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Effects of Stilbene Constituents from Rhubarb on Nitric Oxide Production in Lipopolysaccharide-Activated Macrophages

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Abstract—Two new anthraquinone glucosides [chrysophanol 8-*O*-β-D-(6′-galloyl)-glucopyranoside, aloe-emodin 1-*O*-β-D-glucopyranoside] together with various known stilbenes and their glucosides, anthraquinone glucosides, and a naphthalene glucoside were isolated from the rhizome of *Rheum undulatum* L. Three stilbenes (rhapontigenin, piceatannol, resveratrol), a naphthalene glucoside (torachrysone 8-*O*-β-D-glucopyranoside), and two stilbene glucoside gallates (rhaponticin 2″-*O*-gallate, rhaponticin 6″-*O*-gallate) showed inhibitory activity of NO production in lipopolysaccharide-activated macrophages. (IC_{50} = 11–69 μM). The oxygen functions (-OH,-OCH_3) at the benzene ring were found to be essential to show the activity. Whereas, the glucoside moiety reduced the activity, while the α,β-double bond did not affect the activity. Furthermore, the active stilbenes (rhapontigenin, piceatannol, resveratrol) inhibited iNOS induction.

A Korean rhubarb, the rhizome of *Rheum undulatum* L., is used as the remedy for the blood stagnation syndrome ('Oketsu syndrome' in Japanese traditional medicine) as well as a purgative agent. This rhubarb is considered to have a less purgative effect but more potent effect on the Oketsu syndrome than other rhubarbs such as *R. palmatum*.[1] Previously, anti-allergic and anti-inflammatory effects of the hot water extract were reported as its anti-Oketsu effect.[1] However, its pharmacological property and bioactive constituents have not been studied sufficiently.

Nitric oxide (NO), an inorganic free radical, has been implicated in the physiological or pathological process, such as vasodilation, non-specific host defense, ischemia reperfusion injury, and chronic or acute inflammation. NO is produced by the oxidation of L-arginine by a NO synthase (NOS). In the family of NOS, especially inducible NOS (iNOS) is involved in the pathological aspect with overproduction of NO, and can be expressed in response to pro-inflammatory agents [interleukin-1β, tumor necrosis factor-α, lipopolysaccharide (LPS)] in various cells including macrophages, endothelial cells, and smooth muscle cells.[2] Inhibition of iNOS enzyme activity or the induction may have provided therapeutic benefits in various types of inflammation.[3]

In the course of our studies on the bioactive constituents from the traditional medicine, the methanolic extract from the dried rhizome of *R. undulatum* was found to show inhibitory activity on NO production in LPS-activated mouse peritoneal macrophages. Through bioassay-guided separation, we isolated ten known stilbene constituents from the active fraction together with a known naphthalene glucoside and two known and two new anthraquinone glucosides. This communication deals with the isolation and characterization of active constituents from the rhizome of *R. undulatum*, and structural requirements of active constituents for the activity. In addition, we examined the inhibitory effects of the principal active constituents on iNOS induction.

Materials and Methods

Isolation of chemical constituents from the rhizome of *R. undulatum*

The dried rhizome of *R. undulatum* (5.8 kg, cultivated in Korea) was extracted with methanol at room temperature (24 h, ×3). The methanolic extract (33.8% from the natural medicines) was subjected to Diaion HP-20 column chromatography (H_2O→MeOH→acetone) to give the H_2O-eluated fraction (13.3%), MeOH-eluated fraction (18.7%), and acetone-eluated fraction (1.8%). The active fraction (MeOH eluated fraction) was subjected to silica-gel column chromatography [$CHCl_3$:MeOH (10:1,

