Supporting Information

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Evaluation of Anti-inflammatory and Immunosuppressive Properties of Cynanchum ascyrifolium Matsum. and its Active Secondary Metabolites

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S1: Extraction, fractionation and isolation

The powder of roots (7.2 kg) was heated reflux extraction with 80% ethanol for three times, each of 2hr, to yield ethanol extract. The ethanol extract (EXT, 1.67 kg)was suspended in H_2O and partitioned with ethyl acetate and *n*-buthanol, respectively. The ethyl acetate extract (EXT-E, 28.6 g) was fractionated by silica gel column chromatography (CC) eluted with the solvent system of CH_2Cl_2/MeOH (100:0, 80:20, 70:30, 50:50) to yield six sub-fractions (SFr. A-F) for bioassay.

Then, SFr. B (5.6g) was subjected to silica gel CC (200–300 mesh, Qingdao Marine Chemical Factory, China) and eluted with the mixture of petroleum ether/ethyl acetate (5:1) to obtain **1** and **3**, eluted with mixture of petroleum ether /acetone solvent system (from 8:1 to 4:1) to obtain **2** and **4**, respectively. The structures of isolated determined by spectroscopic NMR (Bruker DRX-300, 500MHz with TMS as the internal standard), ESI-MS (Agilent1100 MSD Trap instrument) spectral analysis. Purity of compounds was controlled by HPLC analysis (>95%). The tested extract / fractions (100μg/ml) or compounds (100 μM) were dissolved in DMSO as stock solutions for bioassay.

S2: Cell culture

A mouse macrophage cell line RAW 264.7 was obtained from the Cell Bank of Chinese Academic of Sciences (Shanghai, China). Cell medium and FBS were obtained from Gibco 106 BRL (NY, USA.) Concanavalin A (Con-A), lipopolysaccharide (LPS), 3-(4, 5-dimethyl-2-thiazo-lyl)-2, 5-diphenyl-2 H-tetrazolium bromide (MTT) in PBS (5 mg/mL) reagents was from Sigma, Germany. Cyclosporin A (CsA) was purchased from Aladdin chemistry Co., Ltd. RAW 264.7 murine macrophage cells were cultured in DMEM supplemented with 10% FBS containing 100 U/mL of penicillinand 100μg/ml of streptomycin at 37°C in a 5% CO_2 humidified incubator.

S3: Cell viability assay of immunosuppressive activity

Male BALB/c mice (18-22g) were provided by the comparative medical center of Yangzhou University, China. Spleen-derived T cells isolated from BALB/c mice were activated by Con A (5 μg/ml) or LPS (10μg/ml) for 24 h, which were indicated as activated cells, whereas those without stimulation were used as non-activated cells. The cells (4×10^5 cells/well) were further incubated in 96 well-plate with or without various concentrations of samples for 24 h. For MTT assay, 20 μL of MTT was added per well and incubate for 4hr. MTT formazan production was dissolved by 150μl of DMSO replacing the medium [1]. The optical density at 570 nm (OD_{570}) was measured.

S4: Assay for nitric oxide production in LPS-stimulated RAW 264.7 cells

Firstly, viability of RAW264.7 cell lines were measured using MTT assay with various concentrations of samples for 24h to evaluate the cytotoxity. The OD_{540} was measured. NO concentration in the cultured medium was determined via the Griess reaction [2]. Specifically, 100 μl of supernatant from each well was mixed with 100μl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylendiamine in 2.5% phosphoric acid) in a separate 96-well plate. After an incubation of 10 min at room temperature, the OD_{540} was measured. The standard curve was obtained using the known concentration of sodium nitrite. In all experiments, NO_2^- concentration in wells containing medium only was measured as a blank control.

[1] H. M. Yuan, J. M. Song, X. G. Li, N. Li and J. C. Dai (2006). Immunomodulation and anti-tumor activity of κ-carrageenan oligosaccharides, *Cancer Lett*. **243**, 228–234.

[2] D. Wang, W. Tang, G. M. Yang and B. C. Cai (2010). Anti-inflammatory, antioxidant and cytotoxic activities of Flavonoids from *Oxytropis falcate* Bunge, *Chin. J. Nat. Med*. **8**, 461-465.

