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Isolation and Antioxidant Activity of Zeylaniin A, a New Macrocyclic Ellagitannin from *Syzygium zeylanicum* Leaves

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ABSTRACT: Methanol extract obtained from *Syzygium zeylanicum* leaves exhibited potent antioxidant activity. The water extract obtained from this methanol extract by sequential extraction with hexane, chloroform, ethylacetate, and *n*-butanol also showed the strongest antioxidant activity among extracts. This water extract was further fractionated by column chromatography with various concentrations of methanol solutions. Among the 6 resultant fractions, the fraction developed with 20% methanol exhibited the most potent antioxidant activity. The one peak among the three major HPLC peaks in this fraction was isolated and purified using a preparative HPLC. The structure of a pure compound was elucidated as a novel macrocyclic ellagitannin using a $^{1}H/^{13}C$ NMR and a high-resolution electrospray ionization mass spectrometer. This newly isolated compound, which was named zeylaniin A, exhibited potent antioxidant activities in the assays of DPPH, oxygen radical absorbance capacity, and malonadehyde/gas chromatography. *S. zeylanicum* leaves can be a possible source of natural antioxidants.

KEYWORDS: *antioxidant, ellagitannin, macrocyclic compound, Syzygium zeylanicum, zeylaniin A*

INTRODUCTION

Medicinal plants have been used to treat various diseases since ancient times. Even after the development of modern medicines, traditional herb therapies have been used clinically, especially in Asia. According to the World Health Organization, 80% of the populations in Asian and African countries depend on traditional medicine for primary health care.[1] In particular, medicinal plants in Vietnam have received attention as new resources for alternative medicines over the last few decades. Approximately 2500 species of exotic plants in Vietnam have been used as medicinally because of their biological activities,[2,3] including diuretic,[4] antioxidant,[5] cytotoxic,[6,7] and antimicrobial. One recent report also demonstrated that several Vietnamese traditional herbs exhibited potent antioxidant activity.[8]

A biological antioxidant has been defined as any substrate that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate.[9] This is important because biological oxidation causes, at least in part, many diseases. Accordingly, there have been many reports on the relationships between oxidative damage and various diseases, including cancer,[1] liver disease, Alzheimer's disease, arthritis, inflammation, diabetes, Parkinson's disease, atherosclerosis, and AIDS, as well as on the relationship to aging.[10] As a results, patients at risk for many diseases have been treated with antioxidants to prevent their occurrence.

Syzygium zeylanicum, called 'Tram Vo Do' in Vietnamese, is a large shrub or tree with pale brown bark and slender shining-purplish twigs. It is one of the edible wild plants, the fresh leaves of which have conventionally been consumed as food in Vietnam. It has been reported that a methanol extract of *S. zeylanicum* leaves contained a high level of polyphenols and possessed α-glucosidase-inhibitory effects, potent antioxidant activity,[11] and anti-inflammatory effects.[12] However, the components responsible for these activities have not yet been identified. Therefore, isolation and identification of the antioxidant components of *S. zeylanicum* leaves were performed in order to find the specific components contributing to the leaves' health- beneficial properties.

MATERIALS AND METHODS

Materials and Reagents. *S. zeylanicum* leaves were purchased from a local market in Ho Chi Minh City, Vietnam in June 2011. The plant was taxonomically identified using the *Dictionary of Vietnamese Medicinal Plants*.[3] All reagent-grade chemicals were purchased from reliable commercial sources.

Sample Preparations of *S. zeylanicum* Leaves. *S. zeylanicum* leaves were freeze-dried (Freezone 6 Plus, Labconco Corp., Kansas City, MO) and ground into a powder using a blender. The powder (100 g) was soaked in 1 L of methanol for 24 h. After the methanol solution was filtered, the residual material was soaked in 1 L of methanol again for 24 h. The two methanol extracts were combined and then filtered. The filtrate was concentrated in vacuo to give a crude methanol extract (29 g). After 20 g of crude methanol extract was dissolved into 500 mL of deionized water, the sample solution was sequentially extracted with 500 mL each of *n*-hexane, chloroform, ethyl

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acetate, and *n*-butanol using a 2 L separatory flask. After the organic solvent extracts were dried over anhydrous sodium sulfate, the solvents were removed with a rotary flash evaporator. The residual water layer was condensed in vacuo. The overall sample preparation scheme is shown in Figure 1. The samples tested for antioxidant activities to screen a fraction with antioxidants are shown in the frame.

