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Supporting Information

Saponins as novel TNF-α inhibitors: Isolation of saponins and a nor-pseudoguaianolide from *Parthenium hysterophorus*

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Experimental Procedures:

MPs were measured in a Buchi-510 apparatus. ^{1}H and ^{13}C NMR spectra were recorded on Bruker 200 and 500 MHz spectrometers with TMS as the internal standard. Chemical shifts are expressed in parts per million (δ ppm); *J* values are given in Hertz. Reagents and solvents used were mostly LR (Laboratory Reagent) grade. Silica gel coated aluminum plates coated on alumina from M/s Merck were used for TLC. MS were recorded on Bruker Esquire 3000 GC-Mass spectrometer. IR was recorded on a FT-IR Bruker (270-30) spectrophotometer. Elemental analyses were performed on Elementar Vario EL-III apparatus. Optical rotations were measured on Perkin-Elmer 241 polarimeter at 25 ^{o}C using sodium D light. Flow cytometeric studies were undertaken in pharmacology division of IIIM for TNF-α estimation in murine isolated neutrophils.

Plant material: The aerial part including flowers of *P. hysterophorus* were collected locally and a voucher specimen (accession number 19513) has been deposited in the herbarium of Indian Institute of Integrative Medicine, Jammu.

Extraction and isolation: The shade dried aerial portion of the plant material including the flowers of *Parthenium hysterophorus* (1 kg) were extracted with MeOH (5 L) in a soxhlet extractor. The crude MeOH extract after concentration (97 g) was defatted with n-hexane (2 L) and the remaining extract was further extracted with chloroform (2.5 L) and re-extracted with MeOH. The crude chloroform extract after concentration (33 g) was subjected to hot water extraction (8 x 50 ml) for the isolation of parthenin (2.1 g, 0.21%). The left over extract combined with re-extracted MeOH extract was subjected to column chromatography over a silica gel (BDH, 60-120 mesh) column. Elution was carried with chloroform: MeOH (99:1 to 65:35) in increasing proportions. The eluants were collected in fractions of 50ml each. Resolution of the components in the mixture was monitored on TLC. The column chromatography was repeated for impure fractions using the same solvent system. The following compounds were isolated in order of increasing polarity: hysterolactone **3** (0.0001%), coronopilin **5** (0.015%), parthenin **4** (0.3%), 2β-hydroxy coronopilin **6** (0.0018%), 3,7-dimethoxy-3',4',5,6-tetrahydroxy flavone **7** (0.01%), saponin **1** (0.0030%) and saponin **2** (0.0050%).

Acid Hydrolysis. The isolated saponins were refluxed in 10% HCl in MeOH (10ml) for 3h. The aglycon was extracted with $CHCl_3$ and identified by comparing ^{1}H NMR, ^{13}C NMR and TLC (using chloroform: methanol, 95:5) with an authentic sample. The aqueous layer was dried and the sugars were extracted with pyridine followed by addition of acetic anhydride to afford pentacetate of glucose which was subsequently identified by ^{1}H NMR and TLC.

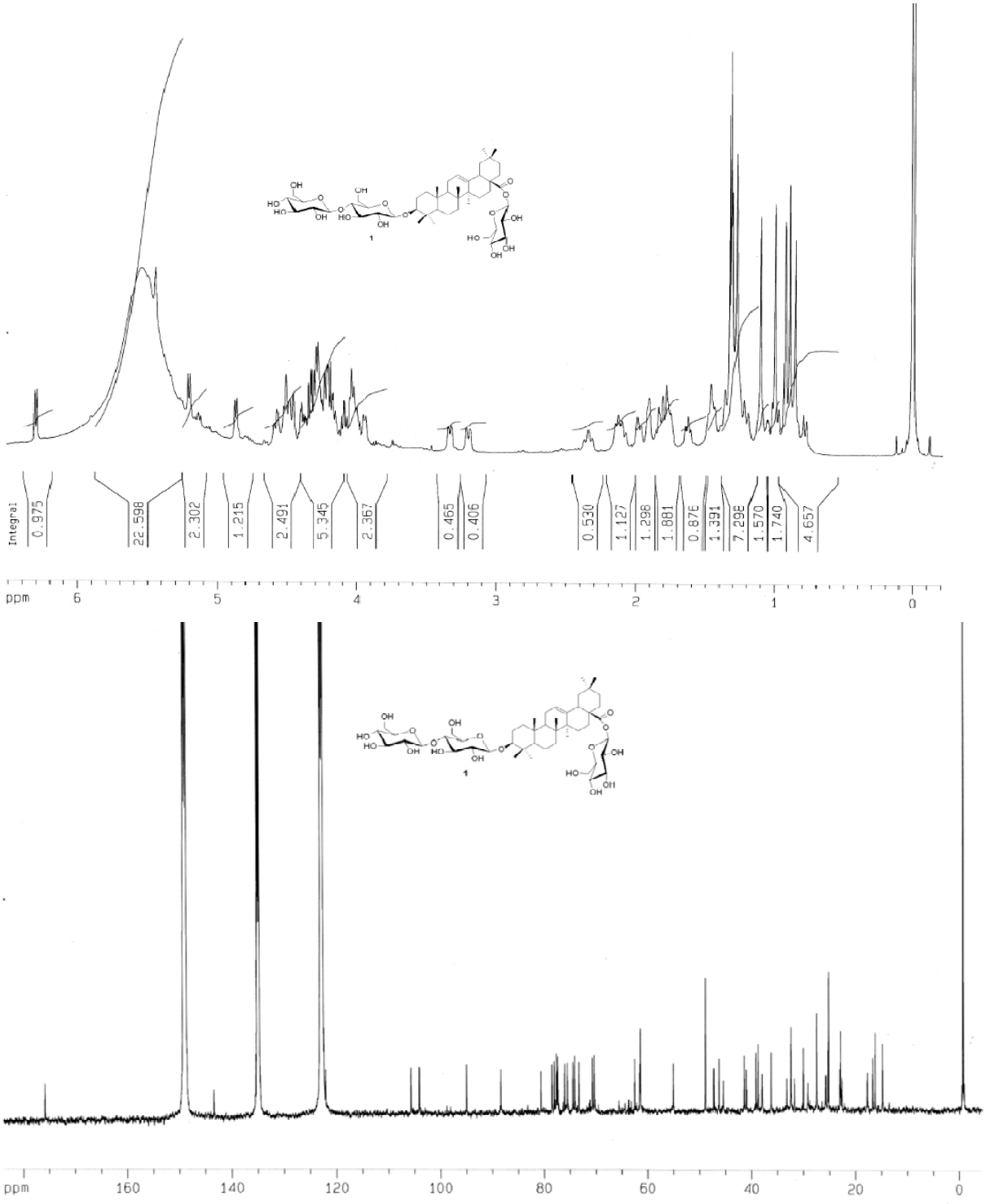
Neutrophil extraction. Blood of mice is taken from retro-orbital plexus and centrifuged at 250g for 20 min. Two layers are formed, the upper layer which is the platelet rich plasma layer is discarded and the lower layer is centrifuged at 1800 g. Three layers were formed upper layer which was the Platelet Poor Plasma layer discarded, the middle layer which appears to be the Buffy Coat layer taken and lower layer which is the RBC rich layer discarded. Middle layer which was taken is layered with Histopaque and incubated for 10 min. The samples were centrifuged at 700 g. After centrifugation two layers were observed upper layer was taken and

