Chemical Constituents of *Morinda citrifolia* Roots Exhibit Hypoglycemic Effects in Streptozotocin-Induced Diabetic Mice

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The hypoglycemic effects of the chemical constituents of *Morinda citrifolia* roots was evaluated in streptozotocin (STZ)-induced diabetic mice. The CHCl_3 , EtOAc, *n*-BuOH and H_2O soluble phases of the MeOH extract of *M. citrifolia* roots were administrated orally to STZ-induced diabetic mice. Only the *n*-BuOH soluble phase showed a significant reduction of the blood glucose levels. From the biologically active *n*-BuOH soluble phase, two iridoids and three anthraquinones were isolated as main constituents. These compounds were identified by spectroscopic analysis to be deacetylasperulosidic acid (1), asperulosidic acid (2), damnacanthol-3-*O*- β -D-primeveroside (3), lucidin 3-*O*- β -D-primeveroside (4) and morindone-6-*O*- β -D-primeveroside (5). 3 and 4 exhibited the hypoglycemic effects, which were anthraquinones with no substituents in one aromatic ring.

Key words *Morinda citrifolia*; streptozotocin; hypoglycemic effect; anthraquinone glycoside

Morinda citrifolia L. (Rubiaceae), known as “noni,” is a small tree that grows widely across Polynesia. In Japan, it is called “Yaeyama-aoki” and grows in Okinawa Prefecture. In Polynesia, *M. citrifolia* has traditionally been used for antibacterial, antiviral, antifungal, antitumor, antihelmin, analgesic, hypotensive, anti-inflammatory, and immune enhancing effects.¹⁾ Furthermore, “noni juice,” which is made from the fruit of this plant, is today widely drunk for its purported ability to prevent lifestyle-related diseases such as diabetes, hypertension, and cardiopathy and cerebral apoplexy caused by arteriosclerosis. In our previous study, six lignanes which inhibit the LDL oxidation process leading to arteriosclerosis were isolated from the fruit of *M. citrifolia*.²⁾ Other pharmacological studies of *M. citrifolia* reported that iridoids isolated from the leaves of this plant inhibited UVB-induced Activator Protein-1 activity³⁾ and the fruit of this plant prolonged the life span of C57BL/6 mice infected with Lewis lung carcinoma.⁴⁾ The above reports suggest that *M. citrifolia* leaves and fruits exhibit some anticancer effects. In addition, *M. citrifolia* root exhibited an analgesic effect.⁵⁾

During the course of our search for natural products capable of preventing lifestyle-related diseases, we have investigated the ability of the chemical constituents of *M. citrifolia* roots to improve hyperglycemia or diabetes. In this study, we used streptozotocin (STZ)-induced diabetic mice, which is known as an insulin-deficiency model, to demonstrate the effects of *M. citrifolia* roots on blood glucose levels.

MATERIALS AND METHODS

Plant Material The roots of *M. citrifolia* were collected at Okinawa in August 2004. A voucher specimen has been retained in our laboratory.

Animals Male ddY mice weighing *ca.* 30 g (5 weeks old) were obtained from SLC (Osaka, Japan). The animals were housed at a temperature of 23–24 °C with a 12 h light–dark cycle (lights on 8:00 a.m. to 8:00 p.m.). Food and water were available *ad libitum*. The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, adopted by the Japan-

ese Pharmacological Society.

Streptozotocin (STZ)-Induced Diabetic Model Mice were injected intraperitoneally (i.p.) with 200 mg/kg STZ (Sigma-Aldrich, St. Louis, MO, U.S.A.) prepared in 0.1 N citrate buffer adjusted to pH 4.3. Only mice with plasma glucose levels greater than 400 mg/dl were considered as diabetic.

Measurement of Blood Glucose Levels The plasma glucose levels in the mice were measured using the Glucose Pilot (Aventir Biotech, CA, U.S.A.) in blood samples obtained from tail vein at 0 or 5 h after a single administration of MeOH extract and CHCl_3 , EtOAc, *n*-BuOH and H_2O soluble phases of *M. citrifolia* roots (each at 3 g/kg, oral route, *p.o.*).

General Procedures Optical rotations were measured using a Jasco DIP-1000 digital polarimeter. IR and UV spectra were measured on a Shimadzu FT-IR 8300 infrared spectrometer and a Hitachi U-3000 spectrometer, respectively. NMR spectra were recorded in CD_3OD and $\text{DMSO}-d_6$ on a Bruker DPX-400 instrument. TLC was performed on Merck precoated TLC plates (Kieselgel 60F₂₅₄ and Rp-18F₂₅₄). Column chromatography was conducted with Kieselgel 60 (70–230 mesh, Merck) and Sephadex LH-20 (Pharmacia). Medium pressure liquid chromatography (MPLC, BUCHI Pump Module C-605 and Manager C-615) was carried out on a CIG column.

