Flavonoids That Mimic Human Ligands from the Whole Plants of *Euphorbia lunulata*

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In our investigation of a cell proliferation-based screening assay using human ligand-dependent cell lines for medicinal herbal extracts, the acetone extract of the whole plants of *Euphorbia lunulata* (EL) was observed for its proliferation activity for insulin- and interleukin-10 (IL-10)-dependent cell lines. Fractionation of the active extract led to the isolation of one new flavonoid galactoside, quercetin 3-*O*-(2″,3″-digalloyl)-β-D-galactopyranoside (1), and four known ones, quercetin 3-*O*-(2″-galloyl)-β-D-galactopyranoside (2), hyperin (3), quercetin (4), and gallic acid (5). Compounds 1 and 2 showed insulin-like activity. Compounds 4 and 5 showed IL-10-like activity. This is the first report of these activities of EL, and 1 and 2 will become the seed compounds for the development of a nonpeptidyl insulin substitutional medicine. Compounds 4 and 5 support the pharmacological use of EL, which has been employed as an herbal medicine for the treatment of bronchial asthma.

Key words *Euphorbia lunulata*; flavonoid galactoside; proliferation assay; ligand; insulin; interleukin-10

Euphorbia lunulata (Euphorbiaceae) is found in the southeast of China. It has long been used as a traditional crude drug for the treatment of bronchial asthma and chronic bronchitis.[1)] Previous investigations of the whole plants of *E. lanulata* have yielded kaempferol, quercetin, kaempferol 3-*O*-L-rhamnoside, quercetin 3-*O*-L-rhamnoside, 6,7-dihydroxycoumarin and maoyancaosu.[2,3)]

More than 30 types of cytokines and growth factors have been identified as ligands to date.[4)] Recently, those genes were cloned one after another, yielding recombinant genes. However, only very few of the peptides and proteins made in this way are used as drugs. The reasons such peptides are hard to utilize as medicines are: metabolization, absorption, neutralization antibody, side effects, *etc.* Screening of low molecular compounds mimicking a ligand of protein is carried out to solve these problems. However, conventional screening methods derive from ligand competent cells and require tracking of the signal after ligands bind to receptors, but signal detection is not easy.

We earlier developed a high-throughput, cell proliferation-based screening assay using the human granulocyte colony stimulating factor (G-CSF)-, erythropoietin (EPO)-, thrombopoietin (TPO)-, interleukin-10 (IL-10)- and insulin-dependent cell lines (BAF/GCSFR, BAF/EPOR, BAF/TPOR, BAF/IL10R and BAF/InsR) to identify low molecular compounds that mimic human ligands.[5)] These cell lines were originally derived from a mouse interleukin-3 (mIL-3)-dependent cell line, BAF/B03. The BAF/GCSFR, BAF/EPOR, BAF/TPOR, BAF/IL10R and BAF/InsR cells proliferate in response to the corresponding ligand or rmIL-3, and undergo apoptosis when deprived of these growth factors.

In the present study, we searched for compounds that mimic human ligands by using this screening method jointly with a process for purification of EL extract.

We discovered that EL showed proliferation activity for BAF/InsR and BAF/IL10R. Therefore, analysis of EL was undertaken to identify the active compounds. Here we describe the isolation and structure elucidation of one new flavonoid galactoside, quercetin 3-*O*-(2″,3″-digalloyl)-β-D-galactopyranoside (**1**), and four known ones, quercetin 3-*O*-(2″-galloyl)-β-D-galactopyranoside (**2**), hyperin (**3**), quercetin (**4**), and gallic acid (**5**) as well as their ligand-like activities.

Results and Discussion

Cell proliferation activities for BAF/InsR and BAF/IL10R after stimulation with EL extract, fractions or isolated compounds are shown in Table 1.

EL showed proliferation activity for BAF/InsR (22.9 mU/ml—corresponding quantity of human recombinant preparation, the following is the same, 30 μg/ml) and BAF/IL10R (0.218 mU/ml, 30 μg/ml). On the other hand, there was no proliferation activity for BAF/GCSFR, BAF/EPOR or BAF/TPOR. EL was partitioned with chloroform, ethyl acetate, *n*-butanol, and water-soluble fraction, successively. Ethyl acetate fraction showed both BAF/InsR (20.6 mU/ml, 30 μg/ml) and BAF/IL10R (0.247 mU/ml, 30 μg/ml) proliferation activities. Therefore, we have focused our efforts on researching the chemical constituents of the ethyl acetate soluble fraction. The ethyl acetate soluble fraction was then separated by Sephadex LH-20 column chromatography to yield 9 fractions. Fractions 5 and 6 showed strong proliferation activities for BAF/InsR; Fractions 3, 4 and 8 showed comparatively strong proliferation activities for BAF/IL10R. Compounds **1**, **2** and **4** were isolated from fraction 6, compound **3** was isolated from fraction 4, and compound **5** was isolated from fraction 3.