Facs Lysing solution added for the lyses of any traces of RBC. The samples were washed with PBS 200 g for 10 min.

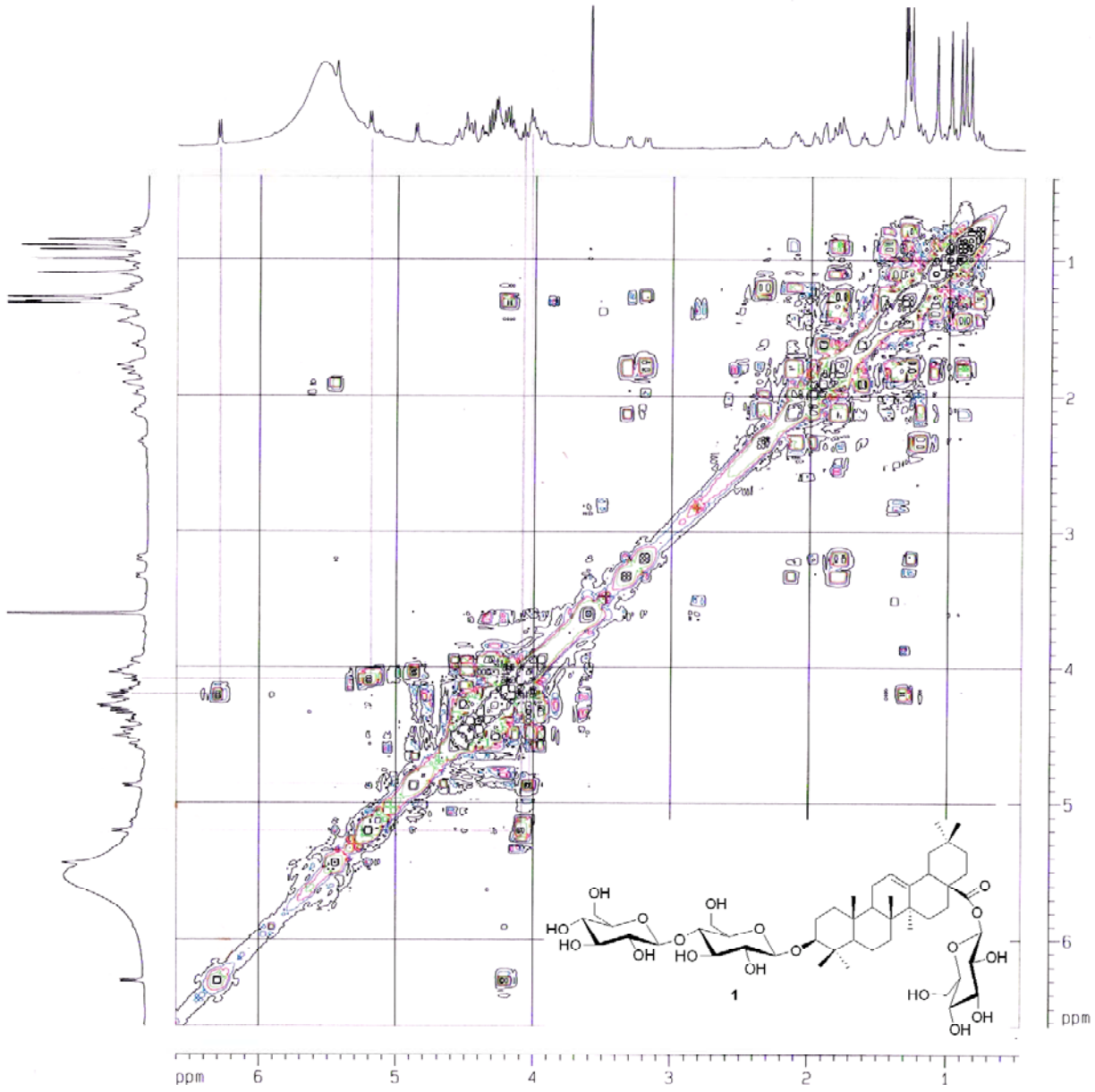
Intracellular TNF-α estimation in murine isolated neutrophils. Neutrophil sample was taken in Falcon tubes. For the stimulation of the cells LPS (10 ng/ml) followed up with the addition of Golgi plug. Test samples of the conc. (1 to 10 μg/ml) were added. Incubation of the samples carried out at 37 ºC for three hours. Permeabilising solution (1X) is added to the treated samples and incubation done for 10 min. The samples were washed with PBS. The cells then labeled with Phycoerythrin (PE) conjugated anti-mouse TNF-α monoclonal antibody. Incubation of the cells was carried out for 30 min. in dark. The cells were washed with PBS. Acquisition is carried out on BD-LSR Flow cytometer.

