Antioxidative Glycosides from the Leaves of *Ligustrum robustum*

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Phytochemical investigation of the ethanol extract of the leaves of *Ligustrum robustum*, monitored by a bioassay involving the hemolysis of red blood cells induced by 2,2′-azo-bis(2-amidinopropane) dihydrochloride, led to the isolation of three new glycosides, ligurobustosides M (**1**), N (**2**), and O (**3**), along with 10 known ones, osmanthuside B (**4**), osmanthuside B6 (**5**), acteoside (**6**), ligupurpuroside A (**7**), ligupurpuroside B (**8**), ligurobustoside C (**9**), ligurobustoside E (**10**), ligurobustoside I (**11**), cosmosiin (**12**), and rhoifolin (**13**). The structures of the new compounds were elucidated by spectroscopic methods. Seven of the glycosides showed stronger antioxidant effects than the standard, trolox.

Tea prepared with the leaves of *Camellia sinensis* (L.) O. Kuntze (Theaceae) is a beverage first developed in China, where it has evolved into a subculture of the elite and affluent class with elaborate writings, ceremonies, selection of cultivars, processing methods, and grading systems. Those who are less affluent, on the other hand, explored and often adopted alternatives. A popular choice among the local people in southern China is teas collectively called "Ku-Ding-Cha". The words "Ku" and "Ding" literally mean bitter (poor) and man, respectively; the two words together also refer to laborers. "Ku-Ding-Cha" is taken to clear "heat" and "toxins" in the body and to quench thirst, and is also used in folk medicine as a diuretic and for the treatment of hypertension, sore throats, and inflammation.[1]

A total of 12 species belonging to six families and six genera were found to have been used as the source plants of "Ku-Ding-Cha" in different parts of the People's Republic of China.[2,3] In Guizhou Province, the leaves of *Ligustrum robustum* (Roxb.) Blume (Oleaceae) are used as the source material of "Ku-Ding-Cha".[2] Two previous studies of this species have led to the isolation and structure elucidation of monoterpenoid glycosides.[4,5]

In our screening program for potent antioxidants,[6−8] the ethanol extract of the processed leaves of *L. robustum* showed a significant inhibitory effect against the hemolysis of red blood cells (RBC) induced by free radicals. Further bioassay-guided analysis led to the isolation of three new (**1**−**3**) and 10 known glycosides (**4**−**13**) from the active fraction of *L. robustum*. This paper describes the structure elucidation of these glycosides and reports the antioxidative activity of the 13 glycosides isolated from this plant against hemolysis of RBC induced by 2,2′-azo-bis(2-amidinopropane) dihydrochloride.

Results and Discussion

The ethanol extract of the processed leaves of *L. robustum* showed strong antioxidant activity, with the concentration exhibiting 50% inhibition (IC_{50}) being 43.9 μg/mL.[8] Further fractionation suggested that the antioxidant components were concentrated in the aqueous fraction (IC_{50} 44.5 μg/mL). Thirteen glycosides (**1**−**13**) were subsequently isolated from this fraction. Three of the glycosides are new, namely, ligurobustosides M (**1**), N (**2**), and O (**3**). The other 10 glycosides, identified by direct comparison with authentic specimens and published data, are previously known: osmanthuside B (**4**), osmanthuside B6 (**5**), acteoside (**6**), ligupurpuroside A (**7**), ligupurpuroside B (**8**), ligurobustoside C (**9**), ligurobustoside E (**10**), ligurobustoside I (**11**), cosmosiin (**12**), and rhoifolin (**13**).[2,4,9,10]

