compound



1 **2** **2a**

Fig. 1. Chemical Structures of **1**, **2**, and **2a**

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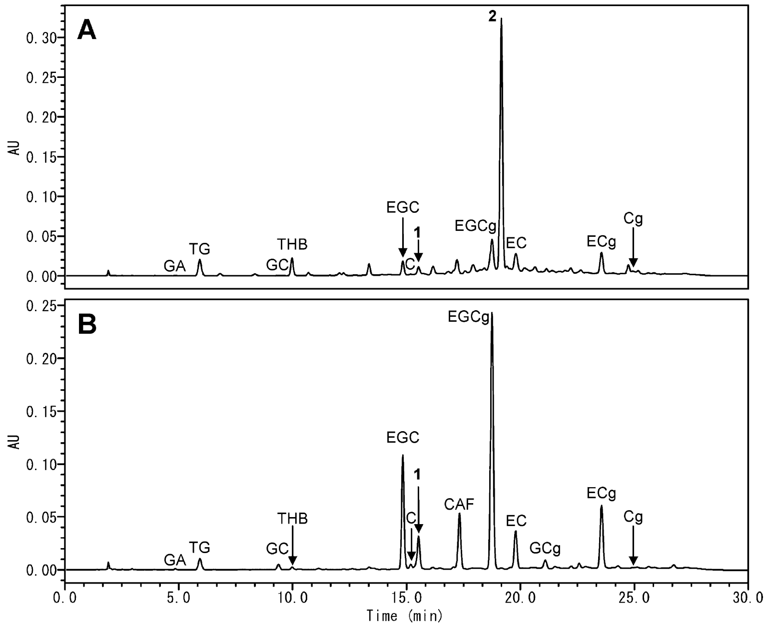

Fig. 2. HPLC Chromatograms of the Leaves of *C. irrawadiensis* (A) and *C. sinesnsis* var. *sinensis* cv. Yabukita (B) Monitored at 230 nm

Peak identification: GA (gallic acid); TG (theogallin); THB (theobromine); CAF (caffeine).

2) at a retention time of 19.2 min and an unknown minor peak (compound **1**) at 15.6 min were detected when the extract from the leaves of *C. irrawadiensis* was analyzed by HPLC-Photodiode array detector (PDA). The UV spectrum of the major peak showed absorption maxima at 276 and 215 nm, and the UV spectrum of the minor peak at 15.6 min was very similar to that of the major one. In order to isolate the two peaks, the concentrated 80% MeOH extract was extracted successively with $CHCl_3$ and EtOAc. Purifications of the EtOAc fraction by a DIAION HP20 column chromatography and preparative HPLC using an ODS column yielded **1** and **2**.

Compound **1** was identified as strictinin,[3] 1-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-*β*-D-glucose, by direct comparison of the spectral data (NMR, MS, and circular dichroism (CD)) and specific rotation with those of an authentic sample which was isolated from green tea extract derived from *C. sinensis*.

Compound **2** was obtained as an off-white amorphous powder, and its molecular formula was determined as $C_{34}H_{26}O_{22}$ by HR-FAB-MS spectrometry (*m/z* 785.0859 $[M-H]^-$ Calcd for 785.0843). The molecular weight of **2** was 152 mass units larger than that of **1**. The ^{1}H-NMR spectrum of **2** closely resembled that of **1** except for appearance of an additional 2H singlet signal (Table 1). The ^{13}C-NMR data of **2** were also similar to those of **1** except for the presence of new five signals of which the chemical shifts appeared in the same fields as the galloyl group in **1**. These observations indicated that **2** has one more galloyl group in the molecule than **1**. In the ^{1}H-NMR spectrum of **2**, anomeric proton signal of glucose moiety (Glc H-1) appeared at δ_H 5.95 (d, $J=8.3$ Hz). The large coupling constant indicated that the glucose moiety of **2** possessed *β*-configuration at the anomeric position. The other proton signals of the glucose moiety were assigned on the basis of the ^{1}H–^{1}H correlation spectroscopy (COSY) correlations from Glc H-1 through Glc H-6. A significant downfield shift was observed for Glc H-2

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of Compounds **1** and **2** (400 MHz for ^{1}H and 100 MHz for ^{13}C in CD_3OD)[a]