Docking Method. The original crystal structure of TNF-α (PDB code: 2az5) was downloaded from Protein Data Bank and was prepared for docking by adding the missing hydrogens, correcting the bond orders and removing the water molecules beyond 5Å from the centroid of the bound ligand using the protein preparation wizard of Schrodinger suite. The binding pocket of the protein was identified based upon the docked ligand. All the docking experiments were performed using Ligandfit module of Accelrys and Glide of Schrodinger suite on Irix based Silicon Graphics Fuel and Linux based Workstation respectively. The ligand molecules **1**, **2** and rolipram were prepared using the Sketcher and Mestro windows of Accelrys and Schrodinger suite respectively. Ligand minimization in Accelrys was done using Conjugate gradient algorithm whereas in Schrodinger suite, the OPLS algorithm was used. In both the cases, the ligands were kept flexible and were evaluated based on the dockscore & consensus score (Accelrys) and glidescore (Schrodinger).

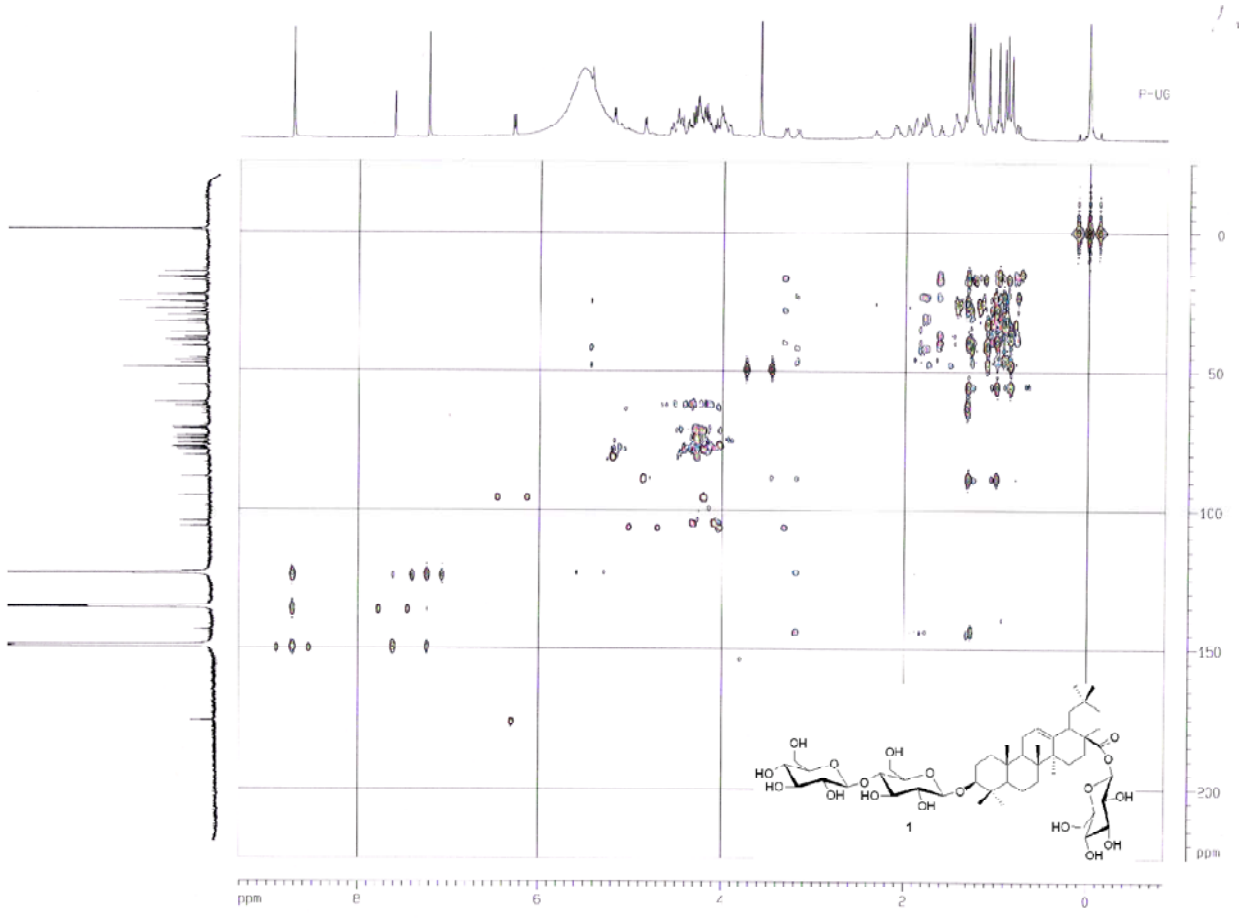
^{1}H & ^{13}C NMR of 3-O-[β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]-28-O-β-D-glucopyranosyl-oleanolic acid (1):



^{1}H-^{1}H COSY of 3-O-[β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]-28-O-β-D-glucopyranosyl-oleanolic acid (1):



HMBC of 3-O-[β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]-28-O-β-D-glucopyranosyl-oleanolic acid (1):