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v/v)→(4:1)→$CHCl_3$:MeOH:H_2O (10:3:1, lower layer)→MeOH] to give five fractions (fr.1–fr.5). Each fraction was subjected to ODS column chromatography (MeOH–H_2O) and finally HPLC [YMC-pack R&D ODS-5-A, 250×20 mm i.d., MeOH–H_2O or CH_3CN–H_2O] to give rhaponticin (**1**, 3.5%),[4] piceatannol 3′-*O*-β-D-glucopyranoside (**2**, 2.0%),[4] desoxyrhaponticin (**3**, 0.048%),[4] isorhapontin (**4**, 0.36%),[5] rhapontigenin (**5**, 0.58%),[4] piceatannol (**6**, 0.073%),[4] desoxyrhapontigenin (**7**, 0.015%),[6] resveratrol (**8**, 0.048%),[7] rhaponticin 2″-*O*-gallate (**9**, 0.12%),[4] rhaponticin 6″-*O*-gallate (**10**, 0.087%),[4] torachrysone 8-*O*-β-D-glucopyranoside (**19**, 0.12%),[8] chrysophanol 1-*O*-β-D-glucopyranoside (**21**, 0.25%),[9] chrysophanol 8-*O*-β-D-glucopyranoside (**22**, 0.16%),[9] and two new anthraquinone glucosides, chrysophanol 8-*O*-β-D-(6′-galloyl)-glucopyranoside (**23**, 0.092%)[10] and aloe-emodin 1-*O*-β-D-glucopyranoside (**25**, 0.0065%).[10] Related compounds **12**, **20** and **24** were derived by enzymatic hydrolysis of **4**, **21** and **25**; **13**–**16** by CH_3I-methylation of **1**, **2**, **5**, **8**; **17** and **18** by methanolysis of **13** and **14**; dihydrostilbenes (**1a**, **2a**, **5a**, **6a**, **8a**, **11a**) by hydrogenation of **1**, **2**, **5**, **6**, **8** and **11**.[11]

Chemical structures of chrysophanol 8-*O*-β-D-(6′-galloyl)-glucopyranoside (23) and aloe-emodin 1-*O*-β-D-glucopyranoside (25)

Chrysophanol 8-*O*-β-D-(6′-galloyl)-glucopyranoside (**23**), yellow needles (MeOH), mp 207–210 °C, $[\alpha]_D^{25}$ +95.0° (c = 0.1, MeOH), $C_{28}H_{24}O_{13}$, UV [MeOH, nm (log ε)]: 220 (4.58), 260 (4.32), 284 (sh, 4.17), 409 (3.79), IR (KBr, cm^{-1}): 3410, 2923, 1709, 1641, 1637, 1466, 1075, showed quasimolecular ion peaks at m/z 591 $(M+Na)^+$ in its positive-ion FAB–MS. The ^1H and ^{13}C NMR (DMSO-d_6) spectra of **23** showed signals due to a galloyl group [δ 6.99 (2H, s, galloyl-2″,6″-H)] and the glucosylanthraquinone (**22**) moiety. Comparison of the ^1H- and ^{13}C NMR data for **23** with those for **22** disclosed an acylation shift at the 6′-position [δ 4.27, 4.50 (1H each, both m, Glc-6′-H_2)]. Furthermore, the position of galloyl group in **23** was determined by the HMBC experiment, which showed long-range correlation between 6′-proton and 7″-carbon. On the basis of this evidence, **23** was characterized as 6′-galloyl-8-*O*-β-D-glucopyranosyl-chrysophanol.

Aloe-emodin 1-*O*-β-D-glucopyranoside (**25**), yellow needles (MeOH), mp 174–177 °C, $[\alpha]_D^{24}$ −56.4° (c = 0.1, MeOH), $C_{21}H_{20}O_{10}$, UV [MeOH, nm (log ε)]: 222 (4.55), 257 (4.37), 282 (sh, 4.06), 407 (3.84), IR (KBr, cm^{-1}): 3410, 2924, 1645, 1638, 1603, 1458, 1072, showed quasimolecular ion peaks at m/z 455 $(M+Na)^+$ in the positive-ion FAB–MS. The ^1H NMR (DMSO-d_6) spectrum of **25** indicated the presence of aromatic protons [δ 7.36 (1H, dd, J = 1.2, 8.2 Hz, 7-H), 7.62 (1H, d, J = 1.2 Hz, 2-H), 7.68 (1H, dd, J = 1.2, 7.6 Hz, 5-H), 7.75 (1H, dd, J = 7.6, 8.2 Hz, 6-H), 7.91 (1H, d, J = 1.2 Hz, 4-H)], hydroxymethyl protons [δ 4.65 (2H, s, 3-CH_2OH)], and an anomeric proton [δ 5.14 (1H, d, J = 7.6 Hz, Glc-1′-H)]. The carbon signals of the aglycone part in the ^{13}C NMR spectrum of **25** were superimposable on those of the aloe-emodin derivative,[12] except for the 1-position. In the HMBC experiment of **25**, long-range correlation was observed between 1′-proton and 1-carbon. Consequently, **25** was determined to be 1-*O*-β-D-glucopyranosyl-aloe-emodin.

