Supporting Information

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Antidiabetic Flavonol Glycosides from *Eryngium caeruleum*

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1 Experimental

1.1 Chemicals and Reagents:

Silica gel (230-400 mesh, and 70-230 mesh, *E. Merck*) was used for column chromatography (CC). TLC was performed with pre-coated silica gel G-25-UV254 plates and detection was done at 254 nm, and by spraying with ammonium cerium-(IV)-sulphate-dihydrate (0.1 g) in 10 % H_2SO_4 (100 mL) solution as a spraying reagent. NADPH was purchased from Calzyme Laboratories (San Luis Obispo, USA), whereas, D,L-glyceraldehyde and *β*-mercaptoethanol were purchased from AppliChem GmbH, Darmstadt, Germany. *p*-nitrophenyl-*α*-D-glucopyranoside, *p*-nitrophenyl-*β*-D-glucopyranoside, *α*-glucosidase extracted from *Saccharomyces cerevisiae* and *β*-glucosidase extracted from sweet almonds were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the other chemicals were of analytical grade.

1.2 Instruments:

Optical rotations were measured on a *JASCO*-DIP-360 digital polarimeter by using 10 cm cell tube. UV and IR spectra were recorded on *Hitachi*-UV-3200 and *JASCO*-320-A spectrometers, respectively. ^{1}H-NMR and ^{13}C NMR spectra were recorded on a *Bruker* AM-500 spectrometer with reference to residual non-deuterated solvent signals (δ_H 3.31 and δ_C 49.0 from CD_3OD), while 2D-NMR spectra were recorded on a *Bruker* AMX 500 MHz NMR spectrometer. Chemical shift (δ) values were reported in ppm, and coupling constants (J) in Hz. Mass spectra (EI) were measured in an electron impact mode on Finnigan MAT 312 spectrometer and ions are given in *m/z*. The absorbance was measured using a Microplate Reader ELx800 (BioTek Instruments, Inc. USA).

1.3 Preparation of Materials for Biological Assays:

1.3.1 Assay for ALR1 and ALR2 inhibition:

The activities of ALR1 and ALR2 were measured spectrophotometrically by monitoring the oxidation of NADPH at 340 nm as a function of time at 37 °C using glyceraldehyde as a substrate [1]. The 200 µL assay mixture contained 70 µL of enzyme, 20 µL of test compound, 20 µL of 100 mM sodium phosphate buffer of pH 6.2, 40 µL of 10 mM D,L-glyceraldehyde, and 0.15 mM NADPH. Enzymatic reactions were run in triplicate with a final volume of 200 µL in each well. Absorbances were noted and IC_{50} values were calculated using statistical software GraphPad Prism 5.00 (GraphPad Software, San Diego, CA, USA). The total percentage inhibition was calculated by the formula:

$$\% \text{ Inhibition} = (100 - [(\text{Absorbance}_{\text{sample}}/\text{Absorbance}_{\text{control}}) \times 100])\%$$

Valproic acid and Sulindac were used as positive controls for ALR1 and ALR2 assays, respectively.

1.3.2 Extraction of ALR1

Bovine kidneys were obtained from a freshly slaughtered animal, within 2 h of killing. The tissues were stored at -20 °C after cutting into smaller pieces. All these operations were carried out at 4 °C. 200 g of tissues were taken and homogenized in 3 volumes of 10 mM sodium phosphate buffer (pH 7.2) composed of 0.25 M sucrose, 2.5 mM 2-mercaptoethanol, and 2.0 mM EDTA in a domestic blender followed by centrifugation at 10,000 g at 4 °C for 20 minutes. The resulting pellet was discarded and the supernatant was again centrifuged at 10,000 g at 4 °C for 30 minutes. Again the supernatant was decanted and added to 0.25

volume of 0.5 M sodium phosphate buffer (pH 7.2). This mixture was saturated up to 35% with powdered $(NH_4)_2SO_4$ and the resulting suspension was stirred for 20 minutes followed by centrifugation at 10,000 g at 4 °C for 20 minutes. The pellet was again discarded and the powdered $(NH_4)_2SO_4$ was added slowly to the supernatant up to 80% saturation to precipitate out the ALR1 [2]. After centrifugation at 10,000 g for 20 minutes, the pellet was re-suspended in a small volume of 10 mM sodium phosphate buffer containing 2 mM EDTA and 2.5 mM 2-mercaptoethanol at pH 7.2. This solution was dialyzed overnight in dialysis membrane against the same buffer to extract out the desired ALR1 enzyme and was stored in small aliquots at -80 °C.