S5: Statistical analysis

All values are presented as mean ± standard deviation of three independent experiments. Statistical analysis for all the results is based on Student's t-test. For determination of IC_{50} value, log concentration and linear response data were analyzed by non-linear curve fitting using the Prism software package 5 (Graphad software Inc.).

S6: Spectral data of four compounds

The ethyl acetate fraction (EXT-E, 28.6g) was separated into six sub-fractions (Fr. A-F） by silica gel CC. SFr. B-D showed higher anti-inflammatory and immunosuppression activities. So, several column chromatographic techniques were used on the best active sub-fraction of SFr. B (5.6g) to find active compounds. As the results, four known compounds, namely ursolic acid (**1**, 120mg), maslimic acid (**2,** 30mg), cynanosideA (**3**, 160mg) and syringaresinol (**4**， 450mg) were isolated from SFr. Band identified as by NMR, ESI-MS spectral analysis and by comparison to literature data. These four compounds were firstly reported in this plant.

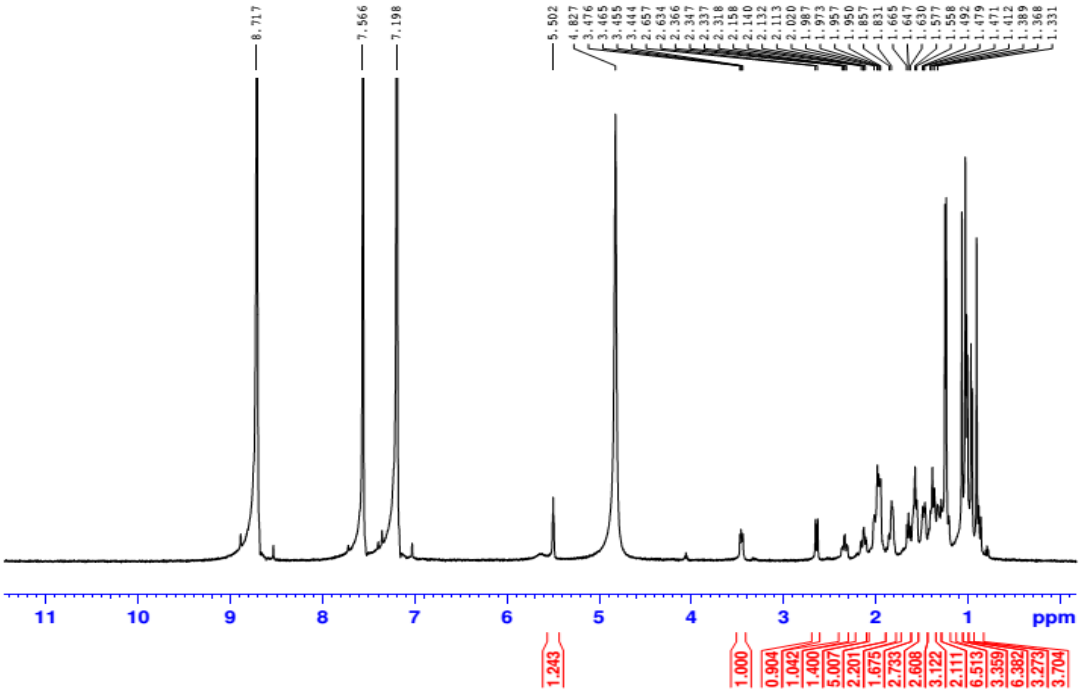
Ursolic acid (**1**): white, amorphous powder. mp. 259-261℃. IR (KBr) ν_{max}: 3477, 3072, 2961, 1698, 1642, 1473, 1386cm^{-1}. ^{1}H-NMR(C_5D_5N, 500MHz): δ3.46 (1H, m, H-3), 5.50(1H, m, H-12), 1.25(3H, s, H-23), 1.06(3H, s, H-24), 0.90(3H, s, H-25), 1.03(3H, s, H-26), 1.23(3H, s, H-27), 1.01(3H, d, J=6.5HZ, H-29), 0.96(3H, d, J=6.0HZ， H-30). ^{13}C-NMR(C_5D_5N, 125MHz): δ39.1(C-1), 28.1(C-2), 78.1(C-3), 39.5(C-4), 55.8 (C-5), 18.8(C-6), 33.6(C-7), 44.0(C-8), 48.1(C-9), 37.4(C-10), 23.9(C-11), 125.6(C-12), 139.2(C-13), 42.5(C-14), 28.8(C-15), 24.9(C-16), 48.1(C-17), 53.6(C-18), 39.4(C-19), 39.4(C-20), 31.1(C-21), 37.3(C-22), 28.7(C-23), 16.5(C-24), 15.7(C-25), 17.5(C-26,23.6 (C-27), 179.9(C-28), 17.5(C-29), 21.4(C-30); ESI-MS m/z: 455.1 $[M-H]^-$.

