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IRIDOID GLYCOSIDES FROM *Morinda tomentosa* AND THEIR ENDOPLASMIC RETICULUM STRESS MODULATION ACTIVITY

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Abstract

Three iridoids **1** - **3**, asperulosidic acid, daphylloside, and asperuloside, were isolated from the methanol extract of the leaves of *Morinda tomentosa*. Their chemical structures were elucidated by 1D- and 2D-NMR spectra and in comparison with those reported in the literature. The effects of these compounds on the endoplasmic reticulum stress in XBP1-eGFP-transfected the 293 T cells were measured. Compound **3** significantly reduced the ER-stress both in DMSO-treated and thapsigargin-treated cells. Unlike this compound, compound **3** selectively reduced thapsigargin-induced ER-stress without any effect on the level of XBP1 splicing in DMSO-treated cells. These results suggested that compounds **2** and **3** can be suggested as new ER stress regulators.

Keywords. *Morinda tomentosa*, iridoid, endoplasmic reticulum.

1. INTRODUCTION

The endoplasmic reticulum (ER) is a type of organelle in the cells of eukaryotic organisms that forms an interconnected network of flattened, membrane-enclosed sacs or tubes known as cisternae. Physiological or pathological processes that disturb protein folding in the ER cause the ER stress, which is associated with a wide range of diseases including hypoxia, reperfusion injury, neurodegeneration, heart disease, and diabetes [1]. The ER stress thereby is a probable instigator of pathological cell death and dysfunction. The recent studies suggested that the ER stress elevation may represent a proximal cause of the relation between obesity, hepatic, adipocyte insulin resistance, and type 2 diabetes [2, 3]. Additionally, neurons are sensitive to protein aggregates that the ER stress is involved in neurodegenerative diseases [4, 5]. On the other hand, hepatocytes have a well-developed ER structure that is essential for the vigorous synthesis of secretory proteins, and the ER stress has been reported to be involved in liver-related diseases [6]. Therefore, molecules that regulate the ER stress response would be potential candidates for drug targets in various related diseases.

Morinda tomentosa Heyne ex Roth., a small tree, (Rubiaceae), is commonly found in Vietnam. Its roots, barks, stems, leaves, and fruits have been used in folk medicine for the treatments of diabetes, hypertension, and cancer. A recent study showed the antimicrobial activities of *M. tomentosa* extracts which was highly sensitive with human pathogens, such as *Staphylococcus aureus* and *K. pnemoniae* [7]. In our bioassay screening for the ER stress activity from Vietnamese medicinal plants, the methanol extract of the leaves of *M. tomentosa* was found to reduce tathapsigargin-induced the ER stress in XBP1-eGFP-transfected 293 T cells. To find out the bioactive compounds from this plant, three iridoid glycosides were isolated.

2. MATERIAL AND METHODS

2.1. Plant materials

The leaves of *M. tomentosa* were collected in Tam Dao, Vinh Phuc, Vietnam in June, 2010, and identified by one of the authors, Dr. Ninh Khac Ban. A voucher specimen (MT1006) was deposited at the Herbarium of Institute of Marine Biochemistry.

2.2. General experimental procedures

Chemical shifts are reported in parts per million from TMS. All NMR spectra were recorded on a Bruker AM500 FT-NMR spectrometer (500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR). NMR measurements, including ^{1}H-NMR, ^{13}C-NMR, HSQC, HMBC, experiments, were carried out using 5-mm probe in CD_3OD solutions, TMS as the

internal standard. Optical rotations were determined on a Jasco DIP-1000 polarimeter. Column chromatography was performed using a silica-gel (230 - 400 mesh, Merck) or RP-18 resins (30 - 50 μm), thin layer chromatography using a pre-coated silica-gel 60 F_{254} (0.25 mm) and RP-18 $F_{254}S$ plates (0.25 mm, Merck).