Ligurobustoside M (**1**) was obtained as an amorphous powder, $[\alpha]^{24}_D$ −67.4° (*c* 0.03, MeOH). Its molecular formula was determined as $C_{26}H_{40}O_{15}$ by HRFABMS. The ^{1}H and ^{13}C NMR signals of **1** were assigned using ^{1}H−^{1}H (COSY) and HMQC experiments. The ^{1}H NMR spectrum in the aromatic region exhibited an AA′BB′ system assignable to the aglycon moiety which appeared at δ 6.88 (2H, d, J = 8.5 Hz) and 7.25 (2H, d, J = 8.5 Hz), indicating that one hydroxyl group was located at C-4 of the aglycon of **1**. The signals of H-7 and -8 in the phenylethyl moiety appeared at δ 3.02 (2H, t, J = 6.5 Hz, H_2-7), 3.77 (1H, m, H-8), and 4.09 (1H, m, H-8). ^{1}H NMR signals of three anomeric protons at δ 5.35 (1H, d, J = 1.5 Hz), 5.39 (1H, d, J = 1.5 Hz), and 4.47 (1H, d, J = 7.9 Hz), as well as two methyl groups of rhamnose at δ 1.43 (3H, d, J = 6.3 Hz) and 1.46 (3H, d, J = 6.3 Hz), were consistent with the configurations for two α−L-rhamnosyl units and one β-D-glucosyl unit. The chemical shifts of C-3′ in the glucosyl moiety and C-4″ in the rhamnosyl moieties of **1** were shifted downfield to δ 84.3 and 81.4, respectively, as compared with those of the standard glycosyl chemical shift. An HMBC experiment of **1** showed ^3J interactions between the anomeric proton H-1‴ of the outer rhamnose and the C-4″ of the inner rhamnose, and the anomeric proton H-1″ of the inner rhamnose and C-3′ of the glucose. The two rhamnosyl groups were attached at C-3′ of the glucosyl moiety and C-4″ of the inner rhamnosyl moiety, respectively. Accordingly, the structure of ligurobustoside M (**1**) was elucidated as [2-(4-hydroxyphenylethyl)]-[3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl]-β-D-glucopyranoside.

Ligurobustoside N (**2**), an amorphous powder, $[\alpha]^{24}_D$ −109° (*c* 0.06, MeOH), was determined as $C_{35}H_{46}O_{18}$ from its HRFABMS. The ^{1}H NMR spectrum showed an ABX system and an AA′BB′ system, which were assigned to *trans*-caffeoyl and 4-hydroxyphenylethyl moieties, respec-

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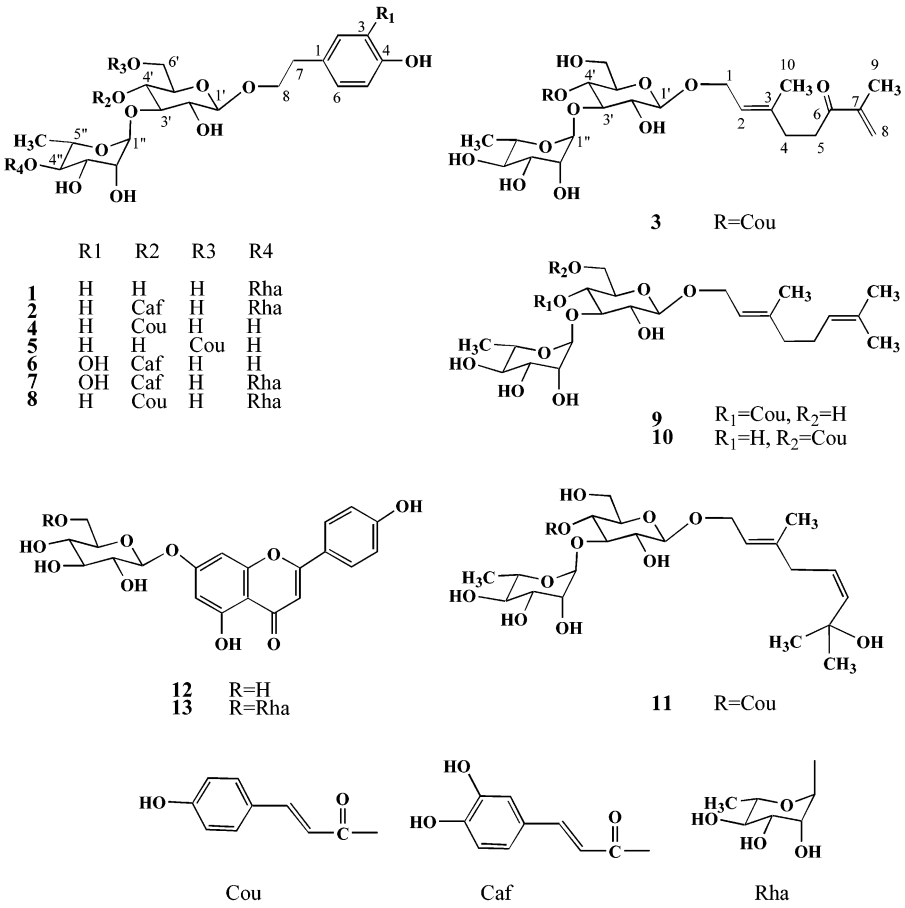
Chart 1. Structures of **1**−**13**



