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′-galloyl)-β-D-galactopyranoside (1), and four known ones, quercetin 3-O-(2′,3′-digalloyl)-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. Previous investigations of the whole plants of *E. lunulata* have yielded kaempferol, quercetin, kaempferol 3-O-β-D-rhamnoside, quercetin 3-O-α-L-rhamnoside, 6,7-dihydroxycoumarin and maoyancaosu. More than 30 types of cytokines and growth factors have been identified as ligands to date. 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 more used as drugs are their low molecular weights, low immunogenicity, and relative stability. They have yielded kaempferol, quercetin, kaempferol 3-O-β-D-rhamnoside, quercetin 3-O-α-L-rhamnoside, 6,7-dihydroxycoumarin and maoyancaosu.

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those of 4C1 galactopyranose (Table 2). These data coupled with those of published data for quercetin revealed an upfield shift of C-3 (δ 3.8) HMBC spectral analysis supported further evidence with the FAB-MS data indicated that 1 may be a digallate of quercetin. Apart from the signals of quercetin, the 1H- and 13C-NMR spectra indicated the presence of two galloyl groups [δH 6.96, 6.93, (each 2H); δC 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 1H–1H shift correlation spectroscopy (COSY), were consistent with those of 4C1 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 α-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 (Δδ 3.0 ppm) and a downfield shift of C-4 (Δδ 1.1 ppm) indicating the placement of the sugar residue at C-3,3,4 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 foregoing 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.7,8,109 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) H2SO4 in ethanol solution and then heating on a hot plate. HPLC was performed on a JASCO PU-2080 apparatus equipped with a JASCO UV-2057, RI-101 (Shodex), and Shodex OR-2. YM Guard pack ODS AI (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, NIH 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: H2O: 50:50:100). 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: H2O: 40:40:60). 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 (YM Guard pack ODS AI, 10×150 mm, MeOH: H2O: 4:6) to yield 1 (21 mg). Portion five was isolated and further purified by HPLC (YM Guard pack ODS AI, 10×150 mm, MeOH: H2O: 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-galactopyranoside: Yellow amorphous powder. [α]D20 = 28.7° (c=0.30, MeOH); UV (MeOH) λmax (log ε) 210 (4.56), 269 (4.17), 290 sh (3.97), 356 (3.87); IR (KBr) νmax 1734, 1616, 1608, 1507, 1450, 1357, 1204, 1091, 1042, 1024, 993, 824, 763 cm−1; 1H- and 13C-NMR data, see Table 2; negative HRFAB-MS m/z 767.1087 (Calcd for C33H30O25: 767.1095); negative FAB-MS m/z 767 [M−H]− (13), 675 [M−H−CO]− (10), 169 (40), 151 (20), 124 (30).

Acid Hydrolysis of 1 Compound 1 (10 mg) was dissolved in 10% H2SO4 and heated at 85 °C for 3 h. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-932U (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:H2O (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, H2O: CH3CN: 20: 80; flow rate 1.0 ml/min; detector, OR and RI. α-Galactoside present in the water layer was identified by comparing its retention time and polarity with that of an authentic sample; tR (min) 7.2 min (α-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 (rIL-10) were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Recombinant human insulin (rhIns, NovoNordisk, Novo Nordisk, Bagsvaerd, Denmark) was purchased from Novo Nordisk.

Plasmds Human G-CSF receptor (GCSFR), EPO receptor (EPO), TPO receptor (TPOR), and IL-10 receptor (IL10R), were described in detail previously.14 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′-ACCGGAGGGCGGGCTGCTGA-3′, InsR reverse 2, 5′-GGATGTCATCACTGTCGTAAC-3′ and InsR reverse 2, 5′-TATGAAGGTGTG-GACGGAGCA-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 Ncol and Sall 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 EcoRI (blunt-ended with Klenow enzyme). Then the full-length of InsR cDNA was ligated into this position of pIRE-Spuro, generating pIRE-Spuro-InsR.

Transfection and Cell Culture The mouse pro-B cell line, BAF/B03, was culured as previously detailed,15 pIRE-Spuro-InsR was electroporated with a GenePulsar 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 limited 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 transfectedcells were cultured as previously detailed.15 BAF/InsR, rhIns-dependent cell line, cells were cultured at 1×106 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)-2H-tetrazolium (WST-1) (5×10−5 M) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) as substrate, and cultured for 3 h at 37 °C in a humidified atmosphere of 5% CO2 in air. Optical density was measured at 450 nm using a microplate reader (CORONA, Hitachi, Japan).

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