1.3.3 Extraction of ALR2

Calf lenses of freshly slaughtered bovine were used for this purpose. All the steps were performed at 4 °C. The enzyme was extracted and purified with some modifications in the protocol developed by Kador [3]. Calf lenses (100 g) were mixed with 10 volumes of 10 mM phosphate buffer (pH 7.0) containing 1 mM 2-mercaptoethanol and 1M ethylene diamine tetra acetic acid (EDTA), and centrifuged after homogenization at 18,000 g at 4 °C for 20 minutes. Powdered ammonium sulfate was added slowly to the resultant supernatant to yield a 35% saturated solution. After 3 h stirring, the solution was centrifuged at 10,000 g for 20 minutes to yield the precipitates. These precipitates were dissolved in phosphate buffer and were dialyzed for 48 h in the same buffer. The required ALR2 enzyme was obtained from the solution present in the dialysis membrane and was stored in small aliquots at -80 °C.

1.3.4 Assay for *α*- and *β*-glucosidase inhibition:

The inhibitory activities of *α*- and *β*-glucosidases were determined by slight modification of the previously published method [4]. The inhibition assays were performed in 96-well plates in a total volume of 100 µL. In case of *α*-glucosidase inhibitory assay the standard solutions of the inhibitors were prepared in methanol while these were prepared in distilled water in case of *β*-glucosidase inhibition. *α*-glucosidase (from *Saccharomyces cerevisiae*) and *p*-nitrophenyl-*α*-D-glucopyranoside as substrate were prepared in 70 mM phosphate buffer (pH 6.8) while *β*-glucosidase (from sweet almonds) and *p*-nitrophenyl-*β*-D-glucopyranoside as substrate were prepared in 0.07 M phosphate buffer having a pH of 6.8. The inhibition assays were conducted by adding 10 µL of inhibitor solution to the 70 µL buffer and 10 µL of enzyme solution (2.5 unit/mL) in 70 mM phosphate buffer (pH 6.8) followed by pre-incubation at 37 °C for 5 minutes. After pre-incubation, 10 µL of 10 mM *p*-nitrophenyl glucopyranoside as a substrate in phosphate buffer was added to the mixture to start the reaction. The reaction mixture was incubated at 37 °C for 30 minutes and then stopped by adding 80 µL of 0.2 M Na_2CO_3. Negative control contained 10 µL of methanol in case of *α*-glucosidase assay and distilled water in case of *β*-glucosidase assay instead of inhibitors. Acarbose was used as a positive control. The *α*- and *β*-glucosidase activities were determined by measuring the *p*-nitrophenol released from *p*-nitrophenyl glucopyranoside at 405 nm. The % inhibition was calculated by the following equation:

$$\% \text{ Inhibition} = (100 - [(\text{Absorbance}_{\text{sample}}/\text{Absorbance}_{\text{control}}) \times 100])\%$$

IC_{50} values were calculated using non-linear curve fitting program GraphPad Prism 5.00. The IC_{50} value was defined as the concentration of sample that inhibited 50% of glucosidase activity.

1.3.5 Antiglycation Assay:

For antiglycation activity, both the compounds were subjected to the method described earlier [5]. In this assay, 500 µL of 1 mg/mL albumin was incubated with 400 µL of 500 mM glucose along with 100 µL of the test compounds. The reaction was proceeded for 24 h at 60 °C, followed by addition of 10 µL of trichloroacetic acid (TCA) in pure form to stop the reaction. The samples were kept for 10 minutes at 4 °C followed by centrifugation for 10 minutes at 10,000 g. The resulting supernatant was discarded and the pellet was re-dissolved in 500 µL phosphate buffer solution at pH 7.4; fluorescence was measured at 370 nm for excitation and 440 nm for emission. The reaction was carried out in triplicate. The following formula was used to determine the percentage inhibition of the compounds:

$$\% \text{ Inhibition} = [1 - (\text{Absorbance}_{\text{sample}}/\text{Absorbance}_{\text{control}}) \times 100]\%$$