Extraction and Isolation The dried roots of *M. citrifolia* (4.7 kg) were extracted with MeOH (51×10) for 6 h at 60 °C. The combined MeOH was evaporated under reduced pressure to give the MeOH extract (705 g), which was suspended in H_2O :MeOH (3:1, 3.6 l) and then was sequentially partitioned with CHCl_3 , EtOAc and *n*-BuOH (each 3.6 l×3). Each solvent was removed under reduced pressure to yield CHCl_3 soluble phase (123 g), EtOAc soluble phase (13 g), *n*-BuOH soluble phase (149 g) and H_2O soluble phase (420 g). The *n*-BuOH soluble phase was subject to column chromatography on Sephadex LH-20 (MeOH) to give fractions 1–4. Fraction 2 was subjected repeatedly to further column chromatography on SiO_2 (EtOAc–MeOH– H_2O solvent system) and Rp-18 (H_2O :MeOH=70:30) to furnish com-

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pounds **1**—**5**.

HPLC Analysis HPLC was performed with an ODS column (Atlantis dC₁₈ 5 μ m, 4.6 $\times$ 250 mm, Waters). The detection wavelength was 254 nm. Elution was carried out with MeOH–H₂O containing 0.1% phosphoric acid (47.5 : 52.5) at a flow rate of 1 ml/min. Injection volume was 10 μ l (1.0 mg/ml, *n*-BuOH soluble phase in MeOH).

Compound 1 (Deacetylasperulosidic Acid): Colorless amorphous powder, $[\alpha]_D^{23} + 22.4^\circ$ ($c=0.13$, MeOH); IR $\nu_{\max}^{\text{KBr}}$ cm⁻¹: 3373, 2928, 1688, 1680, 1634, 1420, 1265, 1200, 1157, 1078, 1049, 991; UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 234 (3.88), 200 (3.72); ¹H-NMR (400 MHz, CD₃OD) and ¹³C-NMR (100 MHz, CD₃OD): Table 1.

Compound 2 (Asperulosidic Acid): Colorless amorphous powder, $[\alpha]_D^{23} + 21.4^\circ$ ($c=0.14$, MeOH); IR $\nu_{\max}^{\text{KBr}}$ cm⁻¹: 3400, 2929, 1720, 1690, 1635, 1369, 1252, 1200, 1158, 1075, 1047, 949; UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 233 (3.80), 201 (3.86); ¹H-NMR (400 MHz, CD₃OD) and ¹³C-NMR (100 MHz, CD₃OD): Table 1.

Compound 3 (Damnacanthol-3-*O*- β -D-primeveroside): Yellow needles, $[\alpha]_D^{23} - 128.62^\circ$ ($c=0.13$, MeOH/H₂O = 1 : 1); IR $\nu_{\max}^{\text{KBr}}$ cm⁻¹: 3380, 2883, 1672, 1580, 1452, 1410, 1327, 1300, 1283, 1070, 1035, 991; UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 266 (4.51), 201 (4.40); ¹H-NMR (400 MHz, CD₃OD) and ¹³C-NMR (100 MHz, CD₃OD): Table 2.

Compound 4 (Lucidin 3-*O*- β -D-primeveroside): Yellow needles, $[\alpha]_D^{23} - 118.9^\circ$ ($c=0.11$, MeOH/H₂O = 1 : 1); IR $\nu_{\max}^{\text{KBr}}$ cm⁻¹: 3379, 2891, 1672, 1632, 1591, 1483, 1420, 1369,

1333, 1296, 1075, 1043; UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 266 (4.45), 246 (4.42), 240 (4.40), 204 (4.52); ¹H-NMR (400 MHz, CD₃OD) and ¹³C-NMR (100 MHz, CD₃OD): Table 2.