Compound **1** was obtained as a yellow amorphous powder and was positive in the Mg–HCl test. The molecular structure of **1** was established as $C_{35}H_{28}O_{20}$ by negative HR-FAB mass spectrometry. The UV spectrum exhibited absorption max at 269 and 356 nm. In the IR spectrum, along with the signal for ester carbonyl (1714 cm^{-1}) and conjugated carbonyl (1654 cm^{-1}), signals for hydroxyl group (3391 cm^{-1}) and aromatic ring (1608, 1507 cm^{-1}) were apparent. The ^{1}H-NMR spectrum of **1** indicated 1,2,4-trisubstituted benzene proton signals at δ 7.69 (dd, J=8.7, 2.5 Hz), δ 7.50 (d, J=2.5 Hz), and δ 6.84 (d, J=8.7 Hz) and *meta*-coupled signals at δ 6.38 (d, J=1.9 Hz) and δ 6.19 (d, J=1.9 Hz). The ^{13}C-NMR data of the aromatic region were in agreement with those of isoquercitrin and hyperin[6,7)] which showed the agly-

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Table 1. Cell Proliferation Activity for BAF/InsR and BAF/IL10R after Stimulation with EL Extract, Fractions and Isolated Compounds

Test substance		Corresponding ligand concentration[a)]	
Name	Concentration (μg/ml)	Insulin (mU/ml)	IL-10 (mU/ml)
Extract	30	22.9	0.218
Chloroform-soluble fraction	30	12.5	0.214
Ethyl acetate-soluble fraction	30	20.6	0.247
n-Butanol-soluble fraction	30	17.8	0.214
Water-soluble fraction	30	14.5	N.D.
Fraction 1	30	6.6	0.203
Fraction 2	30	N.D.	0.243
Fraction 3	30	N.D.	0.289
Fraction 4	30	6.3	0.251
Fraction 5	30	73.6	0.243
Fraction 6	30	153.3	0.226
Fraction 7	30	13.9	0.241
Fraction 8	30	N.D.	0.255
Fraction 9	30	N.D.	0.218
Quercetin 3-*O*-(2″,3″-digalloyl)-β-D-galactopyranoside (**1**)	30	51.1	0.291
	10	51.1	0.280
	3	16.0	0.234
	1	10.4	N.D.
Quercetin 3-*O*-(2″-galloyl)-β-D-galactopyranoside (**2**)	30	46.9	0.263
	10	45.5	0.245
	3	11.6	0.214
	1	6.3	N.D.
Hyperin (**3**)	30	N.D.	0.201
	10	N.D.	N.D.
	3	N.D.	0.211
	1	5.5	N.D.
Quercetin (**4**)	30	4.2	0.323
	10	N.D.	0.251
	3	N.D.	0.216
	1	N.D.	0.208
Gallic acid (**5**)	30	4.9	0.352
	10	3.3	0.247
	3	5.2	0.211
	1	4.2	0.214

a) Corresponding ligand concentration that was calculated from calibration curve of human recombinant preparation ($n=2$; mean). Each value was corrected by a control value. N.D.; not detected.

Table 2. ^{1}H- and ^{13}C-NMR Spectral Data for Compound **1** in DMSO

Position	**1**	
	δ_H	δ_C
2		156.2
3		132.6
4		176.8
5		161.1
6	6.19 (d 1.9)	98.5
7		164.0
8	6.38 (d 1.9)	93.4
9		156.1
10		103.8
1′		120.8
2′	7.50 (d 2.5)	115.7
3′		144.9
4′		148.5
5′	6.84 (d 8.7)	115.1
6′	7.69 (dd 8.7, 2.5)	122.1
1″	5.93 (d 7.9)	98.5
2″	5.63 (dd 7.8, 10.2)	69.6
3″	5.14 (dd 3.1, 10.2)	73.8
4″	4.06 (dd 3.1, 5.3)	65.3
5″	3.37 m	75.5
6″	3.68, 3.50 m	59.6
1‴		118.9, 119.0
2‴, 6‴	6.96, 6.93 s	145.31, 145.30
3‴, 5‴		108.8
4‴		138.6, 138.5
C=O		165.2, 164.9