2.3. Extraction and Isolation

The dried leaves of *M. tomentosa* (3.0 kg) were extracted with MeOH three times to yield 226 g of a dark solid extract, which was then suspended in water and successively partitioned with *n*-hexane and EtOAc to obtain *n*-hexane (MT1, 70.0 g), EtOAc (MT2, 37.0 g), and water (MT3, 115.0 g) extracts after removal solvent *in vacuo*. The water soluble fraction (MT3, 115.0 g) was chromatographed on a Diaion HP-20P column eluting with water containing increasing concentrations of MeOH (0, 25, 50, 75, and 100 %) to obtain five sub-fractions MT3A (40.0 g), MT3B (15.0 g), MT3C (32.0 g), MT3D (12.0 g), and MT3E (15.0 g). MT3C fraction (32.0 g) was chromatographed on a silica gel column eluting with $CHCl_3$–MeOH–water (5:1:0.1, v/v/v) to give three smaller fractions, MT3C1-MT3C3. MT3C1 fraction was chromatographed on a silica gel column eluting with $CHCl_3$–acetone–water (1:2:0.1, v/v/v) to yield **3** (7.0 mg). MT3C3 fraction was chromatographed on an RP-18 column eluting with acetone–water (1:2, v/v) to yield **1** (8.0 mg) and **2** (15.0 mg).

Asperulosidic acid (1): White amorphous powder, $[\alpha]_D^{25}$: +30.0 (*c* = 0.1, MeOH), ESI-MS *m/z* 433 $[M+H]^+$, $C_{18}H_{24}O_{12}$, ^{1}H- and ^{13}C-NMR (CD_3OD), see table 1.

Daphylloside (2): White amorphous powder, $[\alpha]_D^{25}$: -40.0 (*c* = 0.1, MeOH), ESI-MS *m/z* 469 $[M+Na]^+$, $C_{19}H_{26}O_{12}$, ^{1}H- and ^{13}C-NMR (CD_3OD), see table 1.

Asperuloside (3): White amorphous powder, $[\alpha]_D^{25}$: -56.0 (*c* = 0.1, MeOH), ESI-MS *m/z* 437 $[M+Na]^+$, $C_{18}H_{22}O_{11}$, ^{1}H- and ^{13}C-NMR (CD_3OD), see table 1.

2.4. Endoplasmic reticulum assay

To assess whether compounds isolated from the leaves of *M. tomentosa* can modulate ER stress, we assessed the splicing of XBP1, the target of ER stress sensor kinase IRE1*α*, which was well-known to be activated by ER stressed. We used the 293T human embryonic kidney cell line, and transfected them with XBP1-venus DNA (kindly provided by Arthur Kaser, Innsbruck Medical University, Austria) by lipofectamine 2000 (Invitrogen) following manufacturer's instructions. To induce the ER stress in the 293 T cell line, DMSO as negative control or thapsigargin (200nM) were added after plating cells in 96-well plate ($2x10^4$ cells/200 μL/well) and then the layers (5.0 mg/mL), or a positive control, salubrinal (purity > 98% by HPLC, Enzo life science) or compounds (5.0 μM) were added, respectively. After 24 h incubation at 37 ^{0}C, 5 % CO_2, cells were lysed in digitonin buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM EGTA and 10 μM digitonin) at room temperature for 30 min. We used the GEMINI EM (Molecular Device) to measure fluorescence of the spliced XBP1 (emission at 520 nm; excitation at 485 nm).

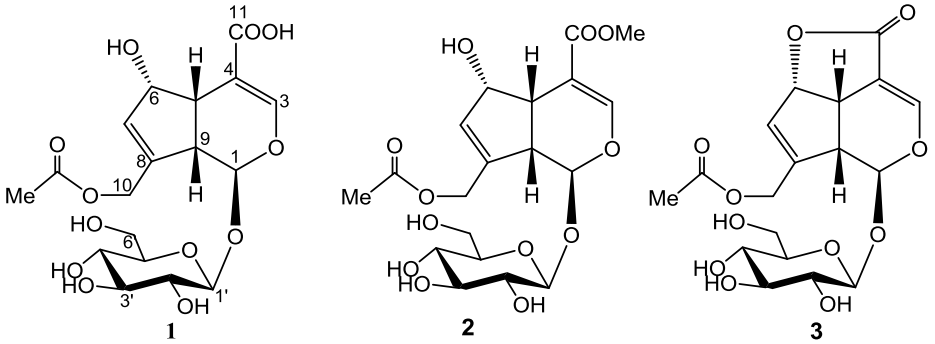

Figure 1: Structures of **1-3** from the leaves of *M. tomentosa*

3. RESULTS AND DISCUSSION

Compound **1** was obtained as a white amorphous powder. The ^{1}H-NMR spectrum of **1** (CD_3OD) exhibited signals for one acetyl group at δ_H 2.11, two olefinic protons at δ_H 6.04 (s) and 7.68 (s), and an anomeric proton at δ_H 4.74 (d, *J* = 8.0 Hz), as listed in table 1. The ^{13}C-NMR and DEPT spectra revealed the signals of 18 carbons, including two carbonyls at δ_C 171.00 and 172.53, two quaternaries at δ_C 108.27 and 145.92, eleven methines at δ_C 42.45, 46.23, 71.57, 74.91, 75.35, 77.87, 78.54,