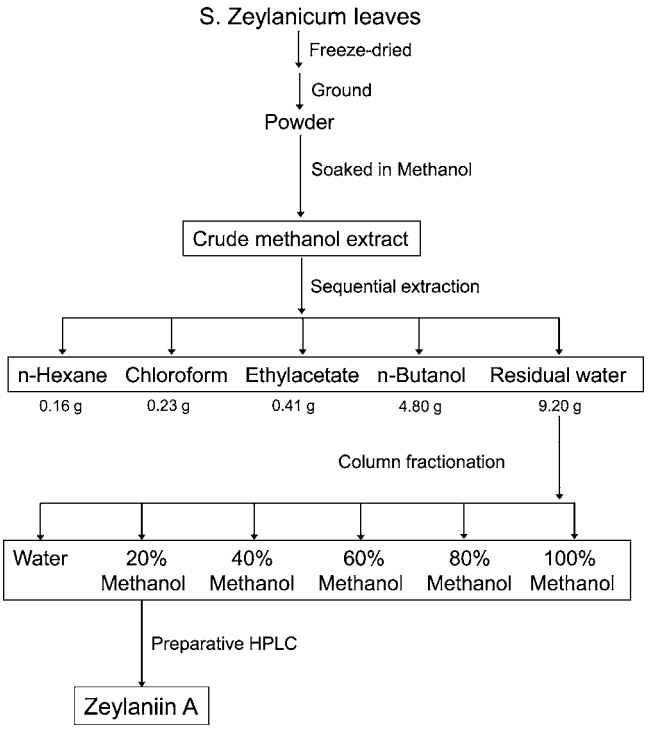

Figure 1. Overall sample preparation schemes. The samples tested for antioxidant activities are shown in the frame.

Column Fractionation of the Residual Water Layer. The residual water fraction (9.2 g), which exhibited the strongest antioxidant activity, was dissolved in 100 mL of deionized water and then placed on a 150 mm × 30 mm Diaion HP-20 column (Mitsubishi Chemical Corp., Tokyo, Japan). After the water was allowed to elute from the column (water fraction), the sample was eluted sequentially with 5 different methanol solutions (20, 40, 60, 80 and 100%). The methanol solvents were removed with a rotary flash evaporator. The water eluate was condensed in vacuo.

Isolation and Purification of the Antioxidant by HPLC. The presence of possible antioxidants in the eluate of 20% methanol, which showed potent antioxidant activity, was confirmed by an HPLC equipped with a 250 mm × 4.6 mm i.d. YMC-Pack ODS-AM column (YMC Co., Ltd., Kyoto, Japan). The gradient mode of the mobile phase was initially set at 0.1% aqueous formic acid/methanol (95/5) and then linearly changed to 30/70 at 40 °C over 30 min. The flow rate was 1.0 mL/min. The diode array detector was set at 220−300 nm and 10 μL of a sample was injected. A typical chromatogram is shown in Figure 2. Subsequently, components in the eluate of 20% methanol (1.2 g) were purified by a preparative HPLC equipped with a 250 mm × 14 mm i.d. Inertsil ODS-3 column (GL Sciences, Tokyo, Japan). The mobile phase was 0.1% formic acid/methanol (80/20) and the flow rate was 6 mL/min at 40 °C. The detector was set at 280 nm and a 100 μL (4 mg) sample of the 20% methanol fraction was injected repeatedly until sufficient pure samples were obtained. The peaks at retention times 13.5 min (**I**), 15.3 min (**II**), and 20.7 min (**III**) were collected. After each sample was lyophilized, pure components (10.6 mg from **I**, 22.2 mg from **II**, and 14.1 mg from **III**) were obtained. Purity of each isolated compound was obtained by the HPLC method described above.

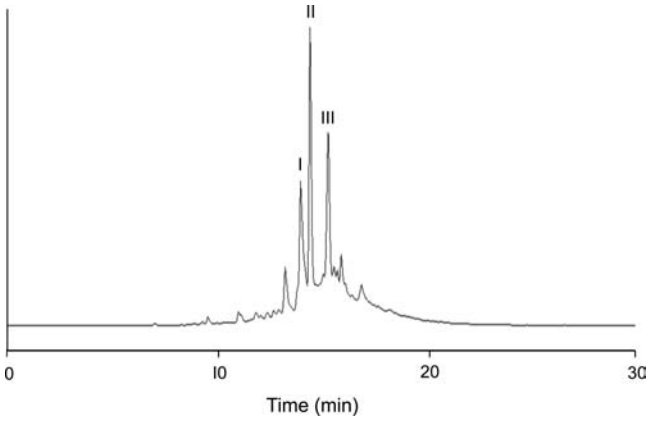

Figure 2. Typical HPLC chromatogram of the 20% methanol fraction.