1.4 Spectroscopic data and characterization

1.4.1 Compound 1

Pale yellow powder; $C_{30}H_{26}O_{13}$; UV (MeOH) λ_{max}: 365, 354, 313, 299, 268. IR (KBr) υ_{max}: 3391 (OH), 1695 (C=O), 1259, 1171, 1080 (ether linkage) cm^{-1}. ^{1}H-NMR (300 MHz, CD_3OD) δ: 6.12 (d, *J* = 1.5 Hz, 1H, H-6), 6.30 (br. s, 1H, H-8), 6.79 (d, *J* = 8.4 Hz, 2H, H-3′/H- 5′), 7.98 (d, *J* = 8.7 Hz, 2H, H-2′/H-6′), 5.23 (d, *J* = 7.2 Hz, 1H, H-1″), 3.43–3.46 (m, 3H, H-2″/H-3″/H-5″), 3.31 (masked, H-4″), 4.27 (dd, *J* = 1.5, 11.7 Hz, 1H, H-6″), 4.17 (dd, *J* = 6.6, 12.0 Hz, 1H, H-6″), 6.06 (d, *J* = 15.9 Hz, 1H, H-2‴), 7.39 (d, *J* = 15.9 Hz, 1H, H-3‴), 7.30 (d, *J* = 8.7 Hz, 2H, H-5‴/H-9‴), 6.79 (t, *J* = 8.4 Hz, 2H, H-6‴/H-8‴). ^{13}C-NMR (125 MHz, CD_3OD) δ: 158.4 (C-2), 135.1 (C-3), 179.4 (C-4), 163.0 (C-5), 99.9 (C-6), 165.9 (C-7), 94.8 (C-8), 159.3 (C-9), 105.6 (C-10), 122.7 (C-1′), 131.2 (C-2′/C-6′), 116.7 (C-3′/C-5′), 161.2 (C-4′), 103.9 (C-1″), 75.8 (C-2″), 75.7 (C-3″), 71.7 (C-4″), 77.9 (C-5″), 64.2 (C-6″), 168.7 (C-1‴), 114.7 (C-2‴), 146.5 (C-3‴), 127.0 (C-4‴), 132.2 (C-5‴/C-9‴), 116.0 (C-6‴/C-8‴), 161.5 (C-7‴). EI-MS *m*/*z*: 594.14 $[M]^+$.

