SUPPLEMENTARY MATERIAL

A immunosuppressive triterpenoid saponin from the stems of *Epigynum griffithianum*

Qun Wang [a*], Wen-Yi Li[b] and Yu-Dan Wang[b*]

[a]*Department of Hepatopancreatobiliary Surgery, Hu bei Cancer Hospital, Wuhan 430079, People's Republic of China*

[b]*Key Laboratory of Chemistry in Ethnic Medicinal Resources, State Ethnic Affairs Commission and Ministry of Education, Yunnan Minzu University, Kunming 650031, Yunnan, People's Republic of China*

Abstract

Chemical investigation of the stems of *Epigunum griffithianum* led to the isolation and identification of a new triterpenoid saponin (**1**) and two known compounds (epigynosides A (**2**) and B (**3**)). These structures were elucidated by means of spectroscopic analysis (1D and 2D NMR, MS, UV, IR) as well as comparison with the reported data. Compound 1 was evaluated in vitro for the immunosuppressive activities on proliferation of mice splenocyte and displayed significant immunosuppressive activities compared to the positive control (dexamethasone) with the concentration at 25 μM.

Keywords: *Epigunum griffithianum*; triterpenoid saponin; immunosuppressive activity

∗ Corresponding authors, Tel: +86-15926270216 E-mail address: swander@126.com (Q.Wang)

Contents

Experimental Section

Plant material

The stems of Epigynum griffithianum were collected from the republic of the union of myanmar, in June 2014, and were identified by Mr. W. Q. Xiao, Xishuangbanna Tropical Plant Garden. A voucher specimen (No. Cao20160411) has been deposited at Kunming University of Science and Technology.

Extraction and isolation

The air dried and crushed stems of E. griffithianum (1.3 kg) were extracted by MeOH at 60℃. After removal the solvent in vacuo, the crud extract (13 g) was suspended in H_2O and partitioned with EtOAc (3×2L) to give a EtOAc fraction (2 g). The EtOAc fraction (2 g) was chromatographed over microporous resin D101 (Average pore size, 9~10nm, Qingdao Marine Chemical Factory, Qingdao, China), eluted with MeOH-H_2O (20:40, 40:60, 60:40, 80:20 and 100:0, v/v) to give five fractions (Fr. A-E). Fr. C (0.2g) was subjected to RP-C18 gel (43-63mm, Merck, Darmstadt, Germany) (MeOH-H2O, 40:60-80:20) to yield three subfractions C1-C3. Fraction C2 (85mg) was subjected to column chromatography over silica gel (200-300 mesh) and eluted with chloroform-acetone (4:1) and further by Sephadex LH-20 (Amersham Biosciences AB, Uppsala, Sweden) (CHCl3-CH3OH, 1:1) to yield compound 1 (5.0 mg). Fr. D (0.7 g) was chromatographed over Sephadex LH-20 (MeOH-H2O, 1:1) to yield four subfractions D1-D4. Fraction D2 (0.2 g) was subjected to MPLC [medium pressure liquid chromatographic was performed on an Agilent C-601 liquid chromatograph (Agilent, San Francisco, CA, USA)] (MeOH-H2O, 40:60-80:20) and further purified by a semi-preparative HPLC (Agilent 1200 liquid chromatograph, Agilent, San Francisco, CA, USA) (MeCN-H2O, 55:45) to obtain compound **3** (3. mg). Fraction D3 (100 mg) was chromatographed over RP-C18 gel (MeOH-H2O, 40:60-80:20) and further by Sephadex LH-20 (CHCl3-CH3OH, 1:1) to yield compound **2** (2.0 mg).