Identification of Isolated Compounds. The structural elucidation of compound **II** was performed using a $^{1}H/^{13}C$ NMR Bruker AvanceIII 600 Spectrometer (Bruker BioSpin, Karlsruhe, Germany) and a JMS-T100LP high-resolution electrospray ionization mass spectrometer (Jeol, Tokyo, Japan).

Antioxidant Assays. The convenient DPPH assay was used to screen fractions with antioxidants. DPPH, ORAC and MA/GC assays were used to confirm the antioxidant activity of the isolated compounds.

DPPH Radical Scavenging Assay. The DPPH radical scavenging assay was conducted according to a previously reported method.[10,13] During the purification process, this assay was conducted on the fractions of the organic solvent extraction and the residual water fraction. Briefly, 100 μL of 300 μM DPPH methanol solution was mixed with 100 μL of various concentrations of samples (50 and 500 μg/mL), α-tocopherol (50 μg/mL), or solvent (blank) in 96-well plates. The mixtures were incubated at room temperature for 30 min, and the absorbance at 517 nm was measured with a microplate reader (Powerscan HT, Dainippon Pharmaceutical, Osaka, Japan).

The same assay was also conducted on the fractions obtained from column chromatography of the residual water layer—the condensed residual water layer was diluted with water to 100-, 1000-, and 10 000-fold and then tested for antioxidant activity. The isolated and purified compound (zeylaniin A) was also examined for antioxidant activity by the same method with a slight modification. Briefly, 1 mL of 300 μM DPPH methanol solution was mixed with 1 mL of various concentrations of zeylaniin A (1, 2, 5, and 10 μg/mL), Trolox, or solvent (blank). The mixture was incubated at room temperature for 20 min, and the absorbance at 517 nm was measured with UV−vis spectrometer (U-0080D, Hitachi High-Technologies, Tokyo, Japan). The standard curve was obtained by plotting Trolox concentrations (5, 10, 15, 20, and 25 μg/mL) against the average inhibition rate for each concentration. Antioxidant activities were calculated using a regression equation between Trolox concentration and inhibition rate, and were expressed as mole Trolox equivalents per mole for zeylaniin A. All assays were conducted in triplicate.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The ORAC assay was conducted according to a previously reported method with slight modifications.[14] All solutions used were diluted with 75 mM phosphate buffer (pH 7.4). Briefly, 25 μL of zeylaniin A solution (2.5, 5, 10, or 20 μM) or Trolox standard solution (6.25, 12.5, 25, and 50 μM) and 150 μL of fluorescein solution (81.6 nM) were placed in the well of the black microplate. The mixture was preincubated for 3 min at 37 °C, and then 25 μL of AAPH solution (306 mM) was added. The microplate was immediately placed in the reader and the fluorescence (485 Ex/520 Em; Powerscan HT, Dainippon Pharmaceutical, Osaka, Japan) was recorded every minute for 70 min at 37 °C. The microplate was automatically shaken prior to each reading. The experiment was done in triplicate. The results of ORAC activity were