Reagents

Lipopolysaccharide (LPS, from *Salmonella enteritidis*), N^G-monomethyl-L-arginine (L-NMMA), and *trans*-stilbene were purchased from Sigma; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Dojin, Japan; caffeic acid phenethylester (CAPE) and guanidinoethyldisulfide (GED) from Calbiochem; RPMI 1640 from Gibco; protease inhibitor cocktail (Complete Mini) from Boehringer Mannheim; fetal calf serum (FCS) from Bio Whittaker; nitrocellulose membrane (0.25 μm) from Bio Rad; 96-well microplate and culture dish (6 cm) from Nunc; anti-mouse iNOS antibody (monoclonal) from Transduction Laboratories; anti-mouse IgG antibody conjugated to horse radish peroxidase and the enhanced chemiluminescense (ECL) kit from Amersham; thioglycolate (TGC) medium from Nissui Seiyaku; and all other chemicals were from Wako.

Bioassay methods

Screening for NO production

Peritoneal exudate cells were collected from the peritoneal cavities of male ddY mice by washing with 6–7 mL of ice-cold PBS, and cells (5×10^5 cells/well) were suspended in 200 μL of RPMI 1640 supplemented 5% fetal calf serum (FCS), penicillin (100 units/mL) and streptomycin (100 μg/mL), and pre-cultured in a 96-well microplate at 37 °C in 5% CO_2 in air for 1 h. Nonadherent cells were removed by washing the cells with PBS, and the adherent cells (more than 95% macrophages using Giemza staining) were cultured in the fresh medium containing 10 μg/mL LPS and various concentrations of test compound for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite (NO_2^-) in the culture medium using Griess reagent.[13] Cytotoxicity was determined by MTT colorimetric assay. Namely, after 20-h incubation with test compounds, MTT (10 μL, 5 mg/mL in PBS) solution was added to the wells. After 4-h culture, the medium was removed, and isopropanol containing 0.04 N HCl was then added to dissolve the formazan produced in the cells. The optical density of the formazan solution was measured by microplate reader at 570 nm (reference: 655 nm). CAPE, L-NMMA, and GED were used for reference compounds. Each test compound was dissolved in DMSO, and the solution was added to the medium (final DMSO concentration was 0.5%). Inhibition (%) was calculated by the following formula and IC_{50} was determined graphically (N = 4).

$$\text{Inhibition (\%)} = \frac{A - B}{A - C} \times 100$$

$A \sim C : NO_2^-$ concentration (μM)

[A : LPS(+), Sample(−); B : LPS(+), Sample(+); C : LPS(−), Sample(−)]

Detection of iNOS

In this experiment, peritoneal exudate cells were obtained from the peritoneal cavities of male ddY mice that had been peritoneally injected with 4% TGC medium 4 days previously to ensure a large number of the cells. Cells (7.5×10^6 cells/3 mL/dish) were pre-cultured in a culture dish (6 cm i.d.) for 1 h, and the adherent cells (more than 95% macrophages) were collected as described above. After washing, culture medium was exchanged for fresh medium containing 5% FCS, 20 μg/ml LPS and test compound for 12 h. Cells were collected in the lysis buffer [100 mM NaCl, 10 mM Tris, Complete Mini (1 tab/10 mL), 0.1% Triton X-100, 2 mM EGTA] and sonicated. After determination of protein concentration of each suspension by the BCA method (BCA™ Protein Assay Kit, Pierce), the suspension was boiled in Laemmli buffer.[14] For SDS–PAGE, aliquots of 50 μg protein of each sample were subjected to electrophoresis in 10% polyacrylamide gel. Following electrophoresis, the proteins were electrically transferred onto a nitrocellulose membrane. The membrane were incubated with 5% nonfat dried milk in Tris buffered saline (TBS, 100 mM NaCl, 10 mM Tris, 0.1% Tween 20, pH 7.4) and probed with a mouse monoclonal IgG (dilution of 1:1000) against iNOS. The blots were washed in TBS and probed with secondary antibody, anti-mouse IgG antibody conjugated to horseradish peroxidase (dilution of 1 : 5000). Detection was performed using ECL kit and X-ray film (Hyper Film, Amersham).