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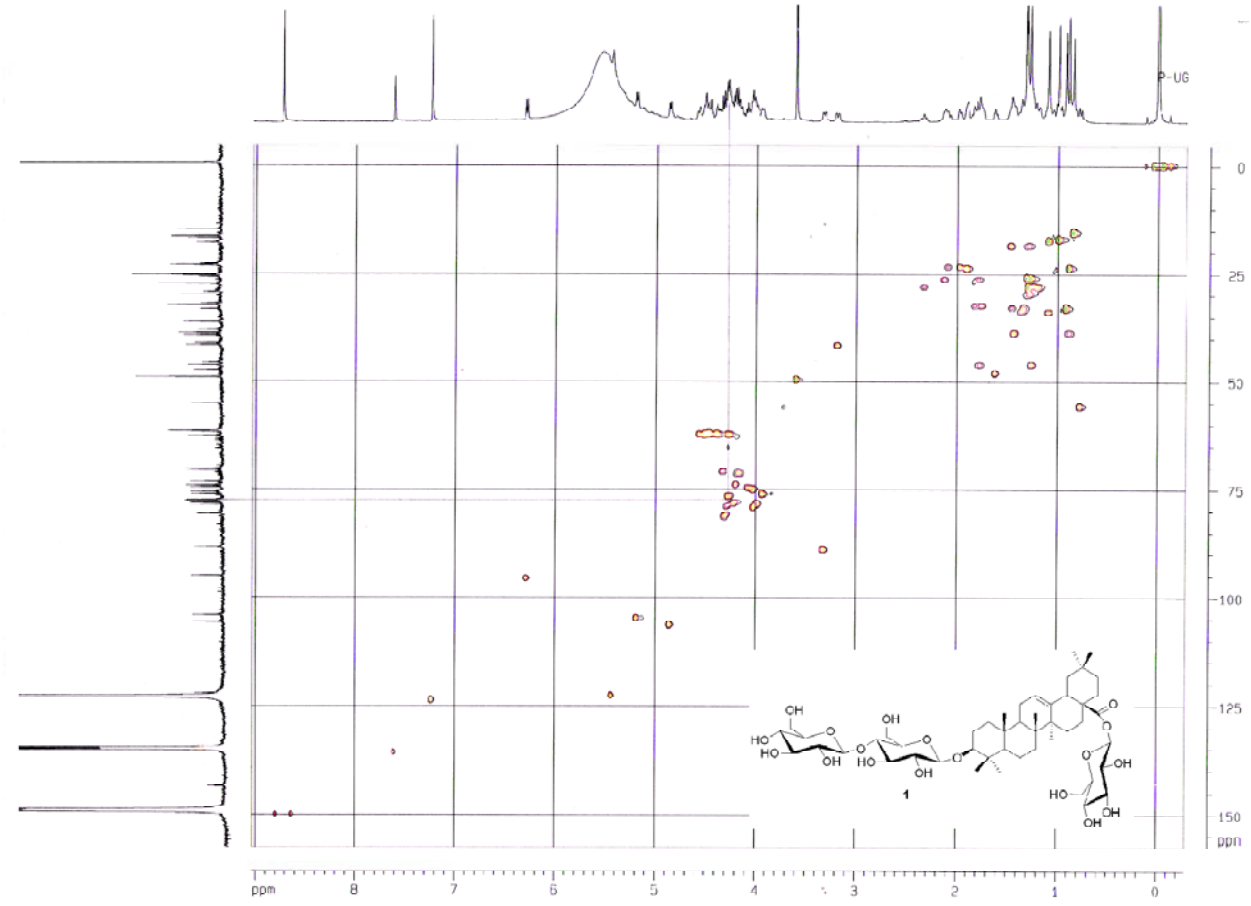