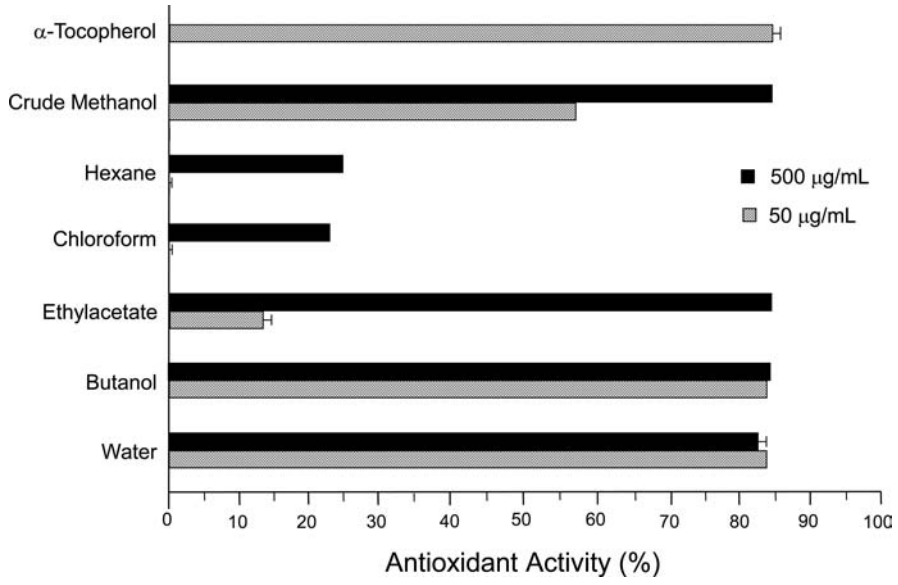

Figure 3. Results of DPPH assay on the samples obtained by sequential solvent extraction.

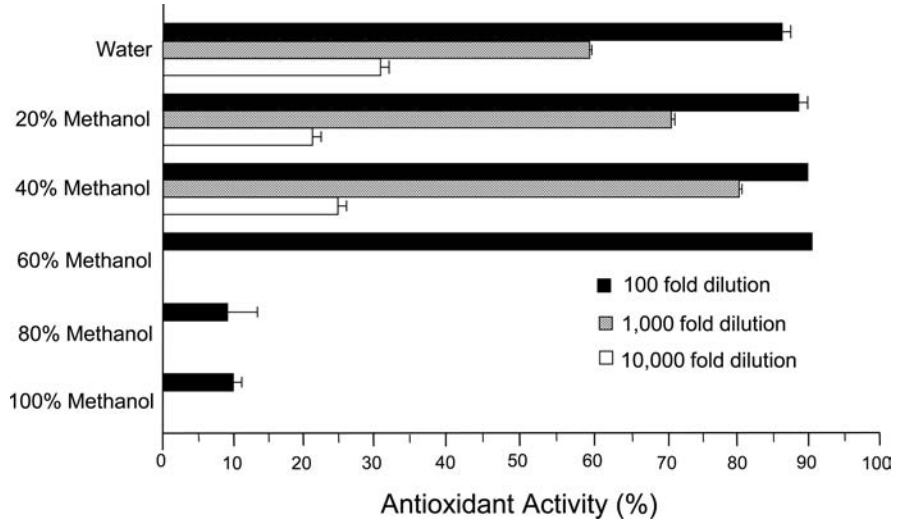

Figure 4. Results of DPPH assay on the samples obtained by HP-20 column chromatography.

estimated on the basis of the standard curve of Trolox, using a quadratic regression equation obtained between the Trolox concentration and net area under the fluorescence decay curve. ORAC activity was expressed as mole Trolox equivalents per mole for samples (mol TE/mol sample).

Malonaldehyde/Gas Chromatography (MA/GC) Assay. The MA/GC assay was performed according to a previously reported method.[15] Briefly, 500 μL of zeylaniin A solution (10, 50, or 100 μg/mL) was added to an aqueous solution (5 mL) containing 10 μL of cod liver oil, 50 mM Tris buffer (pH 7.4), 0.5 μM of H_2O_2, 1.0 μM of $FeCl_2$, 0.75 mM of KCl, and 0.2% SDS in a 20 mL test tube. After the reaction solution was incubated at 37 °C for 18 h, the MA formed in the samples was analyzed as 1-MP using an Agilent Technologies model 6890N GC equipped with a 30 m × 0.25 mm i.d. (d_f = 0.25 μm) DB-wax fused-silica capillary column (Agilent Technologies Inc., Santa Clara, CA) and a nitrogen−phosphorus detector (NPD). The GC oven temperature was programmed from 70 to 120 °C at 4 °C/min. The injector and detector temperatures were 260 and 280 °C, respectively. The helium carrier gas flow rate was 1.4 mL/min.

RESULTS AND DISCUSSION

Results of Antioxidant Assays on Samples. The DPPH assay was used to locate antioxidants in the fractions prepared by various solvent extractions and the results are shown in Figure 3. The crude methanol extract exhibited nearly 90% antioxidant activity at the level of 500 μg/mL, suggesting that *S. zeylanicum* leaves contain potent antioxidants. Among the fractions obtained by the 4 organic solvents, the *n*-butanol fraction as well as the residual water fraction showed comparable activities to α-tocopherol at the level of 50 μg/mL. Whereas the ethylacetate fraction showed only slight activity at the level of 50 μg/mL, the *n*-butanol and water fractions exhibited strong activity at the same level. The amounts recovered from *n*-butanol and water fractions were 4.80 and 9.20 g, respectively. Therefore, the water fraction was further fractionated using column chromatography because the *n*-butanol fraction did not dissolve well in water.