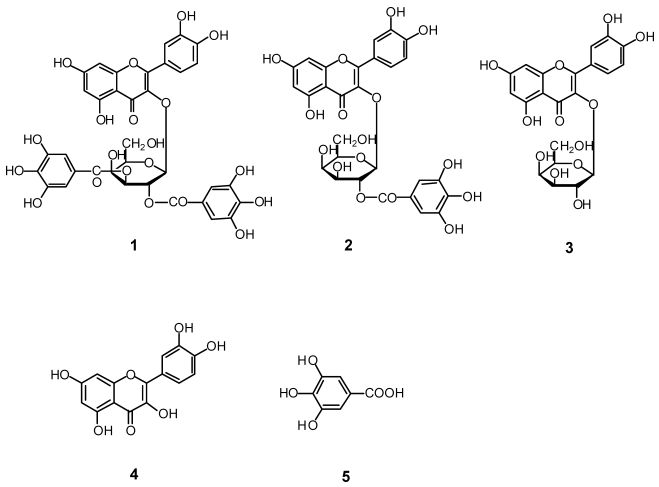

Fig. 1. Structures of Compounds **1**—**5**

cone should be a quercetin. Apart from the signals of quercetin, the ^{1}H- and ^{13}C-NMR spectra indicated the presence of two galloyl groups [δ_H 6.96, 6.93, (each 2H); δ_C 165.2 (CO), 164.9 (CO), 145.31, 145.30, 138.6, 138.5, 118.9, 119.0, 108.8]. The coupling patterns of the aliphatic proton signals, which were assigned with the aid of ^{1}H–^{1}H shift correlation spectroscopy (COSY), were consistent with those of $^{4}C_1$ galactopyranose (Table 2). These data coupled with the FAB-MS data indicated that **1** may be a digallate of quercetin galactoside. Quercetin, gallic acid, and D-galactose were identified by hydrolysis of **1** (see Experimental). Furthermore, comparison of the carbon shifts of the aglycone with those of published data for quercetin revealed an upfield shift of C-3 ($\Delta\delta$ 3.0 ppm) and a downfield shift of C-4 ($\Delta\delta$ 1.1 ppm) indicating the placement of the sugar residue at C-3.[8] HMBC spectral analysis supported further evidence for this assignment. The galloyloxy groups in **1** were linked at C-2 and C-3 of the galactose residue based on the remarkable downfield shifts of H-2″ (δ 5.63, dd, $J=10.2$, 7.8 Hz), and H-3″ (δ 5.14, dd, $J=10.2$, 3.1 Hz) signals. The HMBC NMR spectrum of **1** indicated the carbon signal (δ 165.2, 164.9) of the two galloyl carbonyl units showed correlations with the H-2″ and H-3″ signals, respectively. These data confirmed that the galloyloxy groups were linked at C-2 and C-3 of galactose moiety. On the basis of the forgoing observations, **1** was assigned as quercetin 3-*O*-(2″,3″-digalloyl)-β-D-galactopyranoside.

Compounds **2**, **3**, **4**, and **5** are known compounds whose structures were elucidated by their spectral data and comparison with reports in the literature.[7,9,10]

Compounds **1** and **2** showed proliferation activity for BAF/InsR. Stimulation with **1** or **2** was dose-dependent and

maximal at 30 μg/ml. Compounds **3**, **4** and **5** did not show clear activity.

The proliferative activity for BAF/IL10R was observed in **1**, **2**, **4** and **5**, although the activity of **1** and **2** was weaker than that of **4** and **5**. Stimulation with those four compounds was dose-dependent and maximal at 30 μg/ml. IL-10-like activity of **1** and **2** may derive from a skeleton of **4** and **5**.

Zhang *et al.* found a nonpeptidyl small molecule insulin mimetic from a fungal metabolite.[11] Its structure was a benzoquinone derivative that binds two indolyl groups. In the meantime, the small molecular compounds (**1**, **2**) that mimic insulin that we got from a higher plant were queretin 3-*O*-galactoside bound to galloyl group at the galactosyl moiety. There was no activity in **4**, **5**, or **3**, which do not bind to the galloyl group. Compounds **1** and **2** will become the seed compounds for the development of a nonpeptidyl insulin substitutional medicine.