Position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
Glucose				
1	5.67 (d, 8.2)	96.3	5.95 (d, 8.3)	94.4
2	3.62 (dd, 9.4, 8.2)	74.8	5.36 (dd, 9.7, 8.3)	74.7
3	3.72 (t, 9.4)	76.1	4.08 (t, 9.7)	74.1
4	4.87 (t, 9.4)	73.2	5.03 (t, 9.7)	73.1
5	4.05 (dd, 9.4, 6.3)	73.7	4.21 (dd, 9.7, 6.4)	73.8
6	5.24 (dd, 13.2, 6.3)	64.3	5.31 (dd, 13.1, 6.4)	64.1
	3.82 (d, 13.2)		3.90 (d, 13.1)	
Galloyl				
1		120.5		119.9
2, 6	7.14 (s)	110.6	7.08 (s)	110.6
3, 5		146.6		146.5
4		140.6		140.7
7		166.9		166.4
1′				120.9
2′, 6′			7.09 (s)	110.5
3′, 5′				146.4
4′				140.1
7′				167.3
HHDP				
1		117.0		116.9
2		126.4		126.2
3	6.69 (s)	108.6	6.73 (s)	108.7
4		145.8[b]		145.9[c]
5		137.7		137.7
6		145.2[b]		144.9[c]
7		169.7		169.4
1′		116.8		116.6
2′		126.6		126.5
3′	6.55 (s)	108.3	6.63 (s)	108.5
4′		145.8[b]		145.8[c]
5′		137.5		137.4
6′		145.1[b]		144.8[c]
7′		170.0		169.9

a) Chemical shift values are in ppm and *J* values (in Hz) are presented in parentheses. *b*, *c*) Assignments may be interchanged.

(δ_H 5.36) of **2** as compared with that (δ_H 3.62) of **1**, suggesting that the additional galloyl group of **2** was bound to the 2-position of glucose moiety. Two 2H singlet signals (δ_H 7.09, 7.08) and two 1H singlet signals (δ_H 6.73, 6.63) were assigned to two galloyl groups and a hexahydroxydiphenoyl (HHDP) group, respectively. Attached positions of the two galloyl groups were determined to be at 1- and 2-positions of the glucose moiety on the basis of heteronuclear multiple bond connectivity (HMBC) correlations between the Glc H-1 (δ_H 5.95) and C-7 (δ_C 166.4) of a galloyl group and between the Glc H-2 (δ_H 5.36) and C-7′ (δ_C 167.3) of another one. The HMBC correlations between the Glc H-4 (δ_H 5.03) and C-7 (δ_C 169.4) of the HHDP group and between the Glc H-6 (δ_H 3.90, 5.31) and C-7′ (δ_C 169.9) of the HHDP group demonstrated that the HHDP group was linked to the 4- and 6-positions of the glucose moiety. The absolute configuration of the HHDP group in **2** was established to be *S* by the strong positive Cotton effect at 234 nm in the CD spectrum because similar Cotton effect has been reported to be observed in ellagitannins which possess (*S*)-HHDP group.[18] Moreover, selective hydrolysis of the galloyl groups in **2** with tannase yielded gallic acid and **2a**. The product **2a** was identified as 4,6-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose by direct comparison of spectral data (MS, NMR, and CD) and specific rotation with those of an authentic specimen prepared from **1**, which was isolated from green tea extract, by similar tannase hydrolysis. Consequently, the structure of **2** was established as 1,2-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucose. It has been reported that this compound was isolated from *Camellia taliensis*[13] belonging to the section *Thea* of genus *Camellia*, *Camellia oleifera*,[19] and *Juglans regia*.[15] However, the structure of **2** has been not well-characterized because of the absence of spectral data such as NMR, MS, and CD in the previous papers. This is the first report to clearly elucidate the structure of **2**.