Figure 4 shows the results of the DPPH assay on the fractions from column chromatography of the water layer. The

Table 1. ^{1}H and ^{13}C NMR Data of Zeylaniin A (Compound II)[a]

position	δ_H	δ_C	position	δ_H	δ_C	position	δ_H	δ_C
glucose-I			1-1		114.18			
1	6.26 (d, 8.4 Hz)	92.65	1-2		142.38			
			1-3		138.57			
2	5.03 (m)	71.61	1-4		140.42			
			1-5		140.65			
3	5.67 (t, 9.6 Hz)	74.08	1-6	6.68 (s)	108.75			
			1-7		166.22			
4	5.04 (m)	70.43	2-1		119.80	2′-1		118.34
			2-2	6.77 (s)	109.98	2′-2		142.93
5	4.51 (m)	71.30	2-3		145.12	2′-3		139.09
			2-4		138.61	2′-4		134.62
6	3.87 (d, 12.0 Hz)	63.05	2-5		145.12	2′-5		139.14
	5.27 (m)		2-6	6.77 (s)	109.98	2′-6	6.18 (s)	107.65
			2-7		164.64	2′-7		167.14
glucose-II			3-1		119.40	3′-1		120.05
1′	4.29 (d, 8.4 Hz)	95.03	3-2	6.76 (s)	110.15	3′-2	6.79 (s)	109.90
			3-3		144.91	3′-3		145.19
2′	4.92 (t, 9.0 Hz)	74.88	3-4		138.72	3′-4		138.70
			3-5		144.91	3′-5		145.19
3′	5.19 (t, 9.0 Hz)	73.33	3-6	6.76 (s)	110.15	3′-6	6.79 (s)	109.90
			3-7		166.94	3′-7		165.71
4′	5.05 (m)	71.07	4-1		124.01	4′-1		124.26
			4-2		114.95	4′-2		117.94
5′	4.25 (m)	71.79	4-3		144.49	4′-3		144.55
			4-4		136.53	4′-4		140.51
6′	3.76 (d, 12.6 Hz)	64.92	4-5		144.99	4′-5		145.82
	5.07 (m)		4-6	6.46 (s)	107.52	4′-6	6.82 (s)	111.37
			4-7		168.87	4′-7		168.95
			6-1		124.55	6′-1		125.19
			6-2		116.48	6′-2		113.78
			6-3		144.04	6′-3		144.18
			6-4		136.50	6′-4		135.23
			6-5		146.66	6′-5		144.99
			6-6	6.35 (s)	107.31	6′-6	6.50 (s)	106.58
			6-7		168.46	6′-7		169.69

[a]Numbering of the proton and carbon atoms refers to the structure in Figure 7.

fractions from water, 20% methanol, and 40% methanol eluates exhibited strong antioxidant activities with a dose response, which were comparable to that of α-tocopherol. On the other hand, the fractions from 60, 80, and 100% methanol eluates showed antioxidant activities only at the highest level tested, even though the fraction from 60% methanol eluate exhibited strong antioxidant activity at that level. HPLC showed the 20% methanol fraction as containing possible antioxidants (Figure 2). Therefore, this fraction was further purified using a preparative HPLC.

Isolation and Identification of Antioxidants. The 20% methanol fraction gave pure compound **I** (10.6 mg), **II** (22.2 mg), and **III** (14.1 mg). The purities of these compounds were 93.5% (**I**), 92.4% (**II**) and 89.0% (**III**). All these compounds showed the same UV absorption (one maximum at 270 nm), suggesting that they may possess related structures. The structural analysis was started on compound **II** (an amorphous powder) because of its higher yield and stability.

ESI-MS spectrum showed the $[M - H]^-$ peak at m/z 1719, indicating that its molecular weight was 1720. The experimental formula of compound **II** was confirmed to be $C_{75}H_{52}O_{48}$ from the molecular weight, and $^{1}H/^{13}C$ NMR data.

Table 1 shows the ^{1}H and ^{13}C NMR spectra of compound **II**. These data, supported with $^{1}H-^{1}H$ COSY and the HSQC spectra, indicated the presence of two sugar units (hexose), six isolated aromatic methines, three pairs of equivalent aromatic methines, and nine carbonyl carbons. These observations strongly suggested that compound **II** is a hydrolyzable tannin composed basically by 9 gallic acids and 2 sugars. On the basis of the molecular formula and the composition of compound **II**, the presence of four oxidative linkages in galloyl structures was proposed.