1.4.2 Compound 2

Pale yellow powder; $C_{39}H_{32}O_{15}$; UV (MeOH) λ_{max}: 365, 354, 313, 299, 268. IR (KBr) υ_{max}: 3391 (OH), 1696 (C=O), 1259, 1170, 1080 (ether linkage) cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) δ: 6.06 (d, *J* = 2.0 Hz, 1H, H-6), 6.26 (d, *J* = 2.5 Hz, 1H, H-8), 6.80 (d, *J* = 8.5 Hz, 2H, H-3′/H-5′), 7.95 (d, *J* = 9.0 Hz, 2H, H-2′/H-6′), 5.62 (d, *J* = 8.0 Hz, 1H, H-1″), 5.06 (dd, *J* = 8.0, 9.5 Hz, 1H, H-2″), 3.65 (dd, *J* = 9.0, 9.0 Hz 1H, H-3″), 3.40 (dd, *J* = 9.0, 9.0 Hz, 1H, H-4″), 3.53 (ddd, *J* = 2.0, 6.5, 9.5, 1H, H-5″), 4.34 (dd, *J* = 2.5, 12.0 Hz, 1H, H-6″), 4.21 (dd, *J* = 6.5, 12.0 Hz, 1H, H-6″), 6.42 (d, *J* = 16.0 Hz, 1H, H-2‴), 7.71 (d, *J* = 16.0 Hz, 1H, H-3‴), 7.47 (d, *J* = 8.5 Hz, 2H, H-5‴/H-9‴), 6.80 (d, *J* = 8.5 Hz, 2H, H-6‴/H-8‴), 6.06 (d, *J* = 16.0 Hz 1H, H-2⁗), 7.39 (d, *J* = 16.0 Hz, 1H, H-3⁗), 7.30 (d, *J* = 8.5 Hz, 2H, H-5⁗/H-9⁗), 6.80 (d, *J* = 8.5 Hz, 2H, H-6⁗/H-8⁗). ^{13}C-NMR (125 MHz, CD_3OD) δ: 158.9 (C-2), 134.4 (C-3), 179.1 (C-4), 163.0 (C-5), 99.9 (C-6), 165.6 (C-7), 94.6 (C-8), 158.3 (C-9), 105.7 (C-10), 122.8 (C-1′), 132.7 (C-2′/C-6′), 116.1 (C-3′/C-5′), 161.4 (C-4′), 100.3 (C-1″), 75.6 (C-2″), 76.1 (C-3″), 71.9 (C-4″), 75.9 (C-5″), 64.0 (C-6″), 168.8 (C-1‴), 115.2 (C-2‴), 147.0 (C-3‴), 127.0 (C-4‴), 131.2 (C-5‴/C-9‴), 116.8 (C-6‴/C-8‴), 161.2 (C-7‴), 168.7 (C-1⁗), 114.6 (C-2⁗), 146.5 (C-3⁗), 127.0 (C-4⁗), 131.2 (C-5⁗/C-9⁗), 116.8 (C-6⁗/C-8⁗), 161.3 (C-7⁗). EI-MS *m*/*z*: 740.17 $[M]^+$.

2 Results and Discussion

The ethyl acetate soluble fraction of the air dried aerial parts of *Eryngium caeruleum* was subjected to column chromatography which resulted into the isolation of two new source flavone glycosides. The structures of these compounds were established by MS, 1D- and 2D-NMR, IR, and UV spectra.

2.1 Structure elucidation of compounds 1 and 2

Compound **1** (Figure 1 in manuscript) was obtained as a yellow powder. Structure of the compound was established on the basis of ^{1}H- and ^{13}C-NMR spectra, including 2D-NMR techniques, and electron impact (EI) mass spectrometry.

The molecular formula of compound **1** was assigned as $C_{30}H_{26}O_{13}$ on the basis of EI-MS (molecular ion peak at *m/z* 594.1) and the number of carbons were confirmed on the basis of ^{13}C-NMR (BB and DEPT) spectra. The IR spectrum showed the absorption bands typical of OH groups (3420 cm^{-1}), C–H stretching (2926 cm^{-1}), *α*,*β*-unsaturated CO (1690 and 1649 cm^{-1}), and of an *O*-glycosidic linkage (1106–1025 cm^{-1}). The UV-spectrum was recorded in MeOH and indicated the characteristic absorption maxima for kaempferol 3-*O*-glycoside at 266, 312, and 354 nm [6]. The maximum at 314 nm corresponded to the *p*-coumaroyl moiety [7].

The ^{1}H- and ^{13}C-NMR data indicated that compound **1** was a flavone glycoside. The anomeric proton signal of the sugar moiety resonated at δ 5.23 (d, J = 7.2 Hz) with corresponding anomeric carbon signal at δ 103.9. The ^{1}H-NMR spectrum showed two proton signals at δ 6.12 (d, J = 1.5 Hz) and 6.30 (br. s) which correlated to δ 100.5 and 95.3, respectively, in the HSQC experiment. These are two characteristic chemical shift values for C-6 and C-8 in the ring A of flavonoids [8]. Also, the signals at δ 6.79 (d, J = 8.4 Hz) and 7.98 (d, J = 8.7 Hz) in the aromatic region, with integration of two for each proton, confirmed two pairs of chemically equivalent protons in the ring B of flavonoid moiety. The chemical-shifts and *J*-values for the aromatic protons together with their corresponding ^{13}C-NMR chemical shifts confirmed that the flavonoid moiety was a kaempferol [9]. In ^{13}C-NMR spectrum, the relatively upfield chemical shift value of the C-3 (δ 135.1) indicated the point of glycosylation at C-3 position of kaempferol [10]. The coupling constant of the anomeric proton and the corresponding carbon resonance indicated that the molecule contained a *β*-D-glucopyranosyl moiety.