Results

Effects on NO production

Nitrite, a NO product, was accumulated in the medium after 20-h incubation with LPS. Nitrite concentration in the medium without inhibitors (control group) was 28.3 ± 5.7 μM and that in without LPS (un-stimulated group) was 2.9 ± 2.1 μM (mean ± SD of 38 experiments). Reference compounds, CAPE (an inhibitor of nuclear factor κB activation),[15] L-NMMA (a non-selective inhibitor of NOS),[16] and GED (an inhibitor of iNOS)[17] potently inhibited the nitrite accumulation in the medium. The methanolic extract and methanol-eluated fraction showed the inhibition of nitrite accumulation more than the H_2O-eluated fraction and acetone-eluated fraction (Table 1).

Table 1. Inhibitory effects of MeOH extract, H_2O-eluated, MeOH-eluated, and acetone-eluated fractions from the rhizome of *R. undulatum* on NO production in LPS-activated mouse peritoneal macrophages

Compounds	μg/mL	Inhibition (%)	Compounds	IC_{50} (μM)
MeOH extract	100	23	CAPE	4.0
H_2O-eluated fraction	100	27	L-NMMA	28
MeOH-eluated fraction	100	51	GED	1.4
Acetone-eluated fraction	100	−4		

As shown in Table 2, three stilbenes [rhapontigenin (**5**), piceatannol (**6**), resveratrol (**8**)], two stilbene glucoside gallates [rhaponticin 2″-*O*-gallate (**9**), rhaponticin 6″-*O*-gallate (**10**)], and a naphthalene glucoside [torachrysone 8-*O*-β-D-glucopyranoside (**19**)] inhibited the LPS-induced NO production ($IC_{50}=11$–69 μM). Whereas, other stilbene constituents (**1**–**4**) and anthraquinone constituents (**20**–**23**, **25**) showed little activity (IC_{50} >100 μM). Related compounds (**5a**, **6a**, **8a**, **12**–**18**) also showed inhibitory activity ($IC_{50}=5.8$–76 μM), though *trans*-stilbene (**11**) and dihydrostilbene (**11a**) lacking the oxygen functions (-OCH_3 and-OH) showed little activity. Dihydrostilbene derivatives (**5a**, **6a**, **8a**) also showed equipotent activities as compared with their corresponding stilbenes, while the glucosides (**1a**, **2a**) did not. Permethylated stilbene glucosides (**13**, **14**) also showed the activity ($IC_{50}=5.8$, 27 μM). Furthermore, two gallates (**9**, **10**) showed more potent activity than **1** and **26**. Test compounds (100 μM) did not show the cytotoxicity using MTT asssay, except for desoxyrhapontigenin (**7**, 100 μM), **17** (100 μM), **18** (100 μM), and aloe-emodin (**24**, 30 μM).

Effects of stilbene constituents on iNOS induction

iNOS was detected at 130 kDa after 12 h incubation with LPS using SDS–PAGE-Western blotting method. Three principal stilbenes [rhapontigenin (**5**), piceatannol (**6**), resveratrol (**8**)] at 100 μM apparently inhibited iNOS induction, in consistent with above results, as shown in Figure 1.

Discussion

The results in the present study demonstrated that the extract of the rhizome of *R. undulatum* and three stilbenes [rhapontigenin (**5**), piceatannol (**6**), resveratrol (**8**)], two stilbene glucoside gallates [rhaponticin 2″-*O*-gallate (**9**), rhaponticin 6″-*O*-gallate (**10**)], and a naphthalene glucoside [torachrysone 8-*O*-β-D-glucopyranoside (**19**)] inhibited the LPS-induced NO production ($IC_{50}=11$–69 μM). Whereas, other stilbene and anthraquinone constituents showed little activity (IC_{50} > 100 μM). Related compounds (**5a**, **6a**, **8a**, **12**–**18**) also showed inhibitory activity, except for *trans*-stilbene (**11**) and **11a** lacking oxygen functions (-OH and-OCH_3) and two dihydrostilbene glucosides (**1a**, **2a**). Dihydrostilbene derivatives (**5a**, **6a**, **8a**) having the oxygen functions showed equipotent activities as compared with their corresponding stilbenes. Permethylated stilbene glucosides (**13**, **14**) also show the activity. These results indicate the following structural requirements of stilbenes for the activity: (1) the oxygen functions (-OH,-OCH_3)