tively. A comparison of the ^{1}H NMR signals of **2** with those of **1** showed that the chemical shift assignable to H-4′ of the glucosyl moiety was shifted downfield from δ 3.50 to δ 5.13, and there was one more set of caffeoyl signals in **2**. The remaining signals in the ^{1}H and ^{13}C NMR spectra were consistent with the presence of one glucosyl unit and two rhamnosyl units as the sugar moieties. The anomeric protons of one β-D-glucose unit and two α-L-rhamnose units were observed at δ 4.47 (J = 7.9 Hz), 5.25 (J = 1.7 Hz), and 5.39 (J = 1.7 Hz), respectively. From the HMBC experiment of **2**, three anomeric carbons of a glucosyl group and two rhamnosyl groups were shown to be joined to C-8 of the aglycon, C-3′ of the glucosyl moiety, and C-4″ of the rhamnosyl moiety, respectively. An HMBC experiment of **2** showed a ^3J interaction between the carbonyl carbon of the caffeoyl group and H-4′ of glucose. Thus, ligurobustoside N (**2**) was identified as [2-(4-hydroxyphenylethyl)]-[3-*O*-α-L-rhamnosyl(1→4)-α-L-rhamnopyranosyl]-(4-*O*-caffeoyl)-β-D-glucopyranoside.

Ligurobustoside O (**3**) was obtained as an amorphous powder. The ESIMS of **3** exhibited a pseudomolecular ion $[M - H]^-$ at m/z 621, and in its HRFABMS a protonated molecular ion at m/z 623.3343 $[M + H]^+$, compatible with the molecular formula $C_{31}H_{42}O_{13}$. The ^{1}H NMR spectrum showed the following signals belonging to a monoterpene moiety: (1) an AB system of a pair of terminal methylene olefinic protons at δ 6.05 (1H, d, J = 1.5 Hz, Ha-8) and 6.28 (1H, d, J = 1.5 Hz, Hb-8); (2) an olefinic proton at δ 5.59 (1H, t, J = 7.4 Hz, H-2); (3) two sets of signals of CH_2 at δ 2.33 (2H, m, H-4) and 3.09 (2H, m, H-5); (4) two methyl signals at δ 2.05 (3H, s, H-9) and 1.91 (3H, s, H-10); and (5) an allylic alcoholic signal at δ 4.46 (2H, d, J = 7.4 Hz, H_2-1). These ^{1}H NMR signals, together with the ^{13}C NMR data assigned to the monoterpene moiety, were in accordance with those of a 3,7-dimethyl-2,7-octadien-6-one unit. The remaining signals in the ^{1}H and ^{13}C NMR spectra were consistent with the presence of one glucosyl, one rhamnosyl, and one *p*-coumaroyl moieties. The anomeric protons of the β-D-glucose and α-L-rhamnose units were observed at δ 4.55 (1H, d, J = 7.9 Hz) and 5.39 (1H, d, J = 1.3 Hz), respectively. A comparison of the ^{1}H and ^{13}C NMR signals of **3** with those of ligurobustoside G[5] showed that the signal assignable to C-6 of the aglycon moiety in **3** was shifted downfield from δ 76.10 to δ 203.89, which indicated that the hydroxyl group at C-6 in ligurobustoside G was replaced by a carbonyl group in **3**. The chemical shifts and coupling constants of H_2-1 and H-2 of **3** were nearly the same as those of ligurobustoside G, which suggested that the position and configuration of the double bond at $\Delta^{2,3}$ of the former are the same as the latter, as shown in the structural formula. The full ^{1}H and ^{13}C NMR signals of **3** were assigned by ^{1}H−^{1}H (COSY) and HMQC experiments. A HMBC experiment on **3** showed a ^3J correlation between the carbonyl carbon of the *p*-coumaroyl group and H-4′ of glucose, and furthermore the two anomeric carbons of glucosyl and rhamnosyl groups were shown to be joined to C-1 of the aglycon and C-3 of the glucosyl moiety, respectively. Accordingly, ligurobustoside O (**3**) was characterized as 3,7-dimethyl-1-*O*-[(3-*O*-α-L-rhamnopyranosyl)-(4-*O*-*p*-coumaroyl)-β-D-glucopyranosyl]-2,7-octadien-6-one.