The ^{1}H-NMR spectrum of **1** also contained signals at δ 6.06 (d, J = 15.9 Hz), 7.39 (d, J = 15.9 Hz), 7.30 (d, J = 8.7 Hz), and 6.79 (d, J = 8.4 Hz). The ^{13}C-NMR spectrum had the corresponding resonances at δ 114.7, 146.5, 132.2, and 116.0. Furthermore, a carbonyl signal at δ 168.7 and two quaternary carbon signals at δ 127.0 and 161.5 were also observed. These observations confirmed the presence of a (*E*)-*p*-coumaroyl moiety in compound **1**. As the H-6″ signals of the glucopyranosyl moiety were deshielded at δ 4.27 (br. dd, J = 1.5, 11.7 Hz), 4.17 (br. dd, J = 6.6, 12.0 Hz), C-6″ must be the point of attachment of the (*E*)-*p*-coumaroyl moiety. Accordingly, the C-6″ chemical shift was shifted downfield at δ 64.2. All the attachments were further confirmed by HMBC experiments. Consequently, the structure of compound **1** was established as kaempferol 3-*O*-[6-*O*-*E*-*p*-coumaroyl]-*β*-D-glucopyranoside

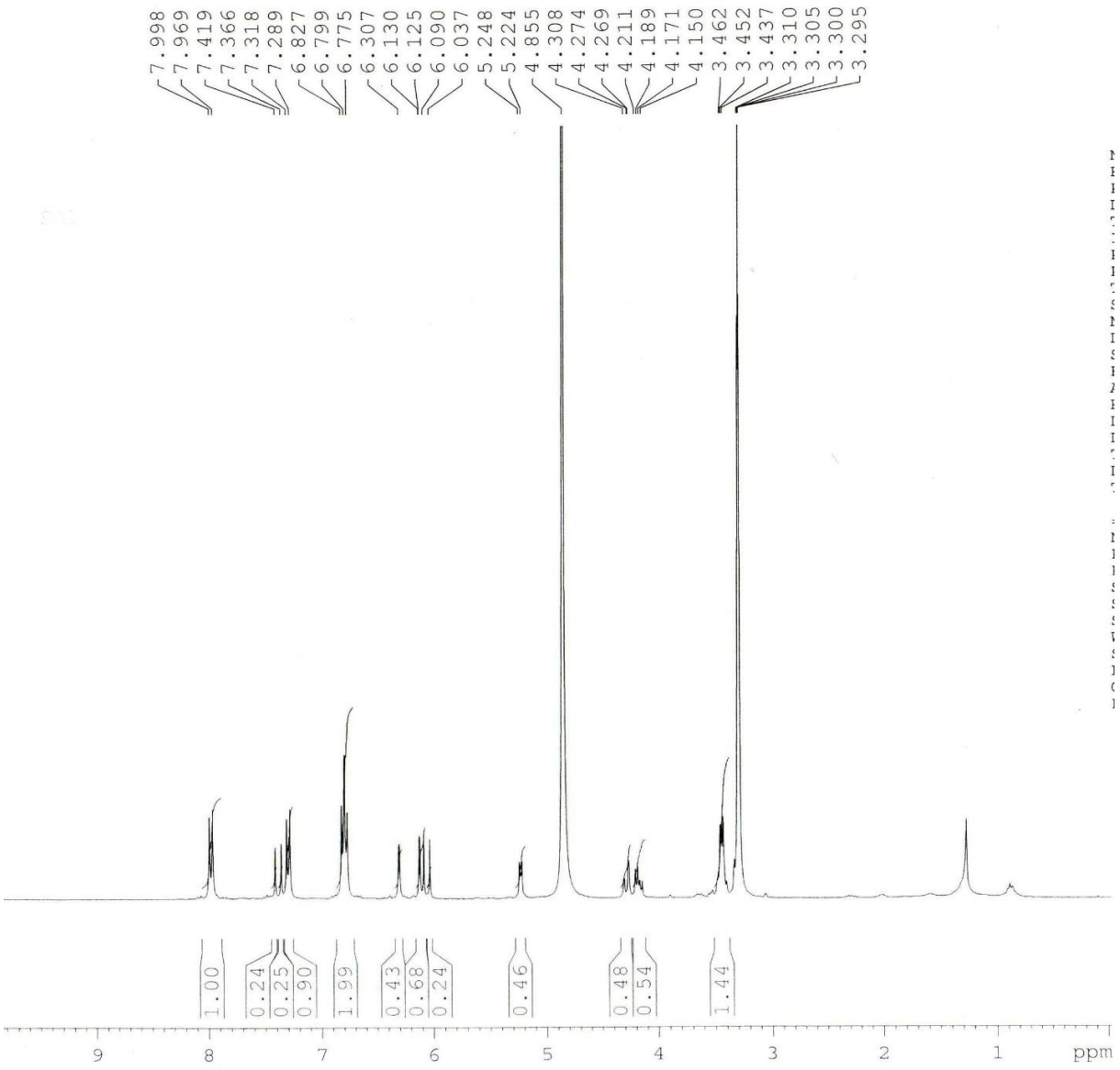
which was in agreement with the reported literature [11] and is being first time reported from the genus *Eryngium*. The isolation of this compound is a new step in the taxonomical correlation of *E. caeruleum* with other members of the genus.