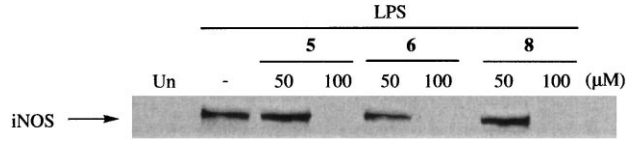
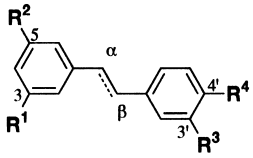
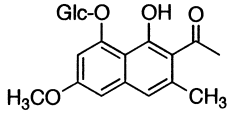


Figure 1. Effects of stilbenes (**5**, **6**, **8**) on iNOS induction in LPS-activated macrophages. Un: unstimulation.

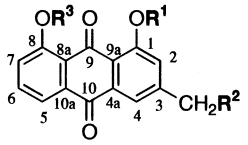
Table 2. Effects of stilbene, naphthalene, and anthraquinone constituents from the rhizome of *R. undulatum* and related compounds on NO production in LPS-activated mouse peritoneal macrophages

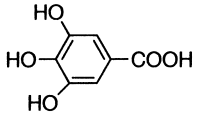


	α–β	R^1	R^2	R^3	R^4	IC_{50} (μM)
Rhaponticin (**1**)	C=C	O-Glc	OH	OH	OCH_3	>100 (25)
1a	C–C	O-Glc	OH	OH	OCH_3	>100 (8)
Piceatannol 3′-*O*-Glc (**2**)	C=C	OH	OH	O-Glc	OH	>100 (10)
2a	C–C	OH	OH	O-Glc	OH	>100 (6)
Desoxythaponticin (**3**)	C=C	O-Glc	OH	H	OCH_3	>100 (13)
Isorhapontin (**4**)	C=C	O-Glc	OH	OCH_3	OH	>100 (5)
Rhapontigenin (**5**)	C=C	OH	OH	OH	OCH_3	48
5a	C–C	OH	OH	OH	OCH_3	49
Piceatannol (**6**)	C=C	OH	OH	OH	OH	23
6a	C=C	OH	OH	OH	OH	32
Desoxyrhapontigenin (**7**)	C=C	OH	OH	H	OCH_3	>30 (13)[#]
Resveratrol (**8**)	C=C	OH	OH	H	OH	68
8a	C–C	OH	OH	H	OH	76
Rhaponticin 2″-*O*-gallate (**9**)	C=C	O-Glc(2-gallate)	OH	OH	OCH_3	13
Rhaponticin 6″-*O*-gallate (**10**)	C=C	O-Glc(6-gallate)	OH	OH	OCH_3	11
trans-Stilbene (**11**)	C=C	H	H	H	H	>100 (21)
11a	C–C	H	H	H	H	>100 (26)
12	C=C	OH	OH	OCH_3	OH	63
13	C=C	O-Glc$(CH_3)_4$	OCH_3	OCH_3	OCH_3	5.8
14	C=C	OCH_3	OCH_3	O-Glc$(CH_3)_4$	OCH_3	27
15	C=C	OCH_3	OCH_3	OCH_3	OCH_3	28
16	C=C	OCH_3	OCH_3	H	OCH_3	22
17	C=C	OH	OCH_3	OCH_3	OCH_3	19[#]
18	C=C	OCH_3	OCH_3	OH	OCH_3	23[#]



torachrysone 8-*O*-Glc (**19**) : IC_{50} (μM) = 69





gallic acid (**26**) : IC_{50} (μM) = 63

	R^1	R^2	R^3	IC_{50} (μM)
Chrysophanol (**20**):	H	H	H	>100 (2)
Chrysophanol 1-*O*-Glc (**21**):	Glc	H	H	>100 (20)
Chrysophanol 8-*O*-Glc (**22**):	H	H	Glc	>100 (31)
Chrysophanol 8-*O*-(6′-galloyl)Glc (**23**):	H	H	Glc(6-gallate)	>100 (31)
Aloe-emodin (**24**):	H	OH	H	>10 (38)[##]
Aloe-emodin 1-*O*-Glc (**25**):	Glc	OH	H	>100 (46)

Glc: β-D-Glucopyranosyl.
( ): Values in parentheses represent the inhibtion (%) at 10 or 100 μM.
#, ##: **7, 17, 18** (100 μM) and **24** (30 μM) showed cytotoxicity.

at the benzene rings are essential for the activity; (2) the glucoside moiety reduced the activity; (3) the α,β-double bond did not affect the activity; (4) the galloyl ester and permethylated glucoside tended to potentiate the activity.