Acid hydro lysis of compound 1

Each glycoside (2 mg) was hydrolysed with 2 M HCl (1, 4-dioxane/H2O 1:1, 2 mL) under reflux at 100 °C for 2 h. After dried in vacuo, the residue was subjected to partitioning between H_2O and EtOAc. The H_2O layer was concentrated to give a monosaccharide residue. This residue was dissolved in pyridine (1 mL), and 2 mg of L-cysteine methyl ester hydrochloride was added. This solution was kept at 60 °C for 2 h, and then trimethylsilylimidazole (0.2 mL) was added. The mixture was maintained at 60 °C for another 2 h. After drying the solution, the residue was partitioned between H_2O (2 mL) and n-hexane (2 mL). The n-hexane layer was analysed by GC (column, DB-5MS, 30 m × 0.32 mm × 0.25 μm; detector, FID; detector temperature, 280 °C; injected temperature, 250 °C; column temperature, from 100 to 280 °C with 10 °C/min, then to 300 °C with 20 °C/min, held for 15 min; carrier gas, N2). The sugar derivatives showed retention time of 19.35 min, identical to the trimethylsilyl-L-cysteine derivatives of authentic D-glucuronic acid.

Splenocyte proliferation assay

Splenocytes were isolated from male BALB/c mice as described previously (Li, Xuan et al. 2014). The cytotoxicity using MTT method proved that compound **1** showed no toxicity of splenocytes with IC50 values > 100 μM. In brief, spleens were rapidly harvested from mice. Splenocytes were obtained by squeezing the organs into the RPMI 1640 medium. Cell suspension was filtered through a 100-μm stainless steel mesh, and erythrocytes were lysed with red blood cell lysing buffer [16.5 mM Tris (pH7.2) and 0.155 M NH_4Cl]. Next, splenocytes were diluted at the density of 1×10^6 cell/mL in RPMI 1640 medium (containing 10% FBS) supplemented with penicillin (100 units/mL) and streptomycin (100 mg/mL), seeded into 96-well flat-bottom microtiter plates (Nunc), and exposed to the test compound at various concentrations in the presence of concanavalin A (Con A, 10 μg/mL), using the Con A-treated splenocytes as the experimental control, dexamethasone (DXM) as a positive control, and splenocytes without Con A-treated as the negative control. After incubation for 72 h at 37 °C in a humidified atmosphere with 5% CO_2, 10 μL of cell counting kit-8 (CCK-8) was added and incubated for another 4 h. The tests were conducted for three independent replicates, and the data were calculated as the mean

of the three individual experiments. The viability of cells was evaluated using the CCK-8 assay by detecting absorbance at 450 nm on a Spectra Max M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen-cultures / the absorbance value for non-stimulated cultures. The mouse cytokine (IL-2 and TNF-α) detecting ELISA kits were purchased from Beijing 4A Biotech Co., Ltd (Beijing, China). IL-2 and TNF-α production were measured by using ELISA kits, the assay procedures were carried out as described in the kit manual recommended.

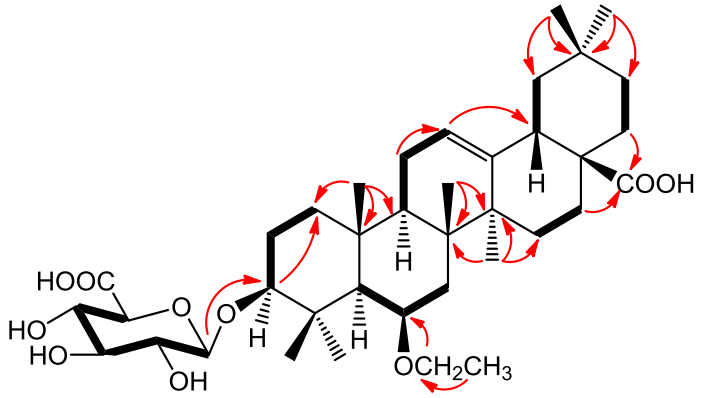


Figure S1. The key $^{1}H–^{1}H$ COSY (bold) and HMBC (arrows) correlations of **1.**