Glycosides **1**−**13** were isolated from the active aqueous fraction B_2 of the EtOH extract of the leaves of *L. robustum*. These glycosides were tested for inhibitory effect on the hemolysis of RBC induced by 2,2′-azo-bis(2-amidinopropane) dihydrochloride (Table 1). The results demonstrated that glycosides **2**, **5**, **6**, **7**, **8**, **12**, and **13** showed stronger

Table 1. Concentrations Exhibiting 50% Inhibition (IC_{50}) of Glycosides **1**−**13** on AAPH-Induced Hemolysis

sample	IC_{50} (μM)
phenylethanoid glycosides	
1	134.1
2	21.8
4	159.6
5	91.7
6	28.0
7	26.3
8	80.4
monoterpenoid glycosides	
3	N.A.[a]
9	N.A.[a]
10	N.A.[a]
11	340.1
flavonoid glycosides	
12	88.4
13	95.9
trolox	101.0

[a] N.A.: no absorption.

antioxidant effects than trolox, the positive control. The most potent compounds were the phenylethanoid glycosides **2**, **6**, and **7**. On the other hand, the monoterpenoid glycosides **3** and **9**−**11** exhibited little or no inhibitory effect on the hemolysis of RBC. Based on the IC_{50} values of glycosides isolated from processed leaves of *L. robustum*, it is evident that the caffeoyl groups at C-4′ of glucose tend to enhance the inhibitory effects observed, but variation in the number of sugar units demonstrated no apparent effects.

Tea and "Ku-Ding-Cha" are the two most popular nonalcoholic beverages in China. Polyphenols in tea leaves have been shown to have strong antioxidant properties.[11] The present results document that the glycosides in the leaves of *L. robustum* also are strong antioxidants. Moreover, thin-layer chromatographic analysis of the extract of leaves of *L. robustum* did not detect any caffeine. *L. purpurascens*, another species of the same genus used as a source material of "Ku-Ding-Cha" in Yunnan Province, People's Republic of China, also showed DPPH radical reducing activity,[12] antiproliferative activity against tumor cells,[13] and inhibitory effects on oxidation of human LDL and α-tocopherol[14,15] and on acyl CoA cholesteryl acyltransferase.[16] Thus, there is good potential for further development of "Ku-Ding-Cha" as caffeine-free substitutes for tea.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were recorded using a Shimadzu UV-3100PC spectrophotometer. IR absorption spectra were obtained with a Shimadzu IR-450 instrument as a film on KBr disks. NMR spectra were obtained with a Bruker 400 spectrometer operating at 400 MHz for ^1H and 100 MHz for ^{13}C, respectively. Chemical shifts are reported in parts per million on the δ scale with TMS as the internal standard. FABMS were recorded on a VG autospec 3000 system, and ESIMS on a Finnigan TSQ 7000. Column chromatography was performed with silica gel (Qingdao Haiyang Chemical Group Co. Ltd., Qingdao, People's Republic of China), Lichroprep RP-18 (40−63 μm, Merck, Darmstadt, Germany), Resin D101 (Tianjin Agricultural Chemical Co. Ltd., Tianjin, People's Republic of China), and Sephadex LH-20 (Pharmacia Fine Chemical Co. Ltd.). TLC was performed on precoated Si gel 60 F_{254} plates (0.2 mm thick, Merck) with $CHCl_3$−MeOH−H_2O (7:3:1), $CHCl_3$−MeOH (10:1 and 9:1), and RP-18 F_{254s} plates (0.2 mm thick, Merck) with MeOH−H_2O (8:2 v/v), and spots were detected by UV illumination and by spraying with 10% ethanolic H_2SO_4 reagent.