As to possible mechanisms of active stilbene constituents, we examined the effects of stilbenes on iNOS induction using three active stilbenes [rhapontigenin (**5**), piceatannol (**6**), and resveratrol (**8**)], since iNOS is responsible for the high-output production of NO by various cells after exposure to LPS. As a result, the stilbenes (**5**, **6**, **8**) inhibited iNOS induction. These results suggest that active stilbenes may, at least in part, inhibit the pathway from stimulation by LPS to iNOS induction, thereby preventing NO production.

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10. The structures of new compounds, **23** and **25**, were elucidated on the basis of chemical and physiochemical evidence. The full characteristics will be presented in our full paper. **23**: High-resolution positive-ion FAB–MS: calcd for $C_{28}H_{24}O_{13}Na$ $(M+Na)^+$: 591.1114, found: 591.1168, ^1H NMR (500 MHz, DMSO-d_6, δ): 2.40 (3H, s, 3-C$\underline{H_3}$), 4.27, 4.50 (1H each, both m, Glc-6′-H_2), 5.24 (1H, d, $J=7.7$, Glc-1′-H), 6.99 (2H, s, galloyl-2″,6″-H), 7.16 (1H, br s, 4-H), 7.46 (1H, br s, 2-H), 7.67 (1H, d, $J=8.6$ Hz, 5-H), 7.72 (1H, dd, $J=7.6$, 8.6 Hz, 6-H), 7.82 (1H, d, $J=7.6$ Hz, 7-H), 12.82 (1H, br s, 1-OH), ^{13}C NMR (125 MHz, DMSO-d_6, δc): 161.5 (C-1), 119.3 (C-2), 147.5 (C-3), 123.9 (C-4), 122.2 (C-5), 138.5 (C-6), 120.5 (C-7), 157.8 (C-8), 187.4 (C-9), 181.9 (C-10), 132.0 (C-4a), 119.3 (C-8a), 114.6 (C-9a), 134.7 (C-10a), 21.4 (3-CH_3), 100.2 (Glc-1′), 73.2 (Glc-2′), 76.3 (Glc-3′), 69.7 (Glc-4′), 74.0 (Glc-5′), 63.3 (Glc-6′), 119.2 (galloyl-1″), 108.6 (galloyl-2″, 6″), 145.5 (galloyl-3″, 5″), 135.6 (galloyl-4″), 165.1 (galloyl-7″), Negative-ion FAB–MS: m/z 567 $(M-H)^-$. **25**: High-resolution positive-ion FAB–MS: calcd for $C_{21}H_{20}O_{10}Na$ $(M+Na)^+$: 455.0954, found: 455.0952, ^1H NMR (500 MHz, DMSO-d_6, δ) : 4.65 (2H, s, 3-C$\underline{H_2}$OH), 5.14 (1H, d, $J=7.6$ Hz, Glc-1′-H), 7.36 (1H, dd, $J=1.2$, 8.2 Hz, 7-H), 7.62 (1H, d, $J=1.2$ Hz, 2-H), 7.68 (1H, dd, $J=1.2$, 7.6 Hz, 5-H), 7.75 (1H, dd, $J=7.6$, 8.2 Hz, 6-H), 7.91 (1H, d, $J=1.2$ Hz, 4-H), 12.90 (1H, br s, 8-OH), ^{13}C NMR (125 MHz, DMSO-d_6, δc) : 158.3 (C-1), 119.2 (C-2), 151.8 (C-3), 118.0 (C-4), 118.3 (C-5), 136.1 (C-6), 124.2 (C-7), 161.3 (C-8), 187.6 (C-9), 182.1 (C-10), 134.5 (C-4a), 116.7 (C-8a), 119.0 (C-9a), 132.4 (C-10a), 62.1 (3-CH_2OH), 100.5 (Glc-1′), 73.2 (Glc-2′), 76.5 (Glc-3′), 69.3 (Glc-4′), 77.2 (Glc-5′), 60.4 (Glc-6′), Nagative-ion FAB–MS : m/z 431 $(M-H)^-$. Known compounds were identified by comparison of their physical data with those of authentic samples (**1**, **6**, **8**, **20**, **24**) or with reported value.[6–9]
11. The details will be presented in our full paper.
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