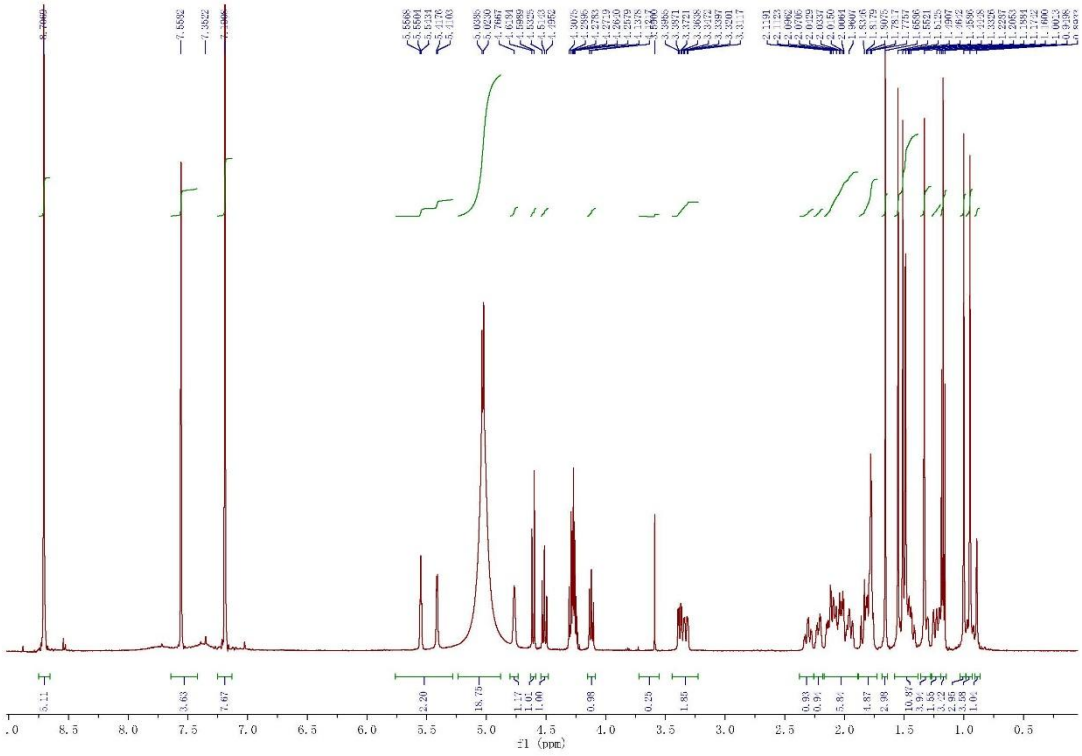


Figure S2. ^{1}H NMR spectrum of Compound **1** recorded at 500 MHz in C_5D_5N

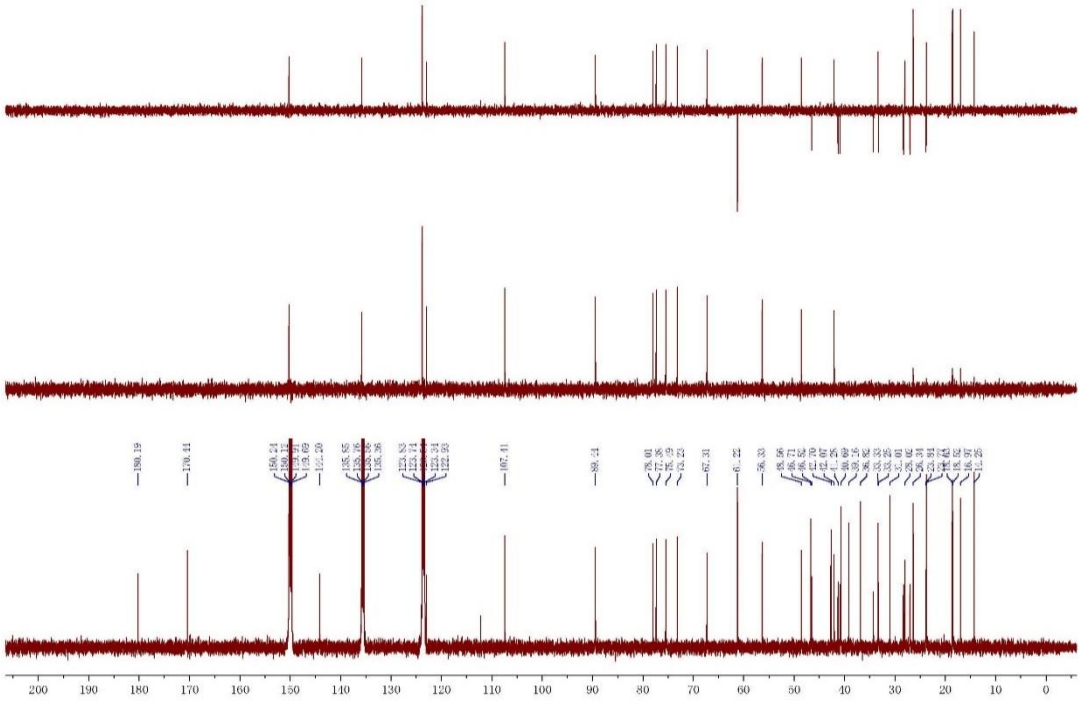