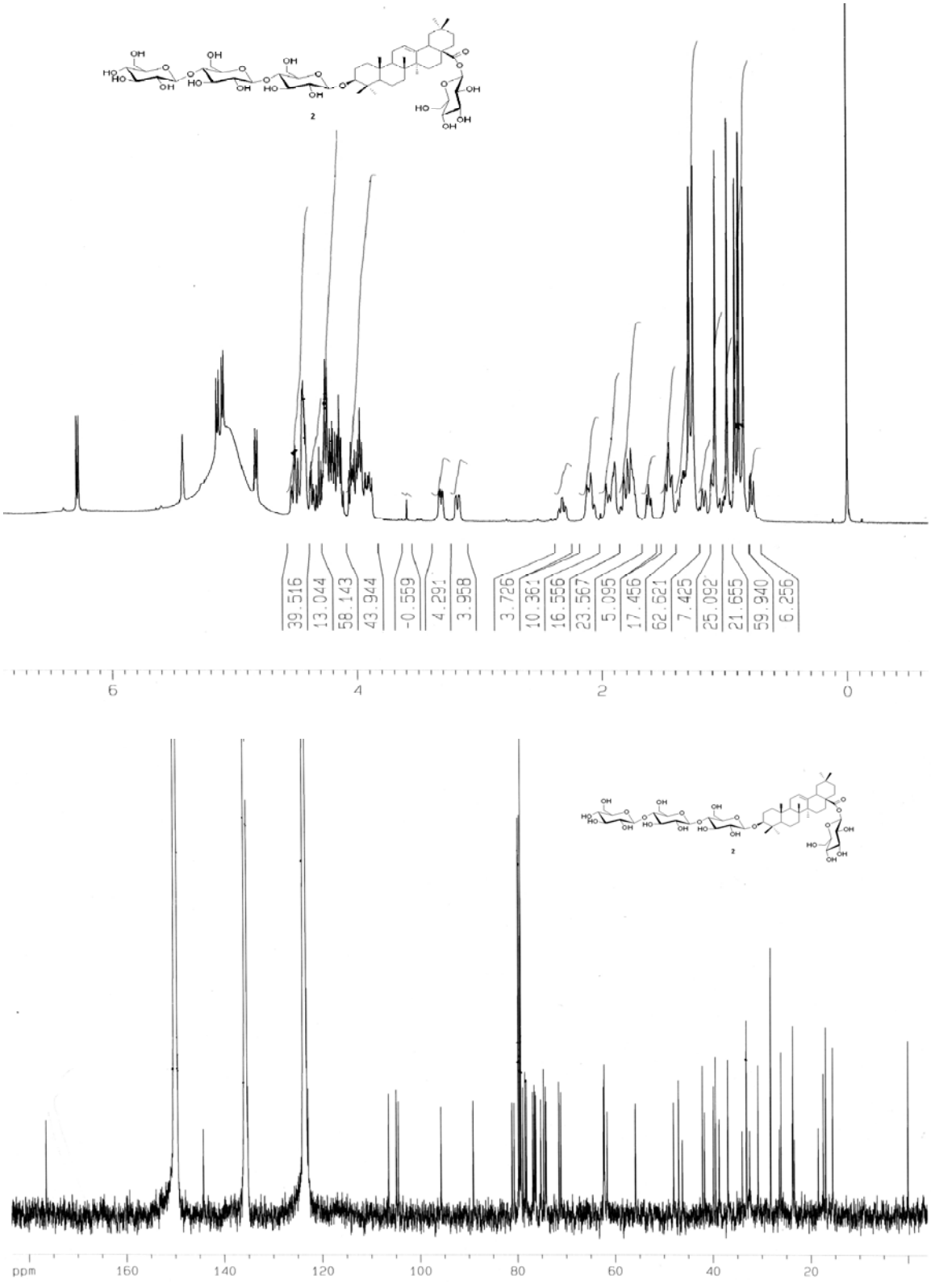
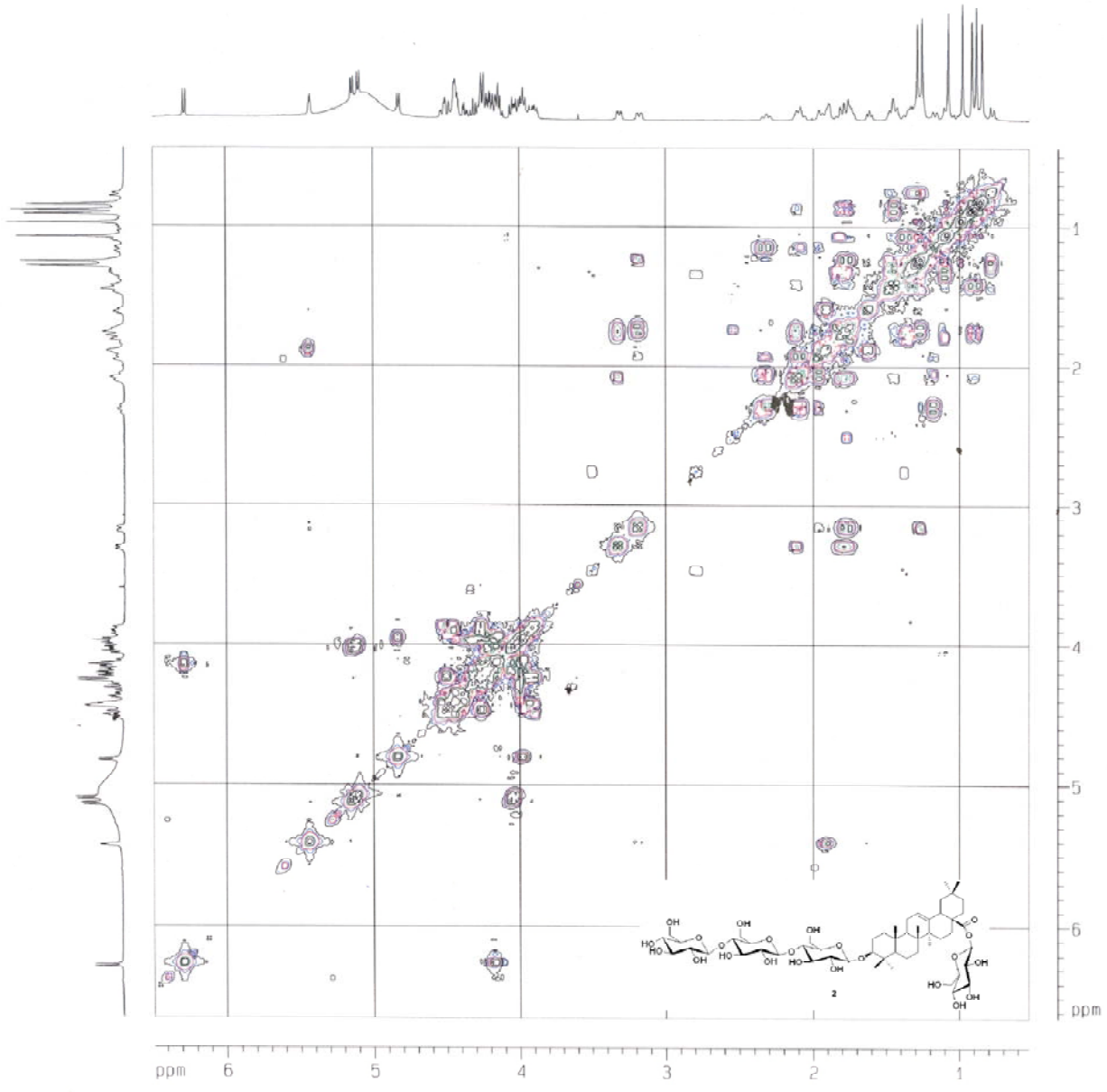