Compound **2** (Figure 1 in manuscript) was obtained as a pale yellow powder, through column chromatography. Its EI-MS showed molecular ion peak at *m/z* 740.17 ($[M]^+$), indicating a molecular formula of $C_{39}H_{32}O_{15}$, which was also supported by its ^{13}C-NMR spectra. The IR spectrum of compound **2** showed the typical absorption bands for hydroxyl groups (3380 cm^{-1}), C–H stretching (2924 cm^{-1}), *α,β*-unsaturated carbonyl (1693 and 1654 cm^{-1}), and of an *O*-glycosidic linkage (1171–1018 cm^{-1}). The UV spectrum recorded in MeOH showed the characteristic absorption maxima for kaempferol 3-*O*-glycoside at 266, 318, and 354 nm [6]. The maximum at 318 nm corresponded to the *p*-coumaroyl moiety [7] and the aromatic portion of flavonoid nucleus.

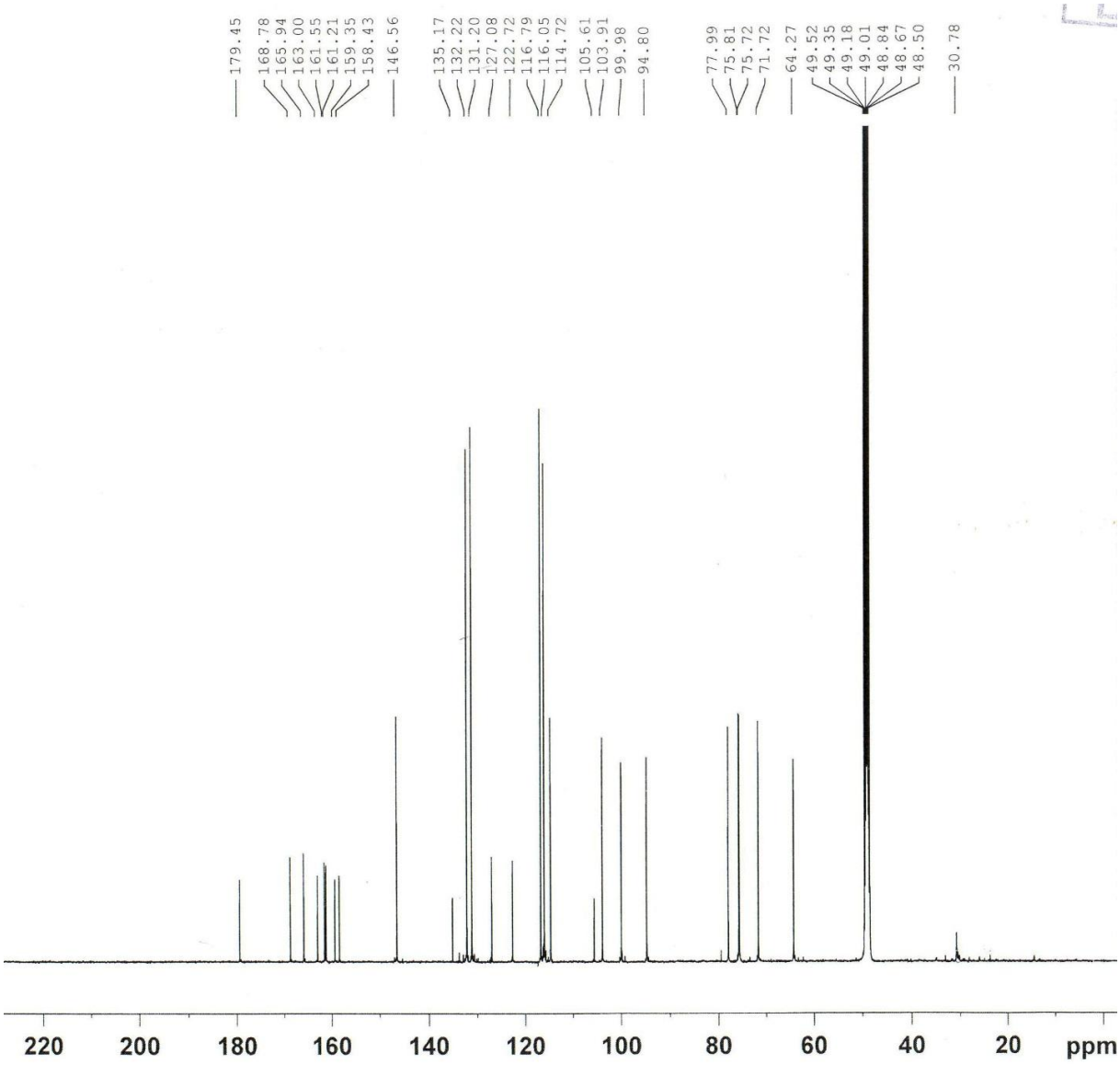
Compound **2** was also a flavone glycoside whose anomeric proton resonated at *δ* 5.62 (d, *J* = 8.0 Hz) with corresponding anomeric carbon signal at *δ* 100.3. The two characteristic ^{13}C-NMR chemical shift values for C-6 and C-8 in the ring A of flavonoid at *δ* 99.9 and 94.6 [8] showed their attachment to the protons at *δ* 6.06 (d, *J* = 2.0 Hz) and 6.26 (d, *J* = 