The large vicinal coupling constants of the ^{1}H signals in two sugars (Table 1) assigned them as $^{4}C_1$ glucopyranose. One glucose unit (glucose-II) was confirmed to be β-form because of the large coupling constant of H-1′ (J = 8.4 Hz), and the anomeric OH was proposed to be free from the chemical shift of H-1′ (δ 4.29), although anomeric OH was free and the proton signals derived from the α-form were not observed. The other glucose unit (glucose-I) was also confirmed to be β-form because of the large coupling constant of H-1 (J = 8.4 Hz), and the acylation of the anomeric OH of this unit was obvious from the chemical shift of H-1 (δ 6.26).

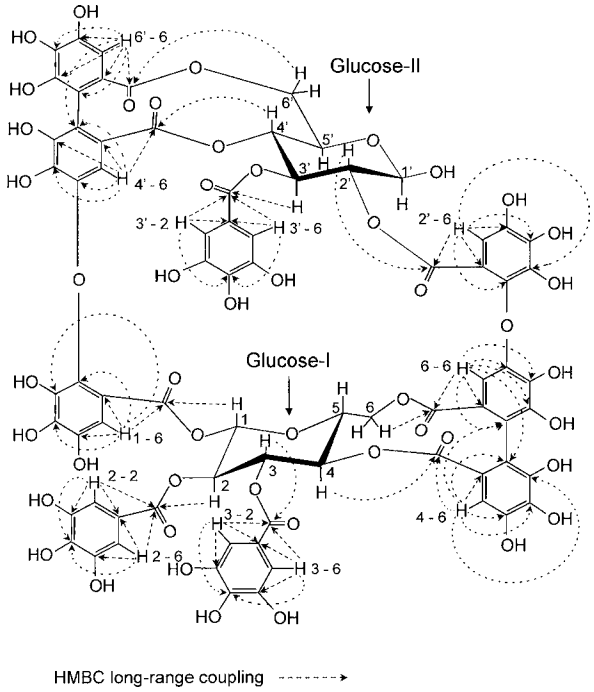

Figure 5. HMBC spectral data of compound **II**.

Figure 5 shows HMBC spectral data of compound **II** taken in D_2O and acetone-d_6. In this spectrum, the long-range couplings from δ 5.03 (H-2, glucose-I) and δ 6.77 (H-2-2 and H-2-6) to the carbonyl carbon at δ 164.64 (C-2-7), from δ 5.67 (H-3, glucose-I) and δ 6.76 (H-3-2 and H-3-6) to δ 166.94 (C-3-7), and from δ 5.19 (H-3′, glucose-II) and δ 6.79 (H-3′-2 and H-3′-6) to δ 165.71 (C-3′-7) were observed. These observation indicated that gallic acid units are attached to glucose-I at C-2 and C-3 and to glucose-II at C-3′ through ester linkage.

Long range couplings from δ 5.05 (H-4′, glucose-II) and δ 6.82 (H-4′-6) to δ 168.95 (C-4′-7), from δ 5.07 (H-6′, glucose-II) and δ 6.50 (H-6′-6) to 169.69 (C-6′-7), and from δ 6.50 (H-6′-6) to δ 117.94 (C-4′-2) were observed. These correlations proved the linkage of H-4′ and H-6′ through a valoneoyl group. A large chemical shift difference (Δ 1.27) in the geminal H-6′ signals also supported this linkage. In the same way, the long-range couplings from δ 5.04 (H-4, glucose-I) and δ 6.46 (H-4-6) to δ 168.87 (C-4-7), from δ 5.27 (H-6, glucose-I) and δ 6.35 (H-6-6) to 168.46 (C-6-7), and from δ 6.35 (H-6-6) to δ 114.95 (C-4-2) proved the linkage of H-4 and H-6 through a valoneoyl group. A large chemical shift difference (Δ 1.40) in the geminal H-6 signals was also observed.