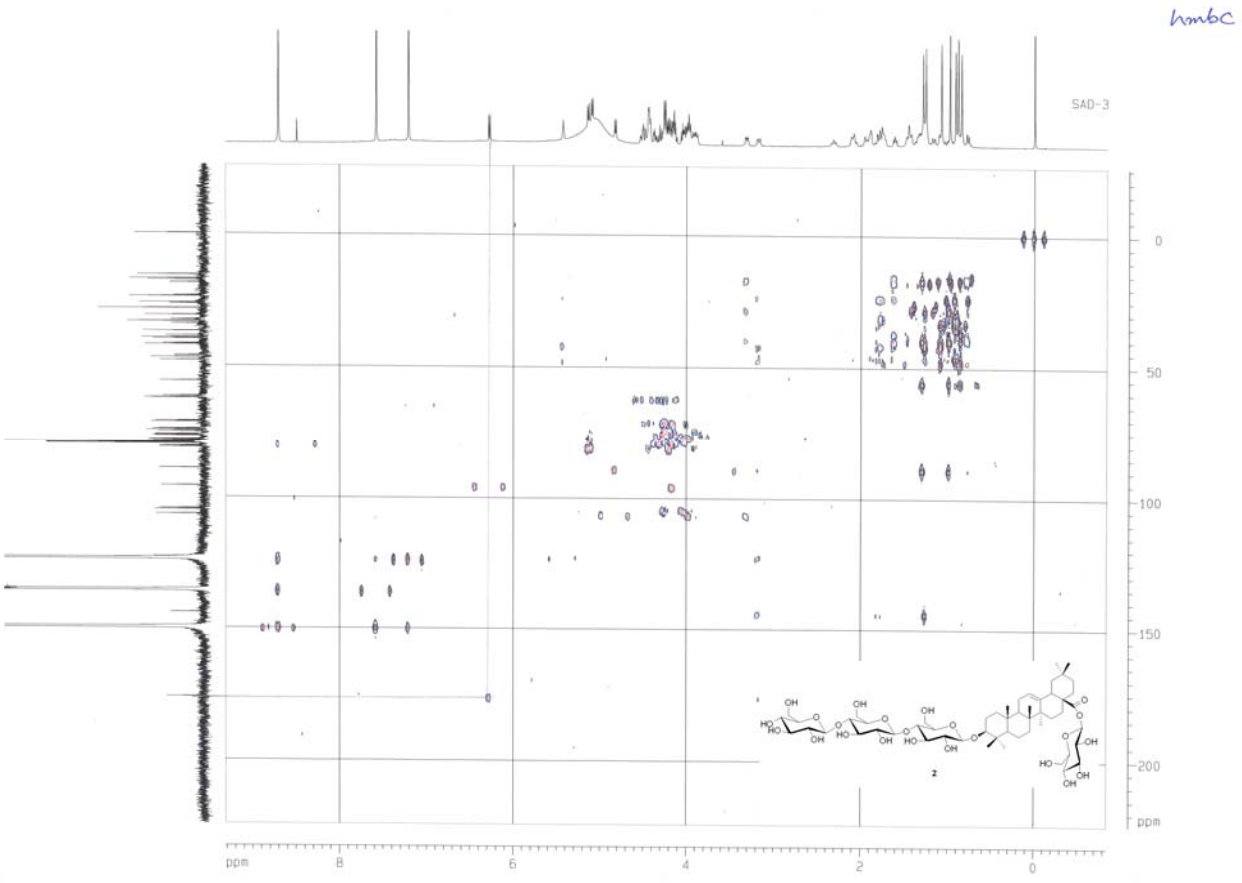
^{1}H & ^{13}C NMR of 3-O-[β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]-28-O-β-D-glucopyranosyl-oleanolic acid (2):