Figure S3. ^{13}C NMR spectrum of Compound **1** recorded at 125 MHz in C_5D_5N

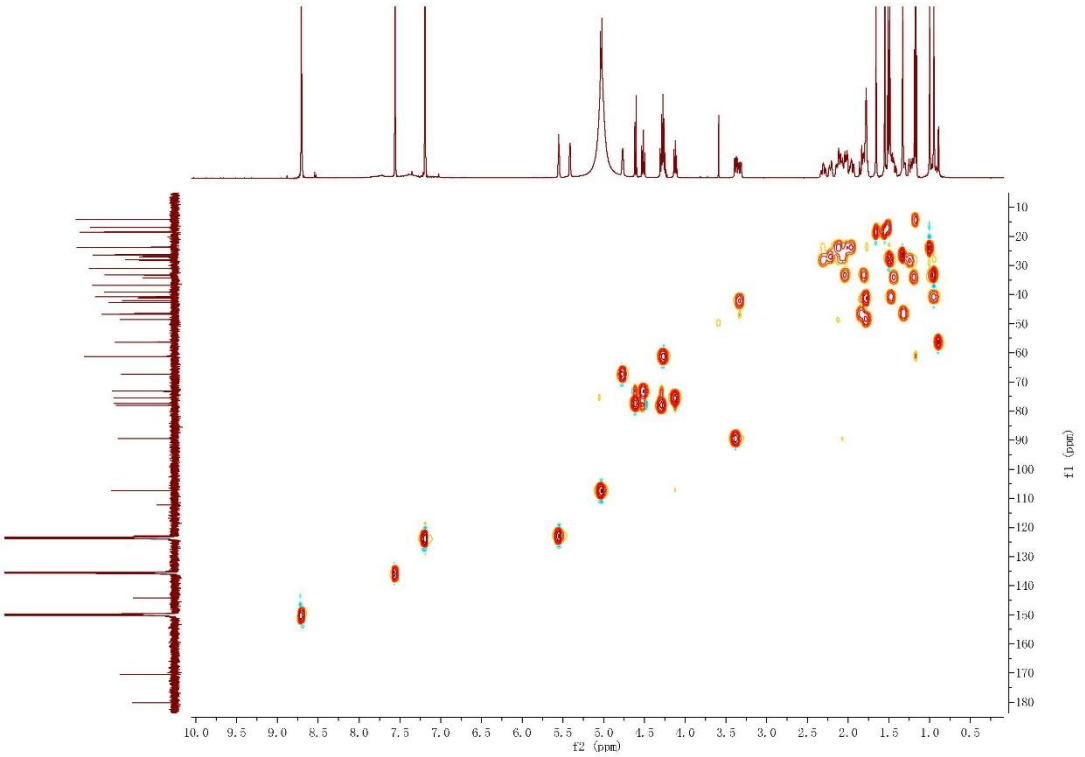