Maslimic acid (**2**): white, amorphous powder. mp. 267-269℃. $[a]_D^{25}$+54° (c1.0, $CHCl_3$-MeOH, 2:1); IR (KBr)ν_{max}: 3386, 2936 2867, 1690 cm^{-1}. ^{1}H-NMR(Acetone-D6, 300MHz,): δ0.81 (6H, s, H-24 and H-26), 0.91(3H, s, H-29), 0.94 (3H, s, H-30), 1.00(3H, s, H-25), 1.02 (3H, s, H-23), 1.17(3H, s, H-27), 3.60(1H, m, H-2), 2.91(1H, m, H-3), 5.25(1H, m, H-12). ^{13}C-NMR(Acetone-D_6,75MHz): δ 48.3(C-1), 69.5(C-2),84.6(C-3), 40.5(C-4), 56.8(C-5), 19.8(C-6), 34.0(C-7), 40.9(C-8), 49.1(C-9), 39.6(C-10), 24.9(C-11), 123.6(C-12), 145.7(C-13), 43.2(C-14), 29.1(C-15), 24.5(C-16), 47.5(C-17), 42.9(C-18), 47.4(C-19), 31.2(C-20), 35.1(C-21), 34.2(C-22), 29.7(C-23), 17.7(C-24), 18.0(C-25), 18.3(C-26), 26.9(C-27), 179.5(C-28), 32.0(C-29), 24.4(C-30); ESI-MS m/z: 495.3 $[M+Na]^+$.