Long range couplings from δ 6.26 (H-1, glucose-I) and δ 6.68 (H-1-6) to δ 166.22 (C-1-7), and from δ 6.68 (H-1-6) to δ 114.18 (C-1-1), δ 142.38 (C-1-2), δ 138.57 (C-1-3), δ 140.42 (C-1-4), and δ 140.65 (C-1-5) were also observed. Therefore, the attachment of 2,3,4,5-tetrahydroxybenzoic acid (2-hydroxy-gallic acid) at C-1 (glucose-I) was confirmed. The long-range couplings from δ 4.92 (H-2′, glucose-II) and δ 6.18 (H-2′-6) to δ 167.14 (C-2′-7), and from δ 6.18 (H-2′-6) to δ 118.34 (C-2′-1), δ 142.93 (C-2′-2), δ 139.09 (C-2′-3), δ 134.62 (C-2′-4), and δ 139.14 (C-2′-5) also proved the attachment of 2,3,4,5-tetrahydroxybenzoic acid (2-hydroxy-gallic acid) at C-2′ (glucose-II).

Figure 6 shows the NOESY spectral data of compound **II** taken in D_2O and acetone-d_6. The cross peaks were observed

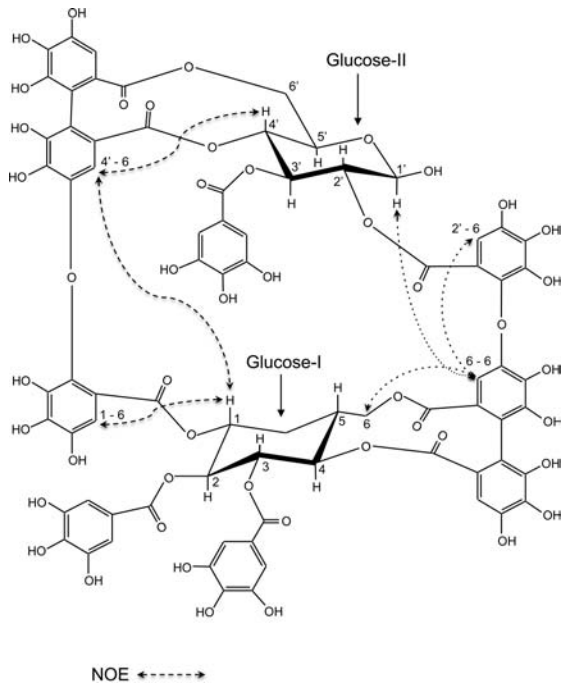

Figure 6. NOESY spectral data of compound **II**.

between δ 6.18 (H-2′-6) and δ 6.35 (H-6-6), and between δ 6.26 (H-1) and δ 6.82 (H-4′-6). These NOEs together with the spectral information of a known compound, camelliin B (Figure 7), strongly suggest the presence of a linkage of C-2′-2 and C-6-5, and C-1-2 and C-4′-5 through ether linkage.[16]

On the basis of these results, compound **II** was identified as a novel macrocyclic ellagitannin and named zeylaniin A. Zeylaniin A (compound **II**) is a regioisomer of camelliin B, which was previously isolated from the flower buds of *Camellia japonica* and *Camellia sasanqua*.[16,17] The structure of zeylaniin A along with that of camelliin B is shown in Figure 7.

It should be noted that macrocyclic ellagitannin, zeylaniin A, was isolated for the first time from *S. zeylanicum*.

Antioxidant Activity of the Isolated Compound, Zeylaniin A (Compound II) from *S. zeylanicum*. The antioxidant activity of zeylaniin A was evaluated by three antioxidant assays: DPPH, ORAC, and MA/GC. Table 2 shows the results of these three assays on zeylaniin A.

The DPPH radical scavenging effect and ORAC activity of zeylaniin A were 19.18 ± 0.24 and 4.37 ± 0.29 (mol TE/mol), indicating that this compound has potent antioxidant activities. In addition, zeylaniin A showed a strong inhibitory effect toward lipid peroxidation with dose-response. It inhibited MA formation by nearly 80% at the level of 100 μg/mL (78. 76 ± 0.21%). The results indicate that zeylaniin A is one of the components contributing to the antioxidant activity of *S. zeylanicum* leaves.