Figure S4. HSQC spectrum of Compound **1** recorded in C_5D_5N

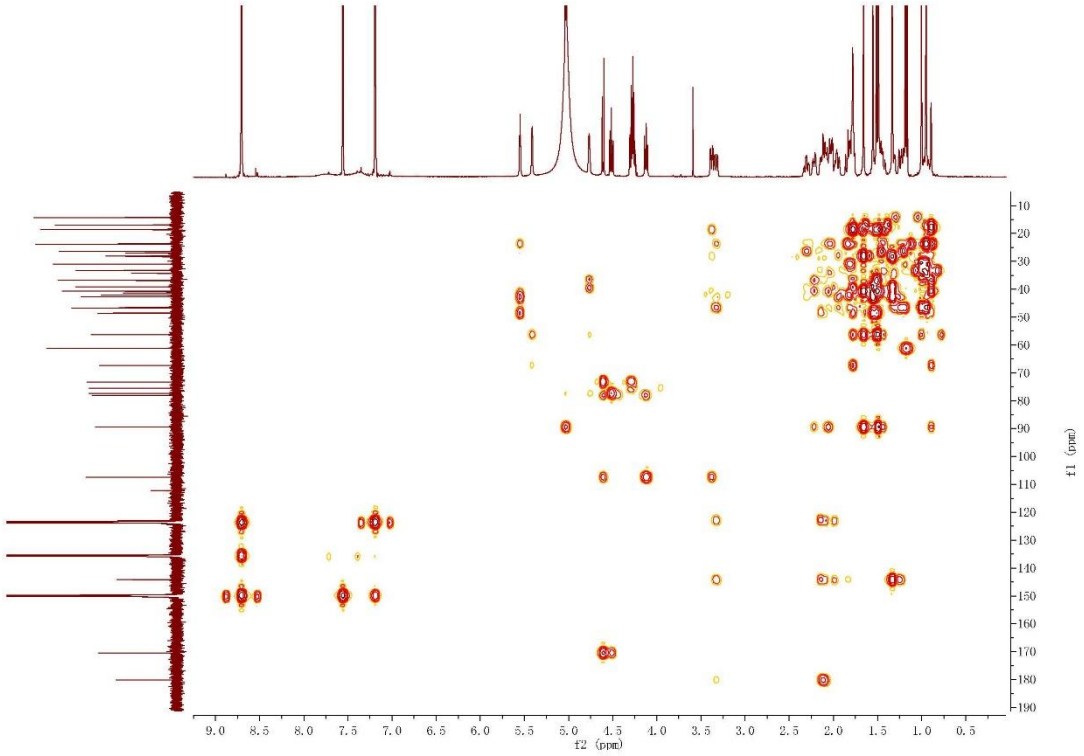


Figure S5. HMBC spectrum of Compound **1** recorded in C_5D_5N