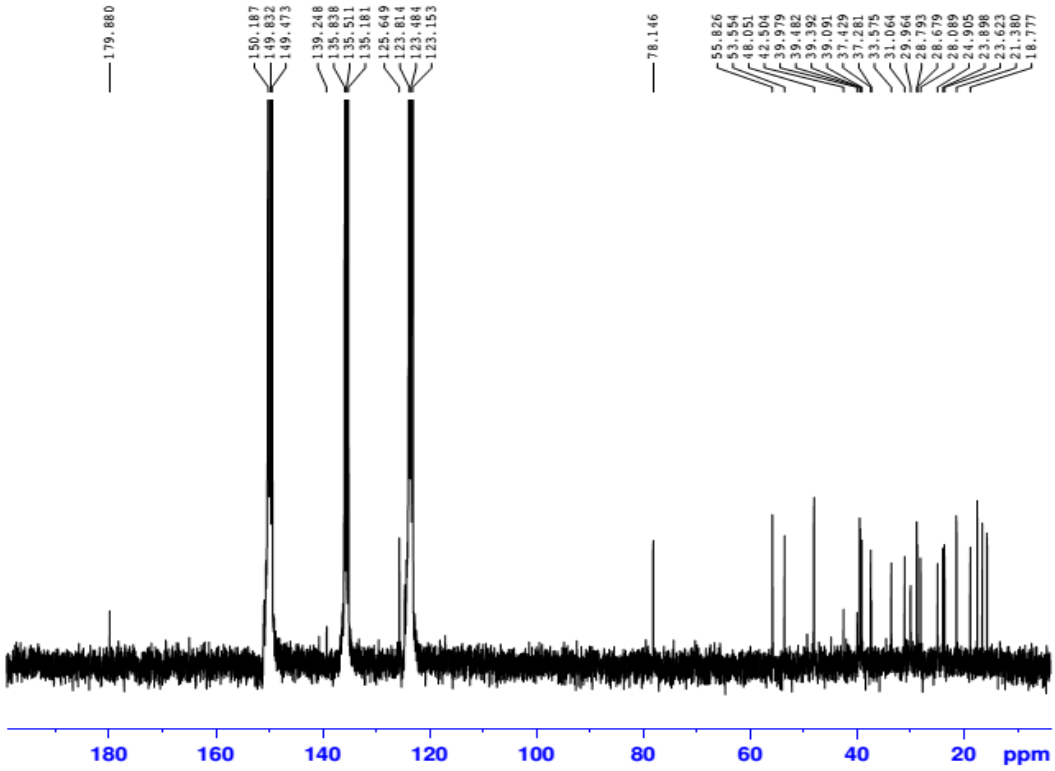
Cynanoside A (**3**): white, amorphous powder. $[a]_D^{26}$ −10.0° (c 0.4, MeOH). ^{1}H-NMR(C_5D_5N, 500MHz): δ0.92(3H, s, H-19), 1.52 (3H, s, H-21), 1.35(3H, s, H-6′), 1.36(3H, s, H-6″), 3.45(3H, s, OCH_3), 3.59(3H, s, OCH_3), 5.21(1H, d, J=9.5HZ, H-1′), 5.06(1H, d, J=9.5HZ, H-1″), 5.44(1H, m, H-2), 5.41(1H, m, H-4″), 3.74(1H,S,H-18), 3.94(1H, t, H-6). ^{13}C-NMR(C_5D_5N, 125MHz): δ44.7(C-1), 69.6(C-2), 85.4(C-3), 37.5(C-4), 139.7 (C-5), 120.7(C-6), 28.4(C-7), 40.2(C-8), 53.0(C-9), 39.4(C-10), 23.8(C-11), 30.0(C-12), 114.3(C-13), 175.3(C-14), 67.7(C-15), 75.5(C-16), 56.1(C-17), 143.8(C-18), 18.9(C-19), 24.7(C-21), 97.8(C-1′), 36.8(C-2′), 77.8(C-3′), 82.9(C-4′), 69.4(C-5′), 18.2(C-6′), 100.4(C-1″), 35.8(C-2″), 74.0(C-3″), 78.7(C-4″), 70.9(C-5″), 18.9(C-6″), 58.7(-OCH_3), 58.0(-OCH_3); ESI-MS m/z: 687.2 $[M+Na]^+$.

Syringaresinol (**4**): colourless, needles. mp. 170-172; $[\alpha]_D^{22}$+11.4° (c 0.7, $CHCl_3$). ^{1}H-NMR(C_5D_5N, 500MHz): δ6.99(4H, s, H-2, 6, 2', 6'), 3.83(12H, s, H-10, ll, 10', ll'), 4.41(2H, dd, J=5.5, 8.0Hz, H-7, 7'), 4.09(2H, dd, 7=2.5, 8.8 Hz, H-9, 9'), 3.32(2H, brs, H-8, 8'), 5.01(2H, brs, OH); ^{13}C-