There are many reports on the biological activities of ellagitannins. Those activities include antioxidant,[18] antiviral,[19] antimutagenic,[20] antimicrobial,[21] anti-inflammatory,[22] and antitumor activities.[23] Moreover, macrocyclic ellagitannins including oenothein B,[24] woodfordin C,[25] and camelliin B[26] have been reported to have host-mediated antitumor activity. Therefore, it is proposed that the zeylaniin A present in *S. zeylanicum*

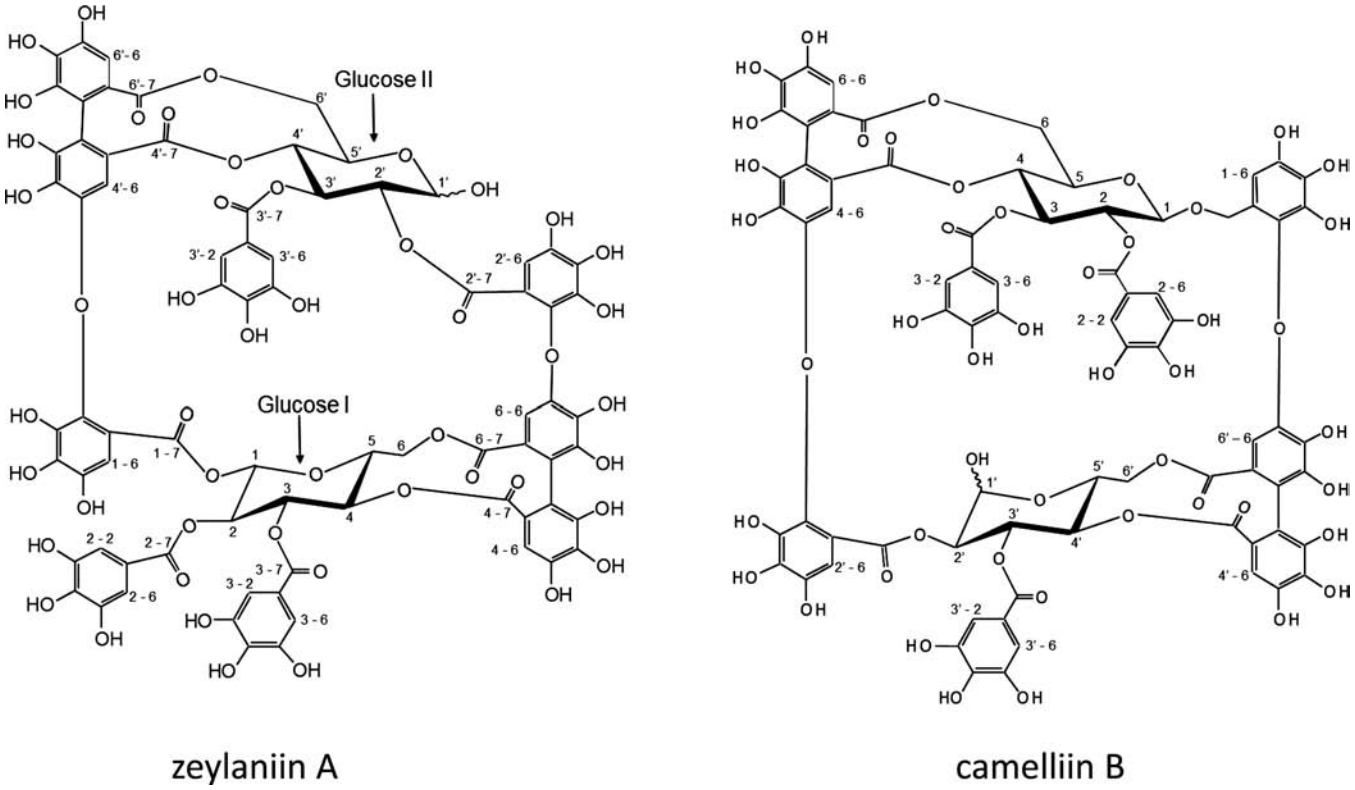

Figure 7. Structures of zeylaniin A and camelliin B.

Table 2. Antioxidant Activities of Zeylaniin A Tested by DPPH, ORAC, and MA/GC Assays

		MA/GC (%)		
DPPH (mol TE/mol)[a]	ORAC (mol TE/mol)[a]	10 μg/mL	50 μg/mL	100 μg/mL
19.18 ± 0.24	4.37 ± 0.29	55.37±3.63	59.35±0.22	78.76±0.21

[a]TE: Trolox equivalents.

leaves also has these biological effects. Further biological studies on zeylaniin A are currently underway.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

DPPH, 1,1-diphenyl-2-picrylhydrazyl; ORAC, oxygen radical absorbance capacity; MA/GC, malonaldehyde/gas chromatography; SDS, sodium dodecyl sulfate; BHT, butylated hydroxytoluene; COSY, ^{1}H−^{1}H correlated spectroscopy; HSQC, heteronuclear single-quantum coherence; HMBC, heteronuclear multiple-bond correlation; NOESY, nuclear Overhauser enhancement spectroscopy.

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