Content of the polyphenols (**1**, **2**, theogallin, gallic acid, and catechins) and the methylxanthines (theobromine and caffeine) in *C. irrawadiensis* and *C. sinensis* var. *sinensis* were analyzed by HPLC-PDA. The content of these components in seven cultivars of *C. sinensis* var. *sinensis* was measured (Table 2) and the mean values of the cultivars were used as the representative values for this species. HPLC chromatograms of *C. irrawadiensis* and *C. sinensis* var. *sinensis* (Yabukita) are illustrated in Fig. 2. The content of polyphenols and methylxanthines in the leaves of the two species together with published data of *C. taliensis*[10,13] are given in Table 2. Nagata and Sakai[10] have reported that leaves of *C. irrawadiensis* contain an abundant amount of theobromine and a trace amount of caffeine. Our results were mostly in agreement with the previous study. Theobromine content in *C. irrawadiensis* (1.26%) was approximately 30-fold higher than that in *C. sinensis* (0.04%), while caffeine was not detected in *C. irrawadiensis* and hence its content was estimated to be less than 0.01% in this study. Theobromine and caffeine content in *C. taliensis* was reported to be 0.14% and 2.32%, respectively. These findings indicate that composition of the methylxanthines in *C. taliensis* is similar to that in *C. sinensis*, but is different from *C. irrawadiensis*. Total catechin content in *C. irrawadiensis* (4.00%) was about three-tenths of that in *C. sinensis* (12.57%) and about one-half of that in *C. taliensis* (7.39%), although there was only a slight difference in the catechin constituents among the three species. The amount of galloylated catechins (EGCg, ECg, GCg, and Cg) in *C. irrawadiensis* was far less than that in *C. sinensis* and in *C. taliensis*. These results were in accordance with those in the previous paper reported by Nagata and Sakai.[12] The most important findings in this study were that compound **2**, which is one of the ellagitannins, predominantly existed in leaves of *C. irrawadiensis*. The content of **2** in *C. irrawadiensis* was shown to be 8.39%, more than three-fold higher than that in *C. taliensis* (2.44%). Although a trace peak was detected at the same retention time of **2** in the HPLC chromatogram of *C. sinensis*, the UV spectrum of the peak did not agree with that of **2**. Therefore, *C. sinensis* was

Table 2. Content of Methylxanthines and Polyphenols in *C. irrawadiensis*, *C. sinensis* var. *sinensis*, and *C. taliensis* (% of Dry Leaf Weight)

Compounds	*C. irrawadiensis*	*C. sinensis* var. *sinensis*								*C. taliensis*[b]
		Yabukita	Kanayamidori	Okumidori	Asanoka	Saemidori	Okuyutaka	Asatsuyu	Average[a]	
Methylxanthines										
Theobromine	1.26	0.08	0.02	0.09	0.07	0.01	0.01	0.01	0.04	0.14[c]
Caffeine	<0.01	2.80	3.16	3.39	3.15	2.64	2.88	2.53	2.93	2.32
Catechins										
EGC	0.46	2.61	4.39	2.16	3.84	3.78	3.29	3.21	3.33	0.26
GC	0.02	0.13	0.27	0.16	0.28	0.13	0.13	0.19	0.18	0.32
EC	1.11	1.05	1.06	0.76	1.21	0.94	0.97	0.95	0.99	0.65
C	0.06	0.14	0.14	0.15	0.21	0.07	0.10	0.09	0.13	+[d]
EGCg	1.38	5.94	6.73	5.86	6.33	6.07	5.89	7.33	6.31	2.47
GCg	ND[e]	0.25	0.22	0.21	0.29	0.11	0.14	0.14	0.20	
ECg	0.87	1.61	1.27	1.12	1.43	1.13	1.29	1.61	1.35	3.69
Cg	0.10	0.06	0.13	0.04	0.13	0.08	0.06	0.10	0.09	
Total catechin[f]	4.00	11.81	14.20	10.46	13.71	12.31	11.86	13.62	12.57	7.39
Ellagitannins										
1	0.32	0.75	0.30	0.74	0.37	0.23	0.40	0.21	0.43	+[d]
2	8.39	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	2.44
Others										
Theogallin	2.00	0.85	0.16	0.57	0.31	0.04	0.18	0.10	0.31	0.21
Gallic acid	0.02	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	+[d]

a) The data are average values for seven cultivars. *b*) Taken from ref. 13 except for theobromine value. *c*) Taken from ref. 10. *d*) This compound has been isolated from *C. taliensis*, but it has not been quantified.[13] *e*) ND: not detected. *f*) Sum of catechins.

considered not to contain **2**. Theogallin content in leaves of *C. irrawadiensis* (2.00%) was much higher than that in *C. sinensis* (0.31%) and that in *C. taliensis* (0.21%). No distinctive difference in the content of **1** and gallic acid was observed between *C. irrawadiensis* and *C. sinensis*. Unfortunately, the contents of the two compounds could not be compared between *C. irrawadiensis* and *C. taliensis* because of a lack of quantitative data for *C. taliensis*.