^{1}H-^{1}H COSY of 3-O-[β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]-28-O-β-D-glucopyranosyl-oleanolic acid (2):



HMBC of 3-O-[β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]-28-O-β-D-glucopyranosyl-oleanolic acid (2):



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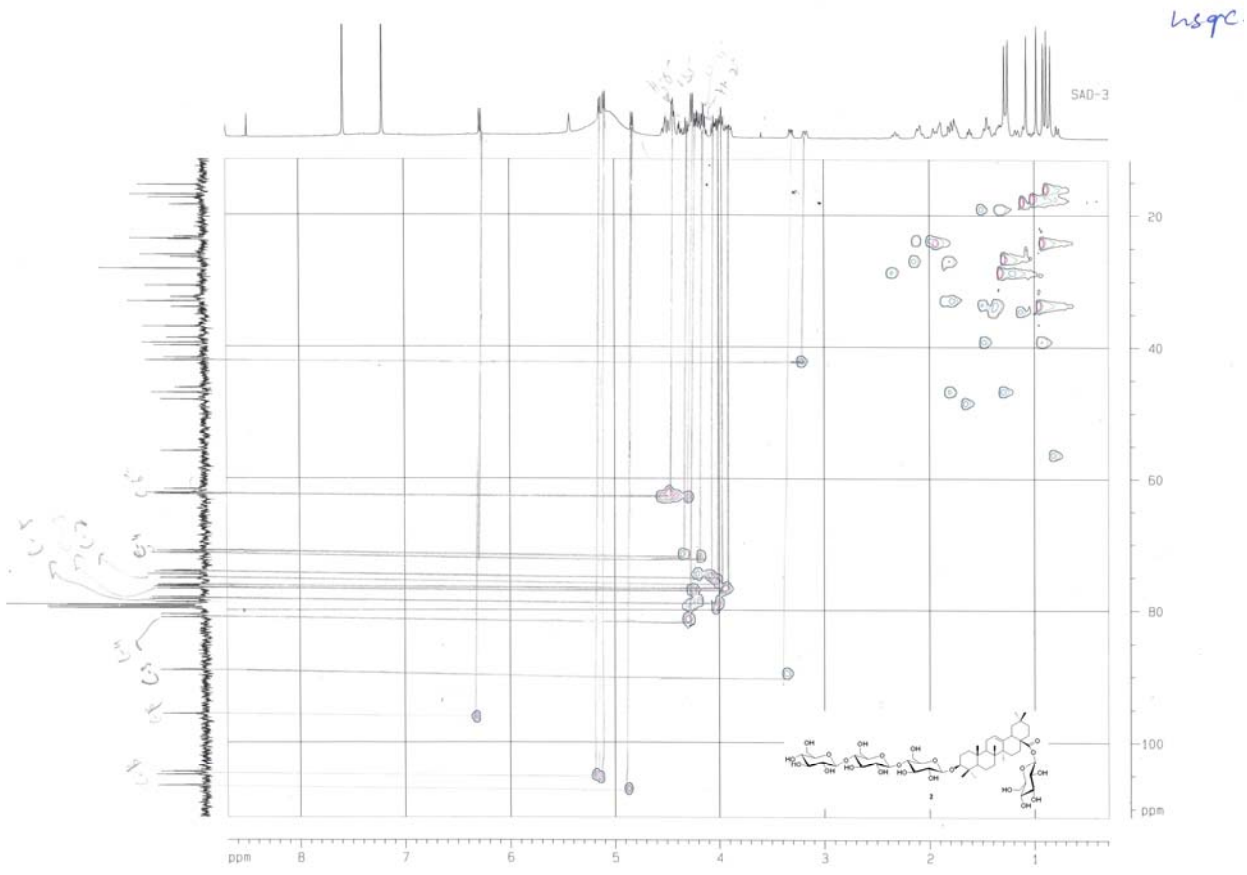
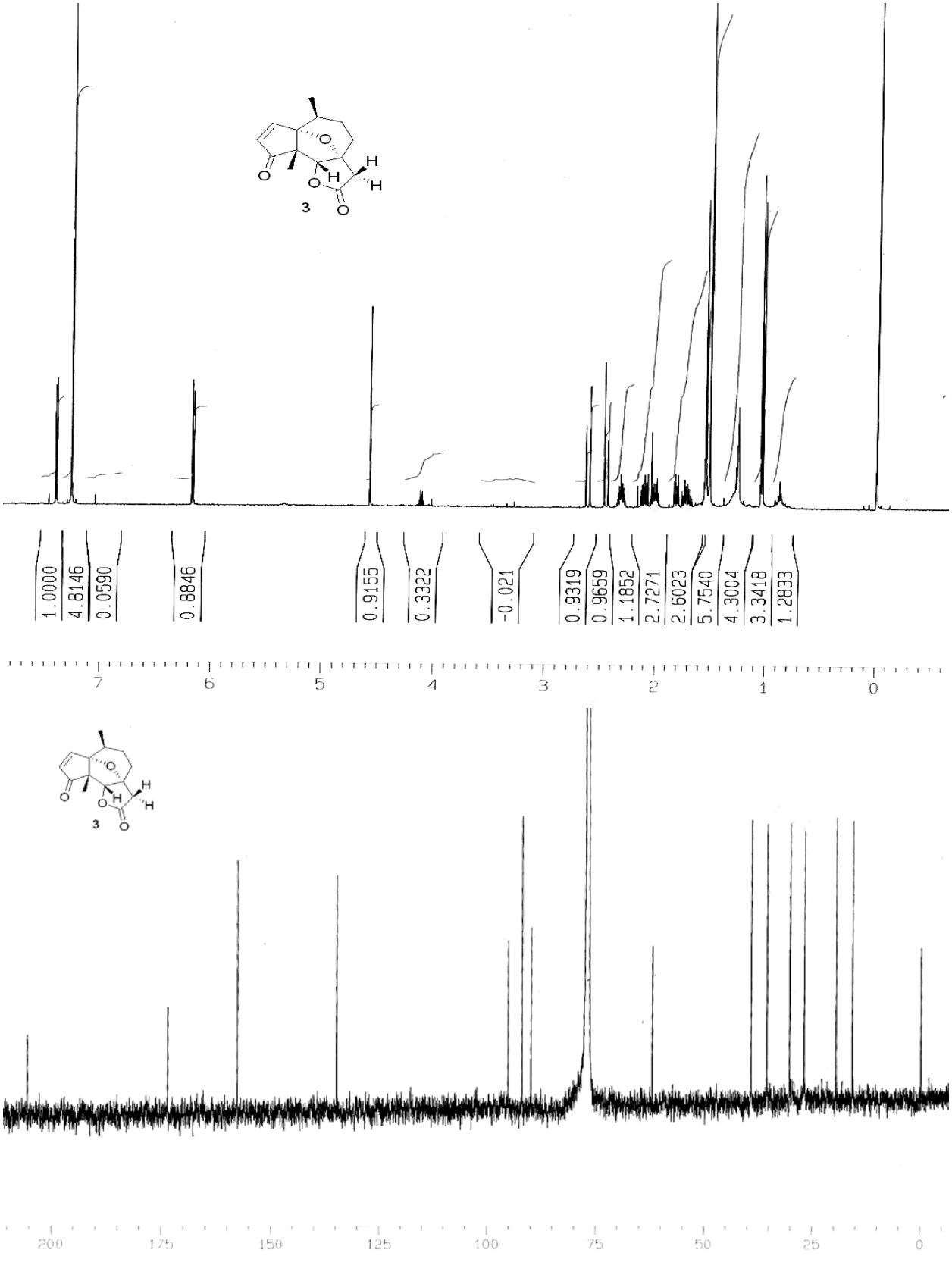
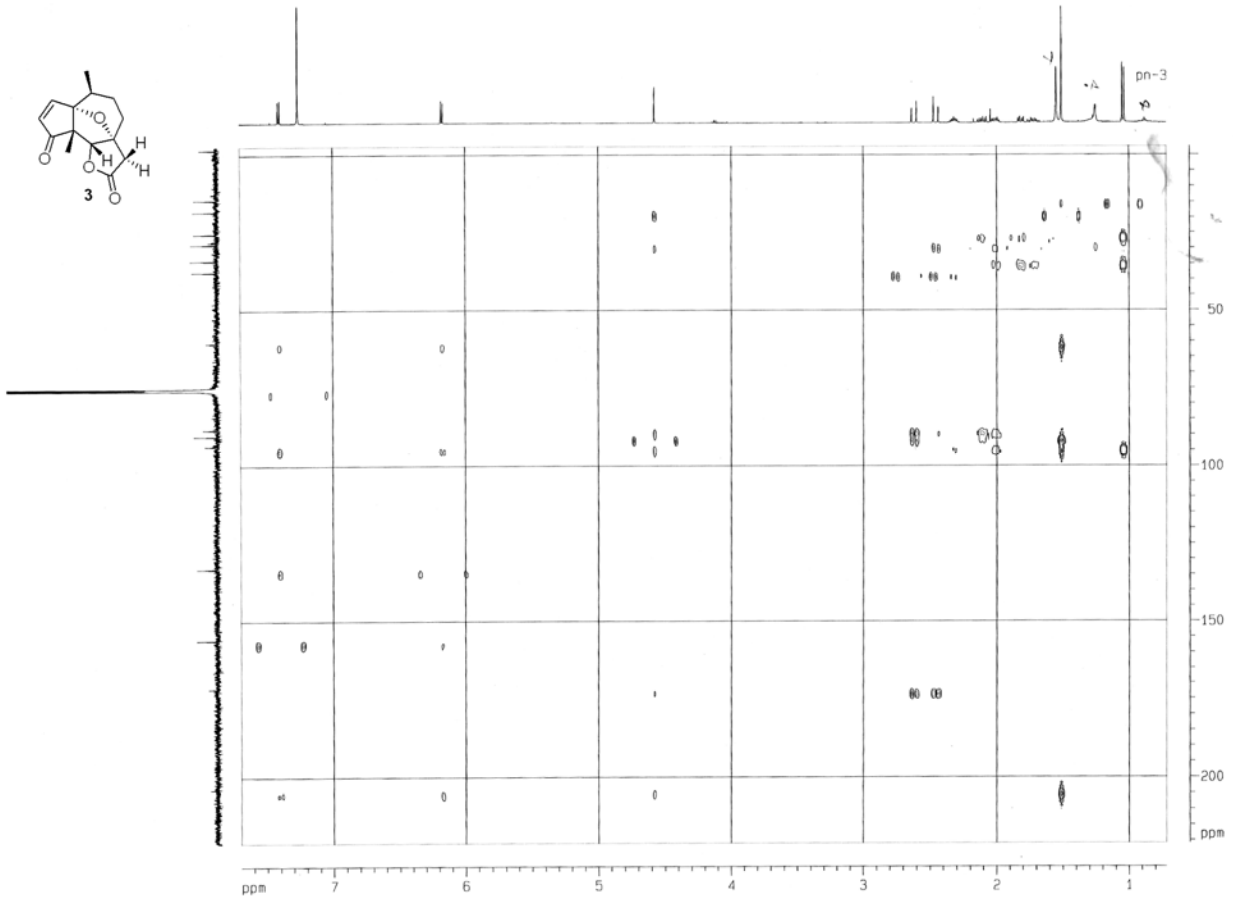