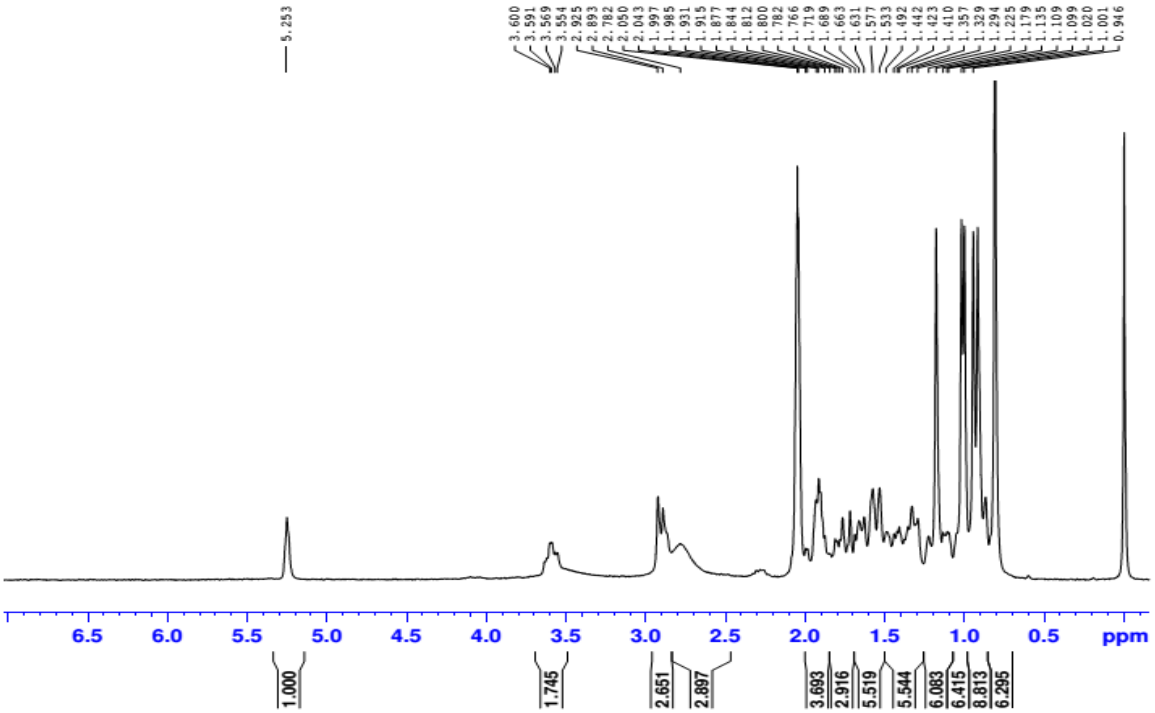
NMR(C_5D_5N,125MHz): δ132.1(C-l, 1'), 104.7(C-2, 2', 6, 6'), 149.4(C-3, 5, 3', 5'), 137.2(C-4,4'), 86.5(C-7,7'), 54.8(C-8,8'), 72.0(C-9,9'), 56.4(C-10,11,10',11'); ESI-MS m/z: 419.1 $[M+H]^+$.



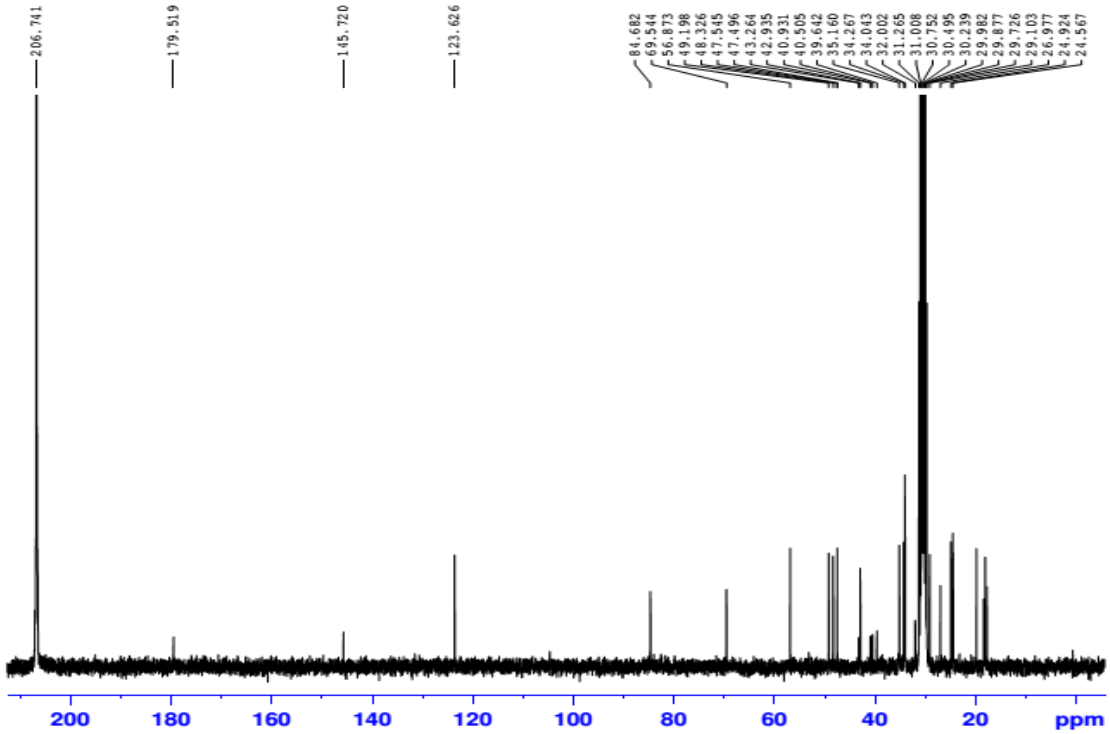
^{1}H-NMR spectrum of compound 1 (Pyridine-d_5, 500MHZ)