These observations revealed that a feature of polyphenolic composition in *C. irrawadiensis* is the abundance of **2**, 1,2-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucose. Past studies have shown that *C. irrawadiensis* is characterized by both a trace amount of caffeine and a large amount of theobromine. Our study shows that compound **2** will be of chemotaxonomic marker for distinction of the three *Camellia* species.

Experimental

General Optical rotations were measured on a Jasco P-1020 polarimeter. CD spectra were taken on a Jasco J-600 spectropolarimeter. HR-FAB-MS spectra were recorded on a JEOL JMS-BU25 mass spectrometer using diethanolamine as a matrix. All 1D and 2D NMR spectra were recorded on a BRUKER AVANCE 400 NMR spectrometer equipped with 2.5 mm probe head operating at 400 MHz for ^{1}H- and 100 MHz for ^{13}C-NMR. Chemical shifts of ^{1}H- and ^{13}C-NMR given in δ value (parts per million) were referenced to tetramethylsilane as an internal standard. ^{1}H–^{1}H COSY, ^{1}H–^{13}C heteronuclear single quantum coherence, and ^{1}H–^{13}C HMBC experiments were carried out using standard pulse sequences. DIAION HP20 (Mitsubishi Chemical Co.), Toyopearl HW-40F (Tosoh Co.), and Chromatorex ODS DU1530MT (Fuji Silysia Chemical Ltd.) were used for column chromatography. Preparative HPLC was performed on a JAI LC-908 HPLC system and a JAI UV-3702 UV detector set at 230 nm with a Mightysil RP-18 GP (20 mm i.d.×250 mm, 5 μm, Kanto Chemical Co., Inc.) at room temperature. Extraction from the leaves of *C. irrawadiensis* and *C. sinensis* was carried out using a BRANSON BRANSONIC 8510 ultrasonic bath.

Chemicals EGCg, EGC, ECg, EC, GCg, GC, and Cg were prepared in our laboratory as previously described.[20,21] Catechin (C) was purchased from Sigma-Aldrich. Authentic sample of **1** was isolated from hot water extract of green tea by chromatographic separations on DIAION HP20 (stepwise elution with H_2O–MeOH), followed by Toyopearl HW-40F (MeOH) and Chromatorex ODS DU1530MT (H_2O/MeOH/MeCN/AcOH; 820 : 160 : 20 : 2, v/v/v/v). Theogallin was also isolated from green tea extract by a combination of DIAION HP20 (stepwise elution with H_2O–MeOH) and Chromatorex ODS DU1530MT (H_2O/MeCN/TFA; 980 : 20 : 1, v/v/v). Gallic acid was purchased from Kanto Chemical Co., Inc. Caffeine and theobromine were purchased from Acros Organics. Tannase was obtained from Sankyo Co., Ltd. HPLC-grade MeOH and MeCN were special grade products of Kanto Chemical Co., Inc. H_2O was purified with a Millipore Milli-Q system. All other reagents were of analytical grade.

Plant Material Leaves of *Camellia irrawadiensis* were harvested at Nittoh Malino Tea (tea garden), Sulawesi, Indonesia, in April 2006 and dried in a microwave oven. The plant material was multiplied by division of "*C. irrawadiensis* No. 2" described in Nagata and Sakai's report.[10] Dried leaves of seven *Camellia sinensis* var. *sinensis* cultivars, cv. Yabukita (plucked in April 2007), Kanayamidori (June 2007), Okumidori (April 2007), Asanoka (June 2007), Saemidori (July 2007), Okuyutaka (June 2007), and Asatsuyu (June 2007) were obtained from green tea suppliers in Japan. The leaves of *C. sinensis* used were so-called Japanese green tea which were made by standard manufacturing process of Sencha under the same conditions. Dried leaves of the samples were ground to fine powder with a Bamix hand-held food blender (ESGE AG).