Plant Material. The processed leaves of *L. robustum* were obtained from Guiyang, Guizhou Province, People's Republic of China. The voucher specimen of the herb sample (No. A2001-206) and of the source plant (*S.Y. Hu & P.P. H. But 23396*) were deposited, respectively, in the Museum of the Institute of Chinese Medicine and the Herbarium of the Department of Biology, Chinese University of Hong Kong.

Extraction and Isolation. The dried leaves of *L. robustum* (4.9 kg) were extracted for 2 h with 95% EtOH (7.4 L × 3). The EtOH extract (50% inhibitory activity IC_{50} 43.9 μg/mL) was concentrated to give a residue (2.2 kg). Part of the residue (72.50 g) was dissolved in H_2O (50 L) and divided into insoluble A (14.55 g) and aqueous B (46.80 g) parts. The aqueous B part (IC_{50} 44.5 μg/mL) was chromatographed on D101 (2 kg), by elution with H_2O (15 L), 60% EtOH (6 L), and EtOH (4 L), to afford three fractions, B_1 (3.60 g), B_2 (41.70 g), and B_3 (1.36 g). The IC_{50} of fractions B_1 and B_2 were 194.25 and 47.4 μg/mL, respectively. Fraction B_3 showed no absorption. Fraction B_2 was then developed on silica gel (1500 g), eluting with $CHCl_3$−MeOH−H_2O (7:3:0.5). The silica gel column was separated into 20 fractions; each fraction was eluted with MeOH (400 mL) and concentrated. The 20 fractions were combined into four fractions, fractions B_{2A}−B_{2D}, based on silica TLC ($CHCl_3$−MeOH−H_2O, 7:3:1) results. Fraction B_{2A} was subjected to silica gel CC with $CHCl_3$−MeOH−H_2O (40:10:1) as eluent and purified by a Sephadex LH-20 column with EtOH−H_2O (6:4), respectively. Fraction B_{2A} yielded three glycosides, **1** (32.1 mg), **12** (3.4 mg), and **13** (3.5 mg). Fractions B_{2B} and B_{2C} were subjected to silica gel CC with $CHCl_3$−MeOH−H_2O (50:10:1) and $CHCl_3$−MeOH−H_2O (60:10:1) as eluents to yield six glycosides, **2** (420.0 mg), **7** (200.0 mg), and **8** (30.4 mg), and **4** (9.1 mg), **5** (14.9 mg), and **6** (20.1 mg), respectively. Fraction B_{2D} was separated by CC on silica gel eluting with $CHCl_3$−MeOH (8:2) and purified by a Lichroprep RP-18 column eluting with MeOH−H_2O (6:5) to afford four glycosides, **3** (45.2 mg), **9** (960.0 mg), **10** (20.8 mg), and **11** (51.8 mg).

Ligurobustoside M (1): amorphous powder, $[\alpha]^{24}_D$ −67.4° (*c* 0.03, MeOH); UV λ_{max} (EtOH, log ϵ) 200 (3.85), 221 (4.10), 283 (3.48), 316 (3.35) nm; ^1H NMR (CD_3OD) δ aglycone 7.25 (2H, d, J = 8.5 Hz, H_2-2, 6), 6.88 (2H, d, J = 8.5 Hz, H_2-3, 5), 3.02 (2H, t, J = 6.5 Hz, H_2-7), 3.77 (1H, m, Ha-8), 4.09 (1H, m, Hb-8), glucosyl 4.47 (1H, d, J = 7.9 Hz, H-1′), 3.58 (1H, t, J = 8.2 Hz, H-2′), 3.58 (1H, t, J = 8.2 Hz, H-3′), 3.50 (1H, t, J = 8.2 Hz, H-4′), 3.52 (m, H-5′), 3.79 (1H, dd, J = 5.5, 11.6 Hz, Ha-6′), 4.04 (1H, dd, J = 3.1, 11.6 Hz, Hb-6′), rhamnosyl 5.39 (1H, d, J = 1.5 Hz, H-1″), 5.35 (1H, d, J = 1.5 Hz, H-1‴), 3.88 (m, H-2″, 2‴, 3″, 3‴), 3.50 (m, H-4″, 4‴), 4.08 (m, H-5″, 5‴), 1.46 (3H, d, J = 6.3 Hz, H_3-6″), 1.43 (3H, d, J = 6.3 Hz, H_3-6‴); ^{13}C NMR (CD_3OD) δ aglycone 131.0 (C-1), 131.2 (C-2, 6), 116.4 (C-3, 5), 157.1 (C-4), 36.6 (C-7), 72.4 (C-8), glucosyl 104.5 (C-1′), 76.0 (C-2′), 84.3 (C-3′), 70.7 (C-4′), 78.2 (C-5′), 63.0 (C-6′), rhamnosyl 103.5 (C-1′), 102.7 (C-1‴), 72.4 (C-2″), 72.7 (C-2‴), 73.2 (C-3″), 73.3 (C-3‴), 81.4 (C-4″), 74.2 (C-4‴), 68.8 (C-5″), 70.5 (C-5‴), 18.9 (C-6″), 18.2 (C-6‴); ESIMS *m*/*z* 591 $[M - H]^-$, 445 $[M - rha]^-$, 299 $[M - rha - O\text{-}rha]^-$; HRFABMS *m*/*z* found 593.3149 $[M + H]^+$ ($C_{26}H_{41}O_{15}$ requires 593.3165).