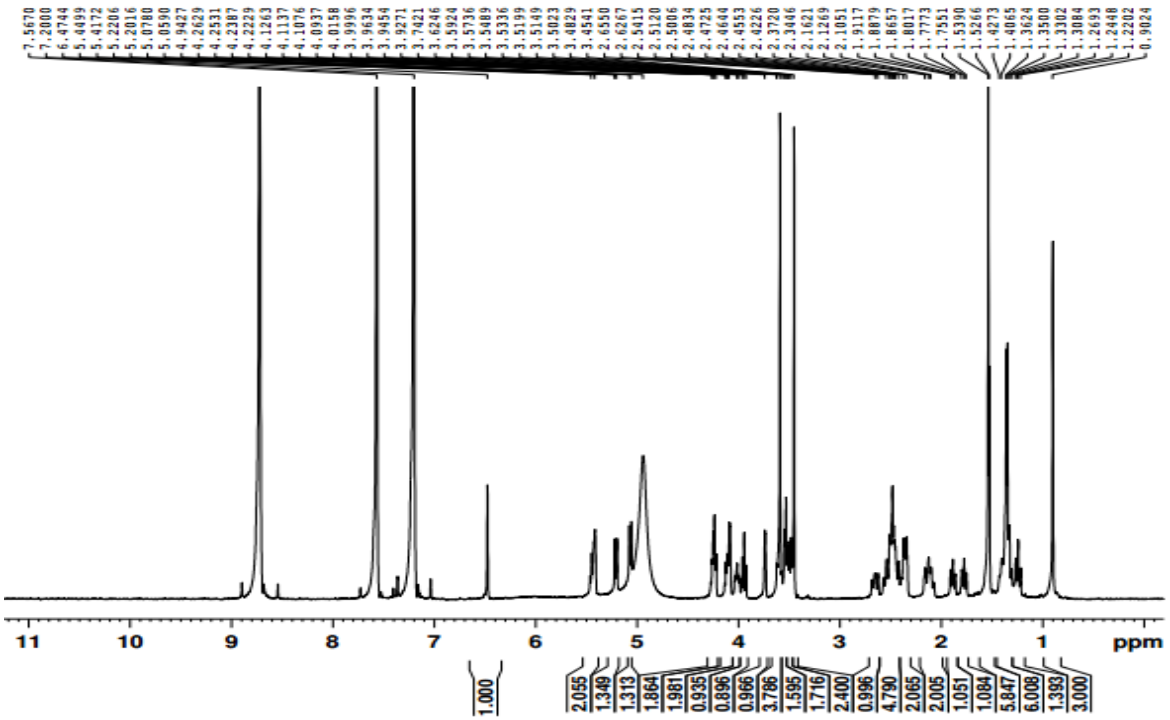
^{13}C-NMR spectrum of compound 1 (Pyridine-d_5, 125 MHZ)