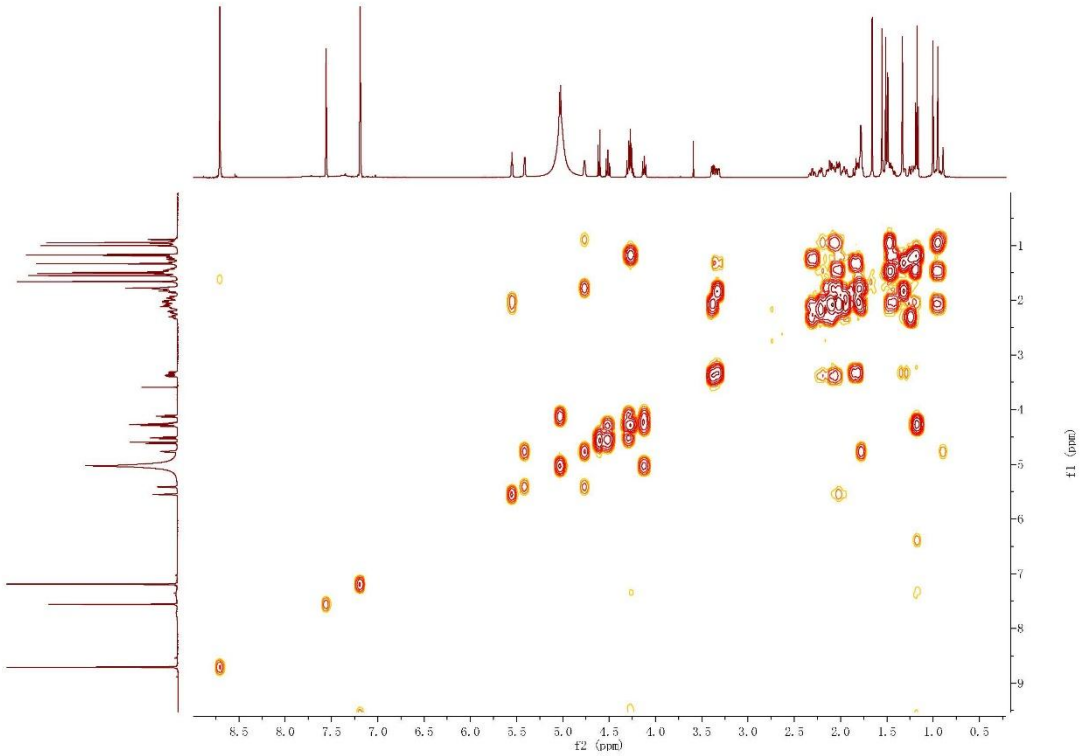


Figure S6. ^{1}H-^{1}H COSY spectrum of Compound **1** recorded in C_5D_5N

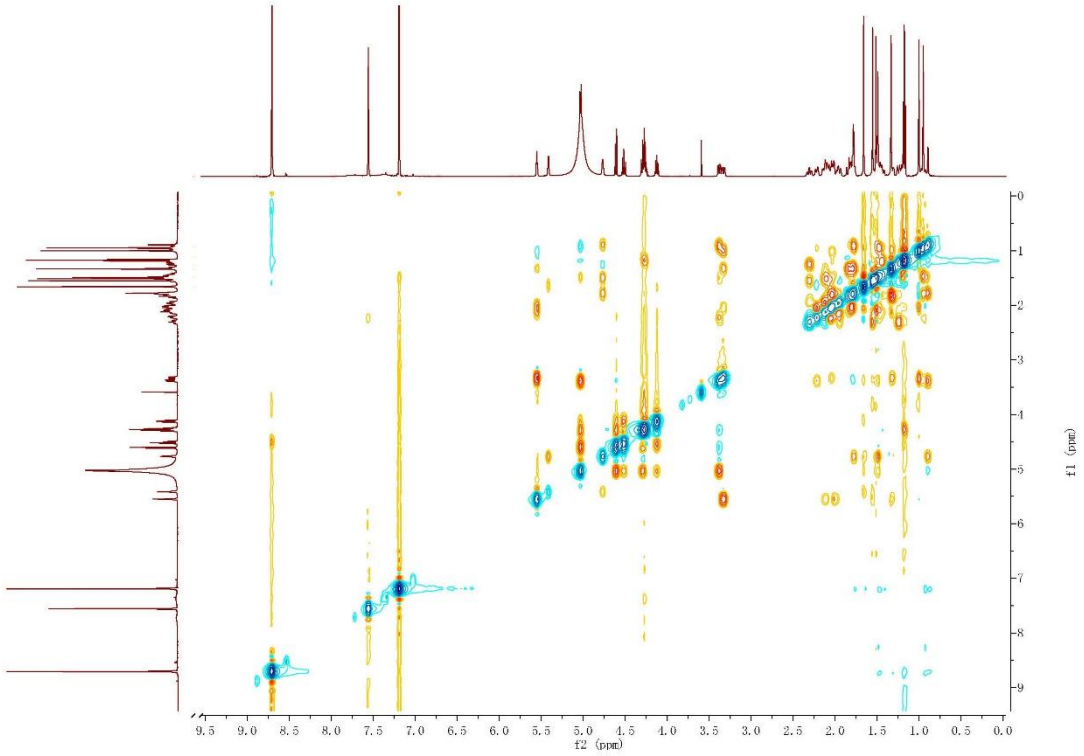