Ligurobustoside N (2): amorphous powder, $[\alpha]^{24}_D$ −109° (*c* 0.06, MeOH); UV λ_{max} (EtOH, log ϵ) 200 (4.11), 212 (4.00), 226 (3.81), 318 (4.19) nm; IR ν_{max} (KBr) cm^{-1} 3400, 2900, 1700, 1618, 1610, 1515, 1450, 1170, 820; ^1H NMR (CD_3OD) δ aglycone 7.27 (2H, d, J = 8.5 Hz, H_2-2, 6), 6.89 (2H, d, J = 8.5 Hz, H_2-3, 5), 3.04 (2H, t, J = 6.8 Hz, H_2-7), 3.87 (1H, m, Ha-8), 4.09 (1H, m, Hb-8), glucosyl 4.47 (1H, d, J = 7.9 Hz, H-1′), 5.13 (1H, t, J = 9.5 Hz, H-4′), rhamnosyl 5.39 (1H, d, J = 1.7 Hz, H-1″), 5.25 (1H, d, J = 1.5 Hz, H-1‴), 1.29 (3H, d, J = 6.3 Hz, H_3-6″), 1.25 (3H, d, J = 6.3 Hz, H_3-6‴), caffeoyl 7.26 (1H, d, J = 2.0 Hz, H-2⁗), 6.99 (1H, d, J = 8.1 Hz, H-5⁗), 7.17 (1H, dd, J = 2.0, 8.1 Hz, H-6⁗), 7.79 (1H, d, J = 15.9 Hz, H-7⁗), 6.45 (1H, d, J = 15.9 Hz, H-8⁗); ^{13}C NMR (CD_3OD) δ aglycone 131.0 (C-1), 131.2 (C-2, 6), 116.6 (C-3, 5), 157.1 (C-4), 36.6 (C-7), 72.6 (C-8), glucosyl 104.5 (C-1′), 76.3 (C-2′), 81.74 (C-3′), 70.6 (C-4′), 76.1 (C-5′), 62.6 (C-6′), rhamnosyl 103.7 (C-1″), 102.9 (C-1‴), 72.7 (C-2″), 74.0 (C-2‴), 70.8 (C-3″), 72.6 (C-3‴), 81.8 (C-4″), 74.2 (C-4‴), 69.2 (C-5″, 5‴),

19.5 (C-6″), 18.0 (C-6‴), caffeoyl 127.8 (C-1‴′), 115.6 (C-2‴′), 147.1 (C-3‴′), 150.2 (C-4‴′), 117.0 (C-5‴′), 123.7 (C-6‴′), 148.2 (C-7‴′), 115.0 (C-8‴′), 168.4 (CO); ESIMS *m/z* 753 $[M - H]^-$, 607 $[M - rha]^-$, 591 $[M - caf]^-$, 444 $[M - rha - caf]^-$; HRFABMS *m/z* found 755.3631 $[M + H]^+$ ($C_{35}H_{47}O_{18}$ requires 755.3624).