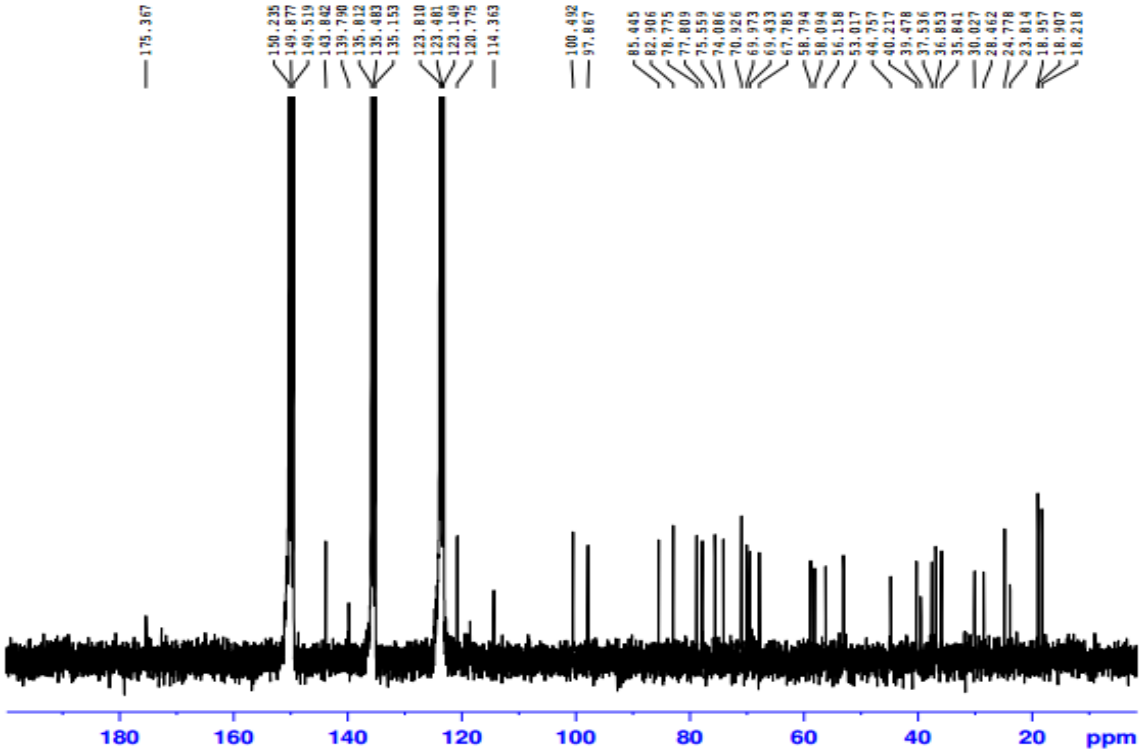
^{1}H-NMR spectrum of compound 2 (Acetone-D_6, 300MHZ)



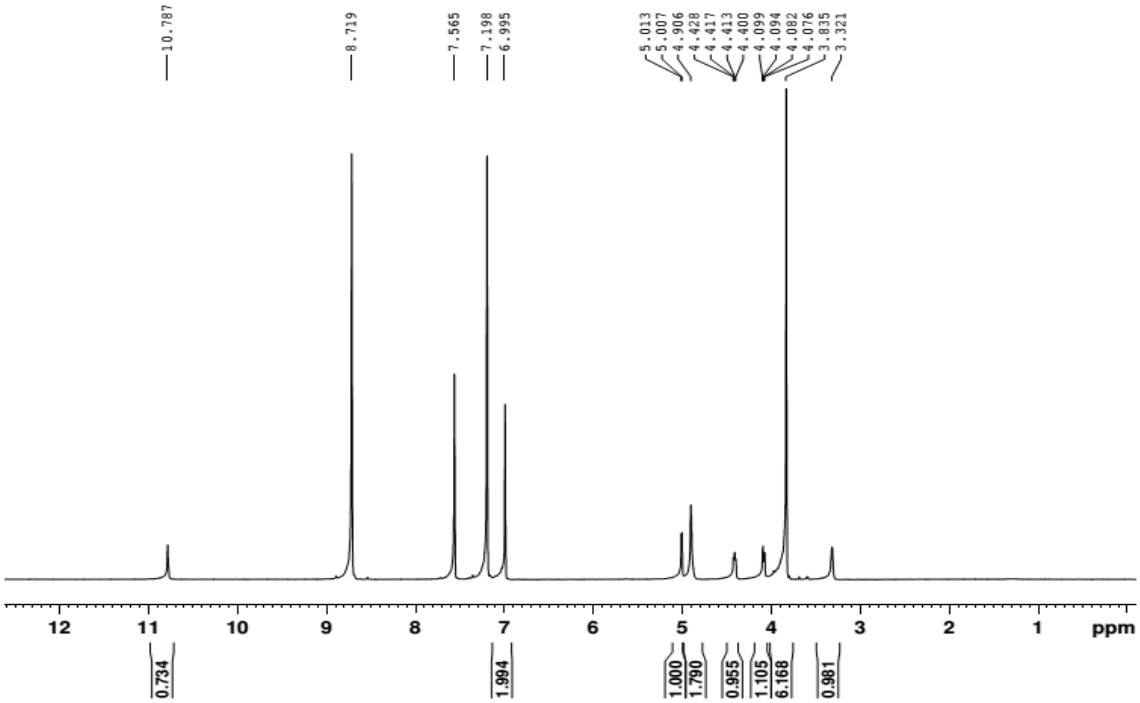
^{13}C-NMR spectrum of compound 2 (Acetone-D_6, 75 MHZ)