Figure S7. ROESY spectrum of Compound **1** recorded in C_5D_5N

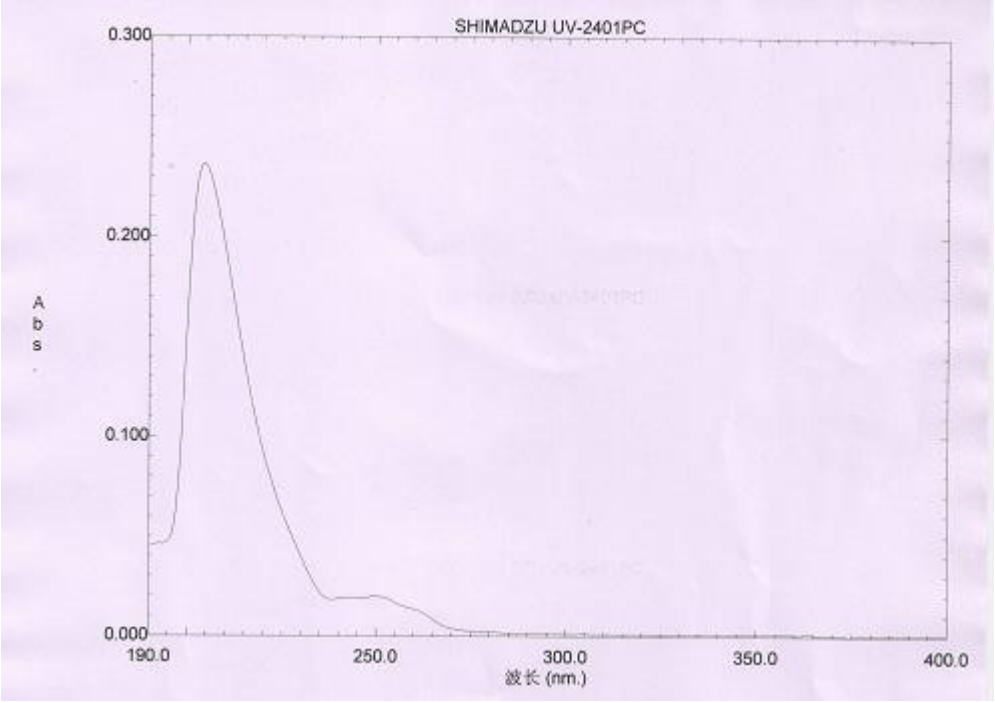
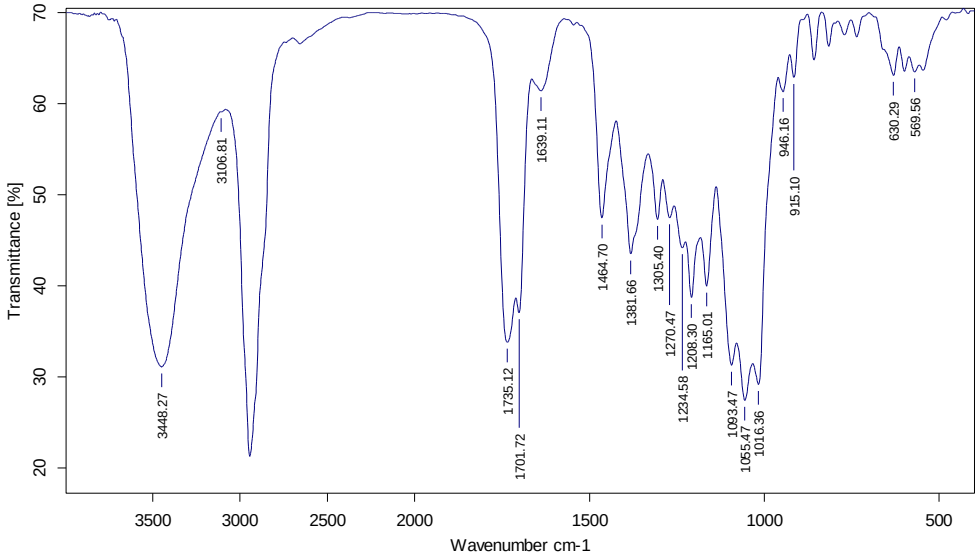