Extraction and Isolation The dried and ground leaves (5 g) of *C. irrawadiensis* were extracted with 80% (v/v) aqueous MeOH (200 ml) for 30 min in an ultrasonic bath and then filtered with a filter paper under suction. The filtrate was concentrated to dryness under vacuum. The residue (2.13 g) was dissolved in H_2O (200 ml), and the resulting solution was partitioned with $CHCl_3$ (200 ml×3) and EtOAc (200 ml×4). After removal of solvent, the EtOAc extract (823 mg) dissolved in H_2O (100 ml) was applied to a column of DIAION HP20 (30 mm i.d.×300 mm) which had previously been equilibrated with H_2O. The column was eluted stepwisely with each 200 ml of H_2O, aqueous MeOH (20, 40, 60, 80%, v/v), and acetone. Each eluate was concentrated and lyophilized. The 40% MeOH fraction (420 mg) was separated into three fractions (Fractions 1—3) by preparative HPLC under the following conditions: solvent A, H_2O/MeCN/AcOH (1000 : 25 : 1, v/v/v); solvent B, H_2O/MeOH/MeCN/AcOH (1000 : 500 : 25 : 1, v/v/v/v); linear gradient from 30 to 100% B in 50 min; flow rate, 15 ml/min. Fraction 1 (65 mg) was further purified by preparative HPLC with H_2O/MeCN/AcOH (900 : 100 : 0.9, v/v/v) at a flow rate of 15 ml/min to yield **1** (4.6 mg). Fraction 2 (269 mg) was further purified by preparative HPLC with H_2O/MeCN/AcOH (700 : 100 : 0.7, v/v/v) at a flow rate of 15 ml/min to yield **2** (206 mg).

Strictinin (**1**): An off-white amorphous powder. $[\alpha]_D^{24}$ $-22.5°$ (c=0.4, MeOH). CD (MeOH) nm ([θ]): 311 (-1.0×10^4), 281 ($+1.4\times10^4$), 260 (-3.5×10^4), 233 ($+9.5\times10^4$). HR-FAB-MS m/z: $[M-H]^-$ 633.0728 (Calcd for $C_{27}H_{21}O_{18}$ 633.0733). ^{1}H- and ^{13}C-NMR: see Table 1.

1,2-Di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucose (**2**): An off-white amorphous powder. $[\alpha]_D^{24}$ $-12.4°$ (c=1.0, acetone). CD (MeOH) nm ([θ]): 296 (-3.3×10^4), 272 ($+1.3\times10^4$), 257 (-1.7×10^4), 234 ($+12.0\times10^4$). HR-FAB-MS m/z: $[M-H]^-$ 785.0859 (Calcd for $C_{34}H_{25}O_{22}$ 785.0843). ^{1}H- and ^{13}C-NMR: see Table 1.

Hydrolysis of Compound 2 with Tannase A solution of **2** (30 mg) in H_2O (2 ml) was incubated with tannase (20 units) for 30 min at 37 °C. The reaction mixture was filtered through a 0.45 μm hydrophilic PTFE filter (ADVANTEC DISMIC-25HP) and then purified by preparative HPLC with H_2O/MeCN/AcOH (1000 : 25 : 1, v/v/v) at a flow rate of 15 ml/min to yield gallic acid (10.5 mg) and **2a** (17.6 mg).