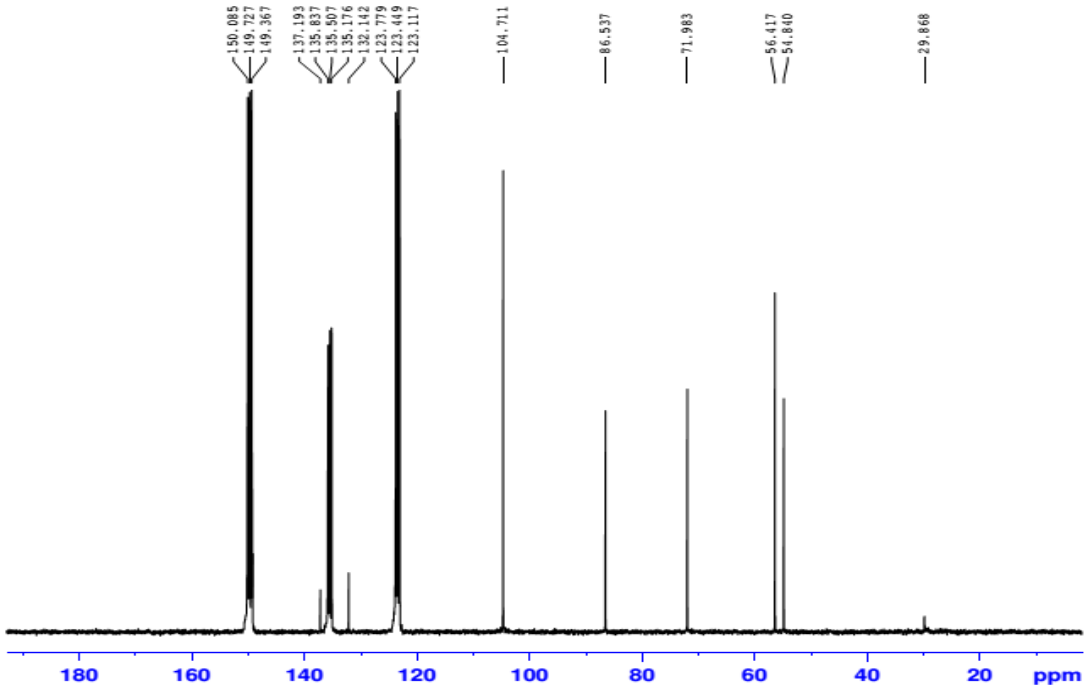
^{1}H-NMR spectrum of compound 3 (Pyridine-d_5, 500MHZ)



^{13}C-NMR spectrum of compound 3 (Pyridine-d_5, 125MHZ)



^{1}H-NMR spectrum of compound 4 (Pyridine-d_5, 500MHZ)



^{13}C-NMR spectrum of compound 4 (Pyridine-d_5, 125 MHZ)