IL-10 is an anti-inflammatory cytokine that may be important in regulating the asthmatic inflammatory response.[12,13] Therefore, The IL-10-like activity found in **4** and **5** is felt to support the pharmacological use of EL.

This is the first report of discovery of insulin mimic compounds and IL-10 mimic compounds from a higher plant. At the same time it was shown that our new cell proliferation-based screening method was useful for a ligand search in natural products.

Experimental

General Procedures The UV spectrum was obtained in MeOH on a Shimadzu UV-160 spectrophotometer, and the IR spectrum was recorded on a JASCO FT/IR 300E spectrophotometer. Optical rotations were measured in MeOH on a JASCO DIP-360 polarimeter. The NMR spectra were recorded on a JEOL JNM-LA400 spectrometer, with TMS as an internal standard. The MS were obtained on a JEOL JMS GCmate spectrometer. Column chromatography was carried out with Sephadex LH-20 (Pharmacia), CHP-20 (75—150 μ, MITSUBISHI CHEMICAL CORPORATION) and ODS-A (YMC GEL). Thin-layer chromatography (TLC) was performed on Merck TLC plates (0.25 mm thickness), with compounds visualized by spraying with 5% (v/v) H_2SO_4 in ethanol solution and then heating on a hot plate. HPLC was performed on a JASCO PU-2089 apparatus equipped with JASCO UV-2075, RI-101 (Shodex), and Shodex OR-2. YMC Guard pack ODS Al (10×150 mm i.d.) was used for preparative purposes. Shenshu Pak TSK gel Aminopak (4.6 mm i.d.×50 mm) was used for the detection of D-galactose.

Plant Materials The whole plants of *E. lunulata* were collected in HeBei Province, People's Republic of China, in October 1998 and were identified by Professor Weichun Wu (Department of Medical Plants, Shenyang Pharmaceutical University, People's Republic of China). Voucher specimens have been deposited at the Laboratory of Pharmacognosy and Natural Product Chemistry, College of Pharmacy, Nihon University.

Extraction and Isolation The whole plants of *E. lunulata* (2.3 kg) were extracted twice with 70% acetone. Evaporation of the solvent under reduced pressure from the combined extract made the 70% acetone extract 675 g. The extract was dissolved and suspended in water (2 l) and partitioned with chloroform (3×2 l), ethyl acetate (3×2 l), and *n*-butanol (3×2 l). The amounts extracted were 65.2, 52.4, and 86.5 g, respectively, and the residual aqueous extract yielded 450.1 g.

The ethyl acetate fraction was subjected to Sephadex LH-20 column chromatography (6.5×35 cm, eluted with MeOH : H_2O 50 : 50→100 : 0). The column chromatographic fractions (200 ml each) were combined according to TLC monitoring into nine fractions. Fraction 6 was subjected to CHP-20 column chromatography (3×20 cm, eluted with MeOH : H_2O=40 : 60). The column chromatographic fractions (100 ml each) were combined according to TLC monitoring into nine portions. Portion eight yielded a yellow solid **4** (354 mg). Portion four was isolated and further purified by HPLC (YMC Guard pack ODS Al, 10×150 mm, MeOH : H_2O, 4 : 6) to yield **1** (21 mg). Portion five was isolated and further purified by HPLC (YMC Guard pack ODS Al, 10×150 mm, MeOH : H_2O, 4 : 6) to yield **2** (210 mg). Fraction four yielded a yellow solid **3** (7.8 mg). Fraction three yielded gray needles **5** (56 mg).

Quercetin 3-*O*-(2″,3″-Digalloyl)-β-D-galactopyranoside: Yellow amorphous powder. $[\alpha]_D^{23}$ −28.7° (c=0.30, MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.56), 269 (4.17), 290 sh (3.97), 356 (3.87); IR (KBr) ν_{max} 3391, 1714, 1654, 1608, 1507, 1450, 1357, 1204, 1091, 1042, 1024, 993, 824, 763 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 2; negative HR-FAB-MS m/z 767.1087 (Calcd for $C_{35}H_{27}O_{20}$, 767.1095); negative FAB-MS m/z 767 $[M-H]^-$ (13), 615 (5), 465 (10), 169 (40), 151 (20), 124 (30).