Figure S8. UV spectrum of compound **1**



Sample : DEP70	Frequency Range : 399.246 - 3996.32	Measured on : 22/08/2016	
Technique : KBr压片	Resolution : 4	Instrument : Tensor27	Sample Scans : 16
Customer : 160826IR11	Zerofilling : 2	Acquisition : Double Sided,Forv	

Figure S9. IR spectrum of compound **1**

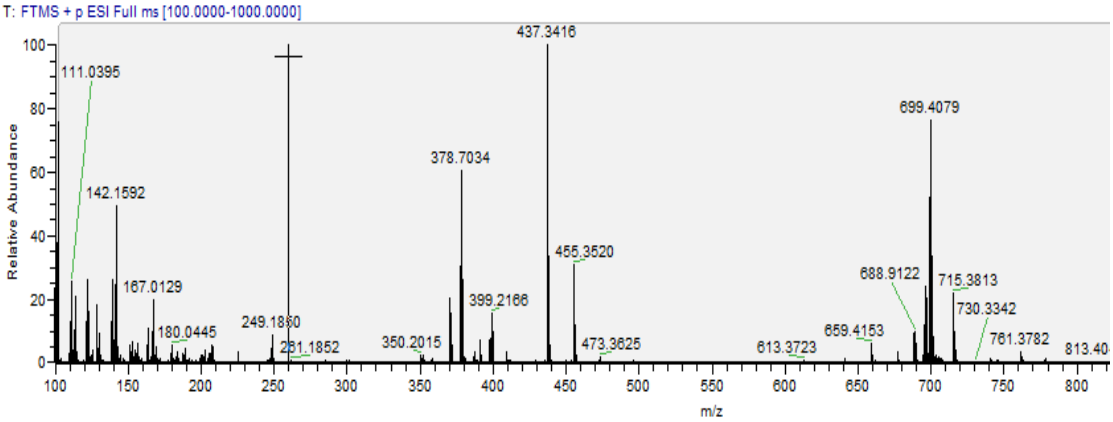


Figure S10. ESI spectrum of compound **1**

Table ***S1***. ^{1}H and ^{13}C NMR data of compound **1 (**δ in ppm**,** *J* in Hz**)**

No.	δ_H (*J* in Hz)	δ_C	No.	δ_H (*J* in Hz)	δ_C
1	1.47 (m)	40.8	19	1.83 (m)	46.5
	0.95 (m)			1.32 (m)	
2	2.21 (m)	26.9	20		31.0
	2.06 (m)		21	1.44 (m)	34.2
3	3.38 (dd, 11.6, 4.2)	89.4		1.20 (m)	
4		40.6	22	2.04 (m)	33.2
5	0.89 (s)	56.3		1.81 (m)	
6	4.77 (s)	67.3	23	1.49 (s)	28.0
7	1.78 m	41.2	24	1.66 (s)	18.5
	1.46 (m)		25	1.51 (s)	16.9
8		39.1	26	1.55 (s)	18.6
9	1.78 (m)	48.5	27	1.34 (s)	26.3
10		36.8	28		180.1
11	2.12 (m)	23.8	29	1.00 (s)	23.7
	1.96 (m)		30	0.95 (s)	33.3
12	5.55 (t, 3.4)	122.9	31	4.27 (m)	61.2
13		144.2	32	1.17 (t, 7.0)	14.5
14		42.6	1′	5.03 (d, 8.0)	107.4
15	2.30 (m)	28.3	2′	4.12 (t, 8.4)	75.4
	1.24 (m)		3′	4.29 (m)	78.0
16	2.12 (m)	23.8	4′	4.51 (t, 9.4)	73.2
	1.96 (m)		5′	4.61 (d, 9.8)	77.3
17		46.7	6′		170.4
18	3.33 (dd, 13.4, 3.8)	42.6			

References

Li, X., B. Xuan, Q. Shou and Z. Shen. (2014). New flavonoids from Campylotropis hirtella with immunosuppressive activity. Fitoterapia 95(10): 220-228.