Ligurobustoside O (3): amorphous powder, $[\alpha]^{24}_D$ −77.3° (*c* 0.05, MeOH); UV λ_{max} (EtOH, log ϵ) 201 (4.32), 214 (4.67), 330 (4.15) nm; IR ν_{max} (KBr) cm^{-1} 3400, 2900, 1700, 1595, 1515, 1432, 1170, 825; ^1H NMR (CD_3OD) aglycone 4.46 (2H, d, J = 7.4 Hz, H_2-1), 5.58 (1H, t, J = 7.4 Hz, H_2-2), 2.33 (2H, m, H_2-4), 3.09 (2H, m, H_2-5), 6.05 (1H, d, J = 1.5 Hz, Ha-8), 6.28 (1H, d, J = 1.5 Hz, Hb-8), 2.05 (3H, s, H_3-9), 1.91 (3H, s, H_3-10), glucosyl 4.55 (1H, d, J = 7.9 Hz, H-1′), 5.11 (1H, t, J = 9.2 Hz, H-4′), rhamnosyl 5.39 (1H, d, J = 1.3 Hz, H-1″), 1.28 (3H, d, J = 6.0 Hz, H_3-6″), *p*-coumaroyl 7.66 (2H, d, J = 8.6 Hz, H-2‴, 6‴), 7.0 (1H, d, J = 8.6 Hz, H-3‴, 5‴), 7.86 (1H, d, J = 15.9 Hz, H-7‴), 6.53 (1H, d, J = 15.9 Hz, H-8‴); ^{13}C NMR (CD_3OD) δ aglycone 66.7 (C-1), 122.2 (C-2), 141.4 (C-3), 37.1 (C-4), 35.5 (C-5), 203.9 (C-6), 147.6 (C-7), 126.3 (C-8), 18.1 (C-9), 17.0 (C-10), glucosyl 103.4 (C-1′), 76.4 (C-2′), 81.9 (C-3′), 70.7 (C-4′), 76.5 (C-5′), 62.7 (C-6′), rhamnosyl 102.9 (C-1″), 72.4 (C-2″), 72.7 (C-3″), 74.1 (C-4″), 70.8 (C-5″), 18.9 (C-6″), *p*-coumaroyl 127.4 (C-1‴), 131.6 (C-2‴, 6‴), 117.2 (C-3‴, 5‴), 161.8 (C-4‴), 147.9 (C-7‴), 115.1 (C-8‴), 168.6 (CO); ESIMS *m/z* 621 $[M - H]^-$, 475 $[M - rha]^-$, 328 $[M - rha - cou]^-$; HRFABMS *m/z* found 623.3343 $[M + H]^+$ ($C_{31}H_{43}O_{13}$ requires 623.3336).

Hemolysis of Red Blood Cells (RBC). Rat blood from the abdominal aorta was collected. The RBC were separated from plasma by centrifugation at 2500 rpm for 10 min. The crude RBC were then washed three times with five volumes of phosphate-buffered saline (PBS, pH 7.4). The packed RBC were then suspended in four volumes of PBS solution.

Oxidative hemolysis in RBC induced by a peroxyl radical initiator, 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH), was chosen as a model for the peroxidative damage in biomembranes. Addition of AAPH to a suspension of RBC caused oxidation of lipids and proteins in cell membranes and thereby induced hemolysis. The AAPH-induced hemolysis in RBC is a function of incubation time and is proportional to the concentration of free radicals. The inhibitory effect against RBC hemolysis is also proportional to the concentration of antioxidants in the incubation mixture. Two milliliters of RBC suspension was mixed with 2 mL of PBS solution containing varying amounts of glycosides. One millliliter of 400 mM AAPH in PBS solution was then added to the mixture. The incubation mixture was shaken gently in a water bath at 37 °C for 3 h. After incubation, 8 mL of PBS solution was added into the reaction mixture followed by centrifugation at 3000 rpm for 10 min.

The absorbance (*A*) of the supernatant at 540 nm was recorded on a Shimadzu 1201 spectrophotometer. Percentage inhibition was calculated by the following equation: % inhibition = $[A_{ctl} - A_{sample}]/A_{ctl}$, in which A_{sample} is the absorbance of the sample containing glycoside and A_{ctl} is the absorbance of the control.

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