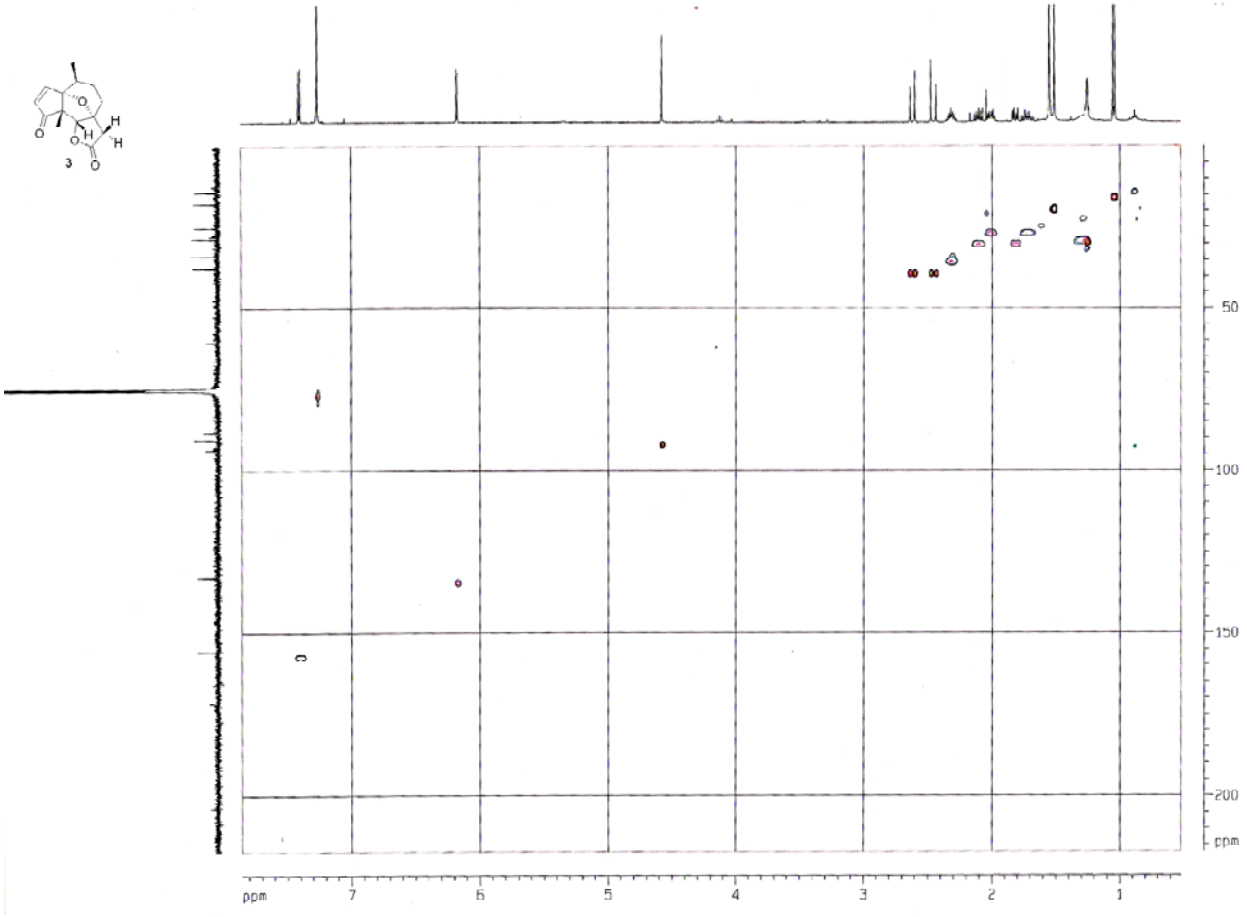
^{1}H & ^{13}C NMR of Hysterolactone (3):



HMBC of Hysterolactone (3):



HSQC of Hysterolactone (3):



NOESY of Hysterolactone (3):