2.5 Hz), respectively, in the HSQC experiment. The ^{1}H- and ^{13}C-NMR spectral data of compound **2** were very similar to those of **1**, except for additional resonances due to a second (*E*)-*p*-coumaroyl moiety in the molecule. These signals were at *δ* 6.06 (d, *J* = 16.0 Hz), 7.39 (d, *J* = 16.0 Hz), 7.30 (d, *J* = 8.5 Hz), and 6.80 (d, *J* = 8.5 Hz). The ^{13}C-NMR spectrum showed the corresponding carbon signals at *δ* 114.6, 146.5, 131.2, and 116.8. In addition, a carbonyl signal at *δ* 168.7 and two quaternary carbon signals at *δ* 127.0 and 161.3 were also observed. These observations and molecular mass difference of 146 between the two compounds was a clear evidence that compound **2** was a (*E*)-*p*-coumaric ester of **1**. The point of attachment of the second coumaroyl moiety was at C-6″ as the protons at C-6″ were deshielded to *δ* 4.34 (dd, *J* = 2.5, 12.0 Hz) and 4.21 (dd, *J* = 6.5, 12.0 Hz). Accordingly, the C-6″ signal was also shifted downfield at *δ* 64.0. The position of attachment of the second coumaroyl moiety was also confirmed by HMBC correlations of C-1‴″ (*δ* 168.7) of *p*-coumaroyl and H-6″ (*δ* 4.34 and 4.21) of glucopyranosyl moiety.