Acid Hydrosis of 1 Compound **1** (10 mg) was dissolved in 10% H_2SO_4 and heated at 85 °C for 3 h. After cooling, the reaction mixture was neutralized by passage though an Amberlite IRA-93ZU (Organo) column and then partitioned between ethyl acetate and water. The ethyl acetate layer was concentrated and the concentrate was pass through an ODS C-18 column, successively eluted with 20% MeOH and 60% MeOH. Gallic acid was recovered from the 20% MeOH fraction and quercetin from the 60% fraction by direct comparison with authentic samples. Cellulose TLC of the water layer in BuOH : AcOH : H_2O (3 : 1 : 1) showed the sugar should be a Galactose (Rf 0.20) by direct comparison with authentic sample. Then the water layer was analyzed by HPLC under the following conditions: column, a Shenshu Pak TSK gel Aminopak (4.6 mm i.d.×50 mm); solvent, H_2O : CH_3CN= 20 : 80; flow rate 1.0 ml/min; detector, OR and RI. D-Galactose present in the water layer was identified by comparing its retention time and polarity with that of an authentic sample; t_R (min) 7.2 min (D-galactose, positive polarity).

Hematopoietic Growth Factor and Reagents Recombinant human G-CSF (rhG-CSF), recombinant human erythropoietin (rhEPO), recombinant human thrombopoietin (rhTPO), and recombinant human IL-10 (rhIL-10) were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Recombinant human insulin (rhIns, NovalinR, Novo Nordisk, Bagsvaerd, Denmark) was purchased from Novo Nordisk.

Plasmids Construction of the expression plasmids, which include human G-CSF receptor (GCSFR), EPO receptor (EPOR), TPO receptor (TPOR), and IL-10 receptor (IL-10R), were described in detail previously.[5] Insulin receptor (InsR) cDNA was amplified from Quick-clone (human spleen, Clontech, Palo Alto, CA, U.S.A.) by PCR. The primer pairs used were: InsR forward 1, 5′-ACCGGGAGCGCGCGCTCTGA-3′, InsR reverse 1, 5′-GATTGGATCCAGGGGCACAGA-3′, InsR forward 2, 5′-TGGATCCAATCTCAGTGTCTAAC-3′ and InsR reverse 2, 5′-TTAGGAAGGATTGGACCGAGGCA-3′. Amplification was performed with a Perkin Elmer DNA Cycler 9600 (PE Applied Biosystems, Foster City, CA, U.S.A.); it involved 30 cycles of denaturation at 94 °C for 15 s, and annealing and extension at 68 °C for 4 min. The PCR products were cloned into vector pCR2.1 (Invitrogen, Carlsbad, CA, U.S.A.), and the nucleotide sequences of the inserts were determined. PCR products were ligated, and digested with *Nco*I and *Sal*I followed by treatment with Klenow enzyme (Takara Shuzo Co., Ltd., Kyoto, Japan) to give blunt ends. The multiple cloning site of pIRE-Spuro (BD Biosciences Clontech, Palo Alto, CA, U.S.A.) was expanded by digestion with *Eco*RI (blunt-ended with Klenow enzyme). Then the full-length of InsR cDNA was ligated into this position of pIRESpuro, generating pIRESpuro-InsR.

Transfection and Cell Culture The mouse pro-B cell line, BAF/B03, was culutred as previously detailed.[5] pIRESpuro-InsR was electroporated with a GenePulser apparatus (Bio-Rad, Hercules, CA, U.S.A.) with a 250-V pulse at 960 μF. The clone resistant to puromycin (2 μg/ml) (Sigma) was selected by limiting dilution in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (FBS) and rhIns (1 U/ml). The stably transfected cell line obtained is referred to as BAF/InsR.

Proliferation Assay Stably transfected cells were cultured as previously detailed.[5] BAF/InsR, rhIns-dependent cell line, cells were cultured at 1×10^5 cells/well for 24 h with various concentrations of rhIns or a test substance in the presence of 0.01% DMSO. Cell proliferation was measured with a Cell Counting Kit (Dojin, Kumamoto, Japan) with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST-1) (5×10^{-3} M) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) (2×10^{-4} M) as substrate, and cultured for 3 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. Optical density was measured at 450 nm using a microplate reader (CORONA, Hitachi, Japan).

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