Compound 5 (Morindone-6-*O*- β -D-primeveroside): Yellow

Table 1. ¹H- and ¹³C-NMR Spectral Data for Compounds **1** and **2** in CD₃OD

No.	1		2	
	¹³ C	¹ H	¹³ C	¹ H
1	101.48	5.06 (d, 9.0)	101.20	5.06 (d, 9.0)
3	155.40	7.66 (d, 0.9)	155.47	7.67 (br s)
4	108.46		108.04	
5	42.70	3.01 (ddd, 7.1, 5.8, 0.9)	42.24	3.02 (br t, 6.4)
6	75.28	4.82 (dd, 5.8, 1.6)	72.73	3.28 (d, 8.7)
7	129.89	6.02 (d, 1.6)	131.78	6.02 (br s)
8	151.42		145.72	
9	45.81	2.56 (dd, 9.0, 7.1)	46.07	2.63 (br t, 8.2)
10	61.67	4.46 (br d, 15.6)	63.67	4.95 (br d, 15.1)
		4.21 (br d, 15.6)		4.83 (br d, 15.1)
11	170.86		170.60	
COCH ₃			20.78	2.10 (s)
COCH ₃			172.55	
1'	100.40	4.73 (d, 7.9)	100.42	4.75 (d, 7.8)
2'	74.92		75.23	
3'	78.41		78.21	
4'	71.58	3.23—3.87	71.35	3.25—3.88
5'	77.75		77.63	
6'	62.79		62.81	

Coupling patterns and coupling constants (*J*) in Hz are given in parentheses.

Table 2. ¹H- and ¹³C-NMR Spectral Data for Compounds **3**—**5** in DMSO-*d*₆

No.	3		4		5	
	¹³ C	¹ H	¹³ C	¹ H	¹³ C	¹ H
1	160.74		161.88		160.18	
1-OH				12.92 (s)		12.92 (s)
2	131.64		123.66		135.07	
3	160.68		162.03		137.03	7.50 (d, 7.8)
4	109.19	7.75 (s)	106.43	7.37 (s)	118.60	7.45 (d, 7.8)
4a	135.75		133.65		130.54	
5	126.30	8.14 (dd, 7.5, 1.3)	126.88	8.06 (m)	152.06	
5-OH						12.70 (s)
6	133.65	7.86 (td, 7.5, 1.3)	134.87	7.84 (m)	151.64	
7	134.70	7.91 (td, 7.5, 1.3)	134.73	7.84 (m)	120.52	7.54 (d, 8.6)
8	126.72	8.16 (dd, 7.5, 1.3)	126.46	8.06 (m)	120.52	7.64 (d, 8.6)
8a	134.36		132.59		125.16	
9	180.43		186.95		186.77	
9a	120.53		111.30		114.53	
10	182.17		181.32		187.35	
10a	132.06		132.74		115.83	
11	51.99	4.68 (dd, 11.2, 6.4)	51.03	4.61 (dd, 11.2, 5.7)	15.74	2.18 (s)
		4.61 (dd, 11.2, 5.8)		4.52 (dd, 11.2, 4.8)		
OCH ₃	62.74	3.89 (s)				
1'	101.00	5.13 (d, 7.2)	100.89	5.09 (d, 6.9)	99.76	5.08 (d, 7.2)
2'	73.29		73.33		73.05	
3'	75.91		75.96		76.50	
4'	69.53	3.01—4.01	69.57	2.99—3.99	69.60	2.93—4.00
5'	76.43		76.48		76.64	
6'	68.06		68.07		68.38	
1''	104.09	4.18 (d, 7.3)	104.13	4.17 (d, 7.3)	104.05	4.15 (d, 7.4)
2''	73.33		73.37		73.48	
3''	75.74		75.77		76.08	
4''	69.24	3.01—4.01	69.21	2.99—3.99	69.53	2.93—4.00
5''	65.66		65.71		65.61	

Coupling patterns and coupling constants (*J*) in Hz are given in parentheses.

needles, $[\alpha]_D^{23} -71.3^\circ$ ($c=0.3$, MeOH/H₂O=1:1); IR $\nu_{\max}^{\text{KBr}}$ cm⁻¹: 3350, 1628, 1603, 1450, 1423, 1371, 1261, 1246, 1057, 991; UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 260 (4.56), 230 (4.61); ¹H-NMR (400 MHz, CD₃OD) and ¹³C-NMR (100 MHz, CD₃OD): Table 2.

Statistical Analysis Data is presented as the mean with S.E.M. The statistical significance was assessed with a one way analysis of variance followed by the paired Student's *t*-test. The differences were regarded as significant when the *p* value was less than 0.05.

RESULTS AND DISCUSSION

The effects of the MeOH extract and CHCl₃, EtOAc, *n*-BuOH and H₂O soluble phases of *M. citrifolia* roots (each at 3 g/kg, *p.o.*) on blood glucose levels in STZ-induced diabetic mice are shown in Fig. 1. Only the *n*-BuOH soluble phase showed a significant reduction of the blood glucose levels at 5 h after administration (303.9 ± 25.6 mg/dl) relative to those at 0 h after administration (455.9 ± 17 mg/dl), while the MeOH extract and other soluble phases did not exhibit any hypoglycemic effect. As shown in Fig. 2, the dose-response curve of the hypoglycemic effect of the *n*-BuOH soluble phase in STZ-induced diabetic mice had a bell-shape with a maximum response at 1.0 g/kg, which decreased for doses over 3.0 g/kg. To elucidate the constituents of the biologically active *n*-BuOH soluble phase, HPLC analysis was performed

Fig. 1. Effects of the MeOH Extract and Each Soluble Phase of *M. citrifolia* Roots on Blood Glucose Levels in STZ-Induced Diabetic Mice

The blood glucose levels were measured at 0 h (□) or 5 h (■) after administration of each sample (3 g/kg, *p.o.*) as described in Materials and Methods. **p*<0.05 vs. 0 h. Control (N); non-treated normal mice; Control (D); STZ-induced diabetic mice.

Fig. 2. The Dose-Dependent Hypoglycemic Effect of *n*-BuOH Soluble Phase of *M. citrifolia* Roots in STZ-Induced Diabetic Mice

The blood glucose levels were measured at 0 h (□) or 5 h (■) after administration of the *n*-BuOH soluble phase (0, 0.1, 0.3, 1, 3, 10 g/kg, *p.o.*) as described in Materials and Methods. ***p*<0.01 vs. 0 h.

(Fig. 3), revealing five main peaks (compounds 1–5). Compounds 1–5 were isolated by repeated column chromatography on Sephadex LH-20, SiO₂ and Rp-18. Compounds 1 and 2 showed brilliant blue spots on TLC upon heating with 10% sulfuric acid, indicating them to be iridoid compounds. The chemical structures of 1 and 2 were confirmed to be deacetyl-asperulosidic acid and asperulosidic acid, respectively, by the comparison with published spectroscopic data. These compounds had previously been isolated from the fruits of the same plant.⁶ Compounds 3, 4 and 5 were obtained as yellow needles and turned red on treatment with ammonia gas, indicating them to be anthraquinone compounds. The chemical structures of 3, 4 and 5 were identified as damnacanthol-3-*O*-β-D-primeveroside,⁷ lucidin 3-*O*-β-D-primeveroside (ophiohayatone-B)⁸ and morindone-6-*O*-β-D-primeveroside,⁹ respectively, by the comparison with published spectroscopic data.

Two iridoids and three anthraquinones were isolated from the biologically active *n*-BuOH soluble phase. The yields of 1, 2, 3, 4 and 5 were 0.18, 0.49, 0.27, 0.20 and 0.14% from the dried root, respectively. These compounds were tested for hypoglycemic effect in STZ-induced diabetic mice. Figure 5 shows the blood glucose levels 0 and 5 h after a single administration of each sample (100 mg/kg, *p.o.*). Although the iridoids did not show a hypoglycemic effect, the two anthraquinones, 3 and 4, showed a significant reduction of the blood glucose levels at 5 h after administration. However, another anthraquinone, 5 did not affect the blood glucose lev-

Fig. 3. HPLC Profile of *n*-BuOH Soluble Phase of *M. citrifolia* Roots

1; deacetylasperulosidic acid, 2; asperulosidic acid, 3; damnacanthol-3-*O*-β-D-primeveroside, 4; lucidin 3-*O*-β-D-primeveroside, 5; morindone-6-*O*-β-D-primeveroside.

Fig. 4. Chemical Structures of Compounds 1–5 Isolated from *n*-BuOH Soluble Phase of *M. citrifolia* Roots

Fig. 5. The Hypoglycemic Effect of Compounds 1–5 in STZ-Induced Diabetic Mice

The blood glucose levels were measured at 0 h (□) or 5 h (■) after administration of compounds 1–5 (each 100 mg/kg, *p.o.*) as described in Materials and Methods. * $p < 0.05$ vs. 0 h.

els. These finding indicate that the hypoglycemic effect of *M. citrifolia* roots in STZ-induced diabetic mice is due to the presence of anthraquinones. In particular, the anthraquinones that have no substituents on one aromatic ring seem to play an important role in the hypoglycemic effect of *M. citrifolia*,

although the detailed mechanism of their hypoglycemic effect remains to be determined.

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