The ^{1}H- and ^{13}C-NMR data were in complete agreement with the proposed structure and the structure of compound **2** was established as kaempferol 3-*O*-(2′′,6′′-di-*O*-*E*-*p*-coumaroyl)-*β*-D-glucopyranoside [12] which is first time reported from the genus *Eryngium*.

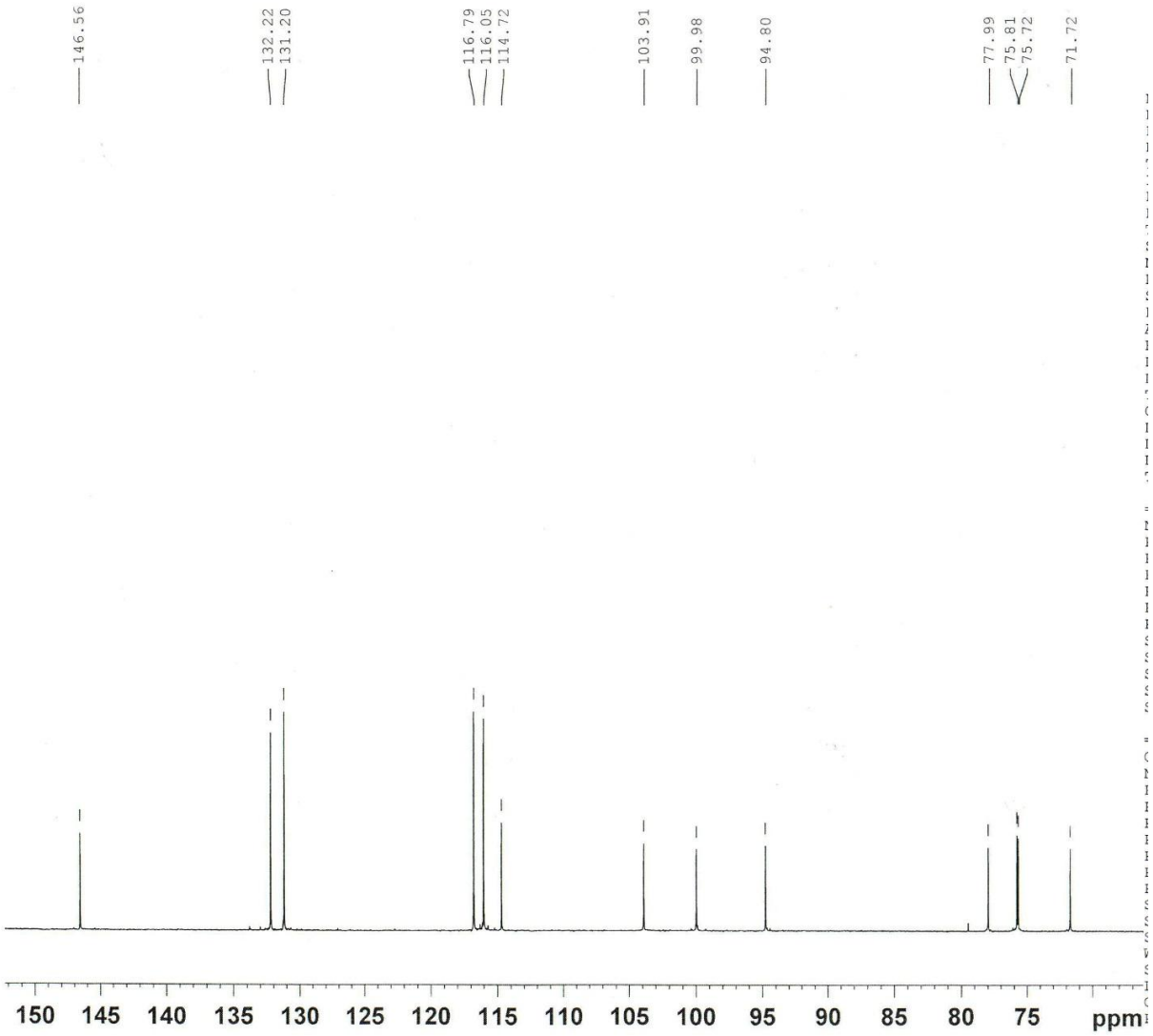
3 ^{1}H-NMR spectrum of compound 1