Table 1: NMR data for compounds **1-3**

C	1			2			3		
	δ_C [$]	δ_C [a,b]	δ_H [a,c] (mult., *J*, Hz)	δ_C [#]	δ_C [a,b]	δ_H [a,c] (mult., *J*, Hz)	δ_C [%]	δ_C [a,b]	δ_H [a,c] (mult., *J*, Hz)
1	101.2	101.26	5.09 (d, 9.0)	101.3	101.27	5.08 (d, 9.0)	93.3	93.29	5.98 (br s)
3	155.0	155.41	7.68 (s)	155.4	155.34	7.68 (d, 1.0)	150.3	150.26	7.32 (d, 2.0)
4	108.1	108.27	-	108.1	108.10	-	106.2	106.14	-
5	42.6	42.45	3.04 (dd,1.0, 6.5)	42.4	42.39	3.06 (dd, 1.0, 7.0)	37.5	37.41	3.72*
6	75.4	75.35	4.85 (m)	75.4	75.35	4.83 (m)	86.3	86.28	5.59 (br d, 6.5)
7	131.9	131.92	6.04 (s)	131.9	131.80	6.04 (d, 1.0)	128.9	128.89	5.75 (br s)
8	145.9	145.92	-	146.0	145.96	-	144.2	144.24	-
9	46.3	46.23	2.66 (dd, 8.0, 9.0)	46.2	46.22	2.67 (dd, 8.0, 9.0)	45.2	45.23	3.30 (m)
10	63.8	63.78	4.82 (d, 15.0) 4.95 (d, 15.0)	63.8	63.74	4.81 (d, 15.0) 4.97 (d, 15.0)	60.9	61.89	4.68 (d, 14.0) 4.80 (d, 14.0)
11	171.2	171.00	-	169.3	169.31	-	172.2	172.23	-
10-Me<u>CO</u>	172.5	172.53	-	172.5	172.50	-	172.5	172.53	-
10-<u>Me</u>CO	20.8	20.74	2.11 (s)	20.8	20.73	2.11 (s)	20.7	20.64	2.10 (s)
11-OMe				52.8	51.83	3.76 (s)			
1′	100.5	100.58	4.74 (d, 8.0)	100.6	100.58	4.75 (d, 8.0)	100.0	99.98	4.71 (d, 8.0)
2′	74.9	74.91	3.24 (m)	74.9	74.90	3.24 (m)	74.6	74.59	3.26 (m)
3′	78.5	78.54	3.30 (m)	78.6	78.56	3.38 (m)	78.3	78.31	3.30 (m)
4′	71.5	71.57	3.28 (m)	71.5	71.55	3.28 (m)	71.5	71.55	3.28 (m)
5′	77.8	77.87	3.40 (m)	77.9	77.87	3.26 (m)	77.8	77.83	3.40 (m)
6′	63.0	62.98	3.64 dd (6.0, 12.0) 3.87 dd (1.5, 12.0)	63.0	62.97	3.64 (dd, 6.0, 12.0) 3.89 (dd, 1.5, 12.0)	62.8	62.76	3.69 (dd, 6.0, 12.0) 3.94 (dd, 2.5, 12.0)

[a)]Measured in CD_3OD, [b)]125 MHz, [c)]500 MHz, [$]$\delta_C$ of asperulosidic acid [8], [#]δ_C of daphylloside, [9], [%]δ_C of asperuloside [9].