4,6-*O*-(*S*)-Hexahydroxydiphenoyl-D-glucose (**2a**): An off-white amorphous powder. $[\alpha]_D^{24}$ $+57.3°$ (c=0.7, acetone). CD (MeOH) nm ([θ]): 311 (-0.8×10^4), 282 ($+1.3\times10^4$), 259 (-3.4×10^4), 234 ($+9.1\times10^4$). HR-FAB-MS m/z: $[M-H]^-$ 481.0614 (Calcd for $C_{20}H_{17}O_{14}$ 481.0624). ^{1}H-NMR (CD_3OD, 25 °C, 0.5 : 0.5 mixture of α/β anomers) δ: 6.69 (0.5H, s, HHDP H-3α), 6.68 (0.5H, s, HHDP H-3β), 6.65 (1.0H, s, HHDP H-3$'\alpha$, 3$'\beta$), 5.19 (0.5H, dd, J=13.0, 6.3 Hz, Glc H-6β), 5.16 (0.5H, dd, J=12.7, 6.4 Hz, Glc H-6α), 5.14 (0.5H, d, J=3.8 Hz, Glc H-1α), 4.79 (0.5H, t, J=9.6 Hz, Glc H-4β), 4.74 (0.5H, t, J=9.7 Hz, Glc H-4α), 4.52 (0.5H, d, J=7.7 Hz, Glc H-1β), 4.38 (0.5H, dd, J=9.7, 6.4 Hz, Glc H-5α), 3.87 (0.5H, dd, J=9.6, 6.3 Hz, H-5β), 3.83 (0.5H, t, J=9.7 Hz, Glc H-3α), 3.82 (0.5H, d, J=13.0 Hz, Glc H-6β), 3.74 (0.5H, d, J=12.7 Hz, Glc H-6α), 3.59 (0.5H, t, J=9.6 Hz, Glc H-3β), 3.53 (0.5H, dd, J=9.7, 3.8 Hz, Glc H-2α), 3.28 (0.5H, dd, J=9.6, 7.7 Hz, Glc H-2β). ^{13}C-NMR (CD_3OD, 25 °C, 0.5 : 0.5 mixture of α/β anomers) δ: 170.2 (HHDP C-7$'\alpha$), 170.1 (HHDP C-7$'\beta$), 169.9 (HHDP C-7α), 169.8 (HHDP C-7β), 145.84 (×2), 145.78, 145.76, 144.9 (×2), 144.8 (×2) (HHDP C-4α, 4β, 4$'\alpha$, 4$'\beta$, 6α, 6β, 6$'\alpha$, 6$'\beta$), 137.6, 137.5 (HHDP C-5α, 5β), 137.3 (×2, HHDP C-5$'\alpha$, 5$'\beta$), 126.7, 126.64, 126.58, 126.5 (HHDP C-2α, 2β, 2$'\alpha$, 2$'\beta$) 116.84, 116.77, 116.70, 116.65 (HHDP C-1α, 1β, 1$'\alpha$, 1$'\beta$), 108.63, 108.60 (HHDP C-3α, 3β), 108.34, 108.32 (HHDP C-3$'\alpha$, 3$'\beta$), 99.0 (Glc C-1β), 94.3 (Glc C-1α), 77.0 (Glc C-2β), 75.9 (C-3β), 74.3 (Glc C-2α), 74.2 (Glc C-4α), 73.7 (Glc C-4β), 73.6 (Glc C-3α), 72.8 (Glc C-5β), 67.9 (Glc C-5α), 64.9 (Glc C-6α), 64.8 (Glc C-6β).

Quantitative HPLC Analysis of Polyphenols and Methylxanthines Quantitative determination of polyphenols (**1**, **2**, theogallin, gallic acid, and eight kinds of catechins) and methylxanthines (theobromine and caffeine) in leaves of *C. irrawadiensis* and *C. sinensis* was carried out by HPLC-PDA.

The dried and ground leaves (200 mg) were weighed and put into a 100 ml volumetric flask, and then 80% (v/v) aqueous MeOH (40 ml) was added to the flask. The mixture was treated for 30 min in an ultrasonic bath. After the addition of 1 M HCl (400 μl), this mixture was diluted to the mark with water, and then filtered through a 0.45 μm hydrophilic PTFE filter (ADVANTEC DISMIC-25HP). The filtrate was subjected to HPLC analysis. All samples were analyzed in duplicate.

Analytical HPLC was performed on a Waters 2695 separations module equipped with a Waters 2996 Photodiode array detector. UV spectra were recorded in the wavelength range of 210—600 nm. Separations were carried out using a Mightysil RP-18 GP (4.6 mm i.d.×150 mm, 5 μm, Kanto Chemical Co., Inc.). The temperature of the column was maintained at 40 °C. The mobile phase consisted of H_2O/MeCN/H_3PO_4 (1000 : 25 : 0.5, v/v/v, solvent A) and H_2O/MeOH/MeCN/H_3PO_4 (600 : 300 : 15 : 0.3, v/v/v/v, solvent B). The gradient elution program was as follows: 0—3 min, 0% B; 3—25 min, 0—100% B; 25—26 min, 100—0% B; 26—30 min, 0% B (re-equilibration to the initial conditions before the next injection). The flow rate was 1 ml/min, and the injection volume was 10 μl.

Peaks were identified by comparison of their retention times and UV spectra with those of authentic standards. Gallic acid and catechins were detected by measuring absorbance at 230 nm and **1**, **2**, theogallin, and methylxanthines at 275 nm. Quantification of the individual compounds was based on calibration curves of the authentic standards.

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