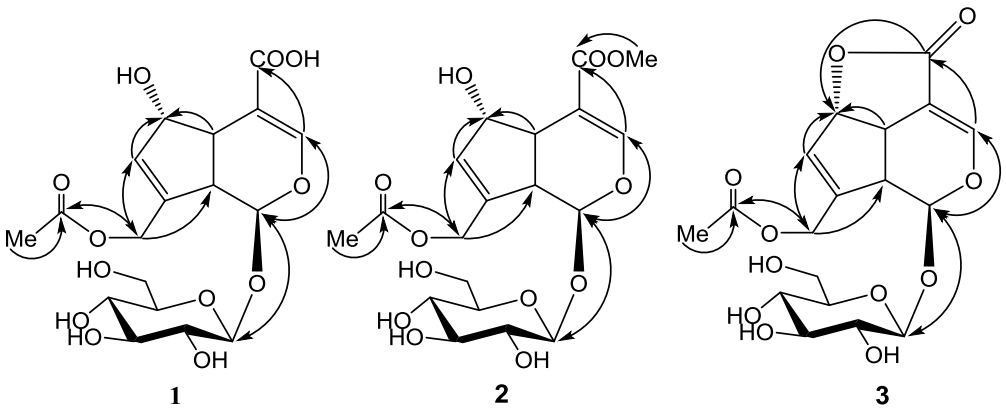

Figure 2: The important HMBC correlations of **1-3**

100.58, 101.26, 131.92, and 155.41, two methylenes at δ_C 62.98 and 63.78, and one methyl at δ_C 20.74 assigned to iridoid and sugar moieties. The ^{1}H- and ^{13}C-NMR data of **1** were similar to those of asperulosidic acid [8]. The HMBC correlations between methyl group (δ_H 2.11)/H-10 (δ_H 4.82 and 4.95) and carbonyl (δ_C 172.5), suggested the position of the acetate group at C-6 and C-10. The coupling constant of H-1′ and H-2′, J = 8.0 Hz and ^{13}C-NMR chemical shifts of sugar at δ_C 100.58, 74.49, 78.54, 71.57, 77.87, and 62.98 confirmed the presence of *β*-D-glucopyranosyl moiety. In addition, the HMBC correlation between glc H-1' (δ_H 4.74) and C-1 (δ_C 101.26) as well as between H-1 (δ_H 5.09) and glc C-1' (δ_C 100.58) suggested the position this sugar moiety at C-1 of the iridoid. Consequently, compound **1** was determined to be asperulosidic acid [8].

The ^{1}H-NMR of **2** (CD_3OD) revealed the signals of one acetyl group at δ_H 2.11, two olefinic protons at δ_H 6.04 (d, J = 1.0 Hz) and 7.68 (d, J = 1.0 Hz), and an anomeric proton at δ_H 4.75 (d, J = 8.0 Hz). The ^{1}H- and ^{13}C-NMR of **2** were almost similar to those of **1** except for an addition of methoxy group at C-11 of asperulosidic acid. The position of the methoxy group at C-11 was confirmed by the HMBC correlation between methoxy group (δ_H 3.76) and C-11 (δ_C 169.31). In addition, its NMR data were identical to those of daphylloside, and found to match [9].

The ^{13}C-NMR and DEPT spectra of **3** revealed the signals of 18 carbons, of which, 10 were assigned to an iridoid moiety, 2 belonged to acetyl, and 6 contributed to a sugar moiety. The ^{1}H- and ^{13}C-NMR data of **3** were similar to those of asperuloside [9]. The acetyl group at C-10 was confirmed by the HMBC correlation between acetyl proton (δ_H 2.10) and C-10 (δ_C 61.89). The HMBC correlations between H-6 (δ_H 5.59) and C-11 (δ_C 172.23) proved the presence of lactone ring at C-11/C-6 of iridoid aglycone. Thus the structure of **3** was elucidated to be asperuloside [9].

To assess the ER stress regulation activity of the methanol extract and compounds **1–3** from the leaves of *M. tomentosa*, relative fluorescence intensities of XBP1-eGFP-transfected 293 T cells were assessed after treating cells with samples at a concentration of 5.0 mg/mL for the methanol extract and 5.0 μM for compounds in the presence or absence of thapsigargin treatment (200 nM). Salubrinal, a widely used ER stress reducer, was used as a positive control. Salubrinal is a selective inhibitor of elF2α dephosphorylation and protects cells from ER stress [10]. Even without thapsigargin, the extract and compound **2** significantly reduced XBP1 splicing in XBP1-eGFP-transfected the 293 T cells with no change in cell viability (data not shown), which implied that there was basal level of the ER stress in cells cultured *in vitro*. Treatment of cells with thapsigargin significantly increased fluorescence intensities as compared with DMSO-treated cells, suggesting that thapsigargin induced ER stress in our *in vitro* system by inhibiting sarco/endoplasmic reticulum Ca^{2+} ATPase [11]. As the results, the extract and compounds **2** and **3** effectively reduced XBP1 splicing in thapsigargin-treated cells. The remaining compounds showed weak or no activity. Although ER stress modulators are getting more attention recently as new drug candidates, until now, a few compounds from natural products have been identified as novel modulators of the unfolded protein response [12]. In conclusion, these results suggested compounds **2** and **3** from the leaves of *M. tomentosa* as new ER stress regulators which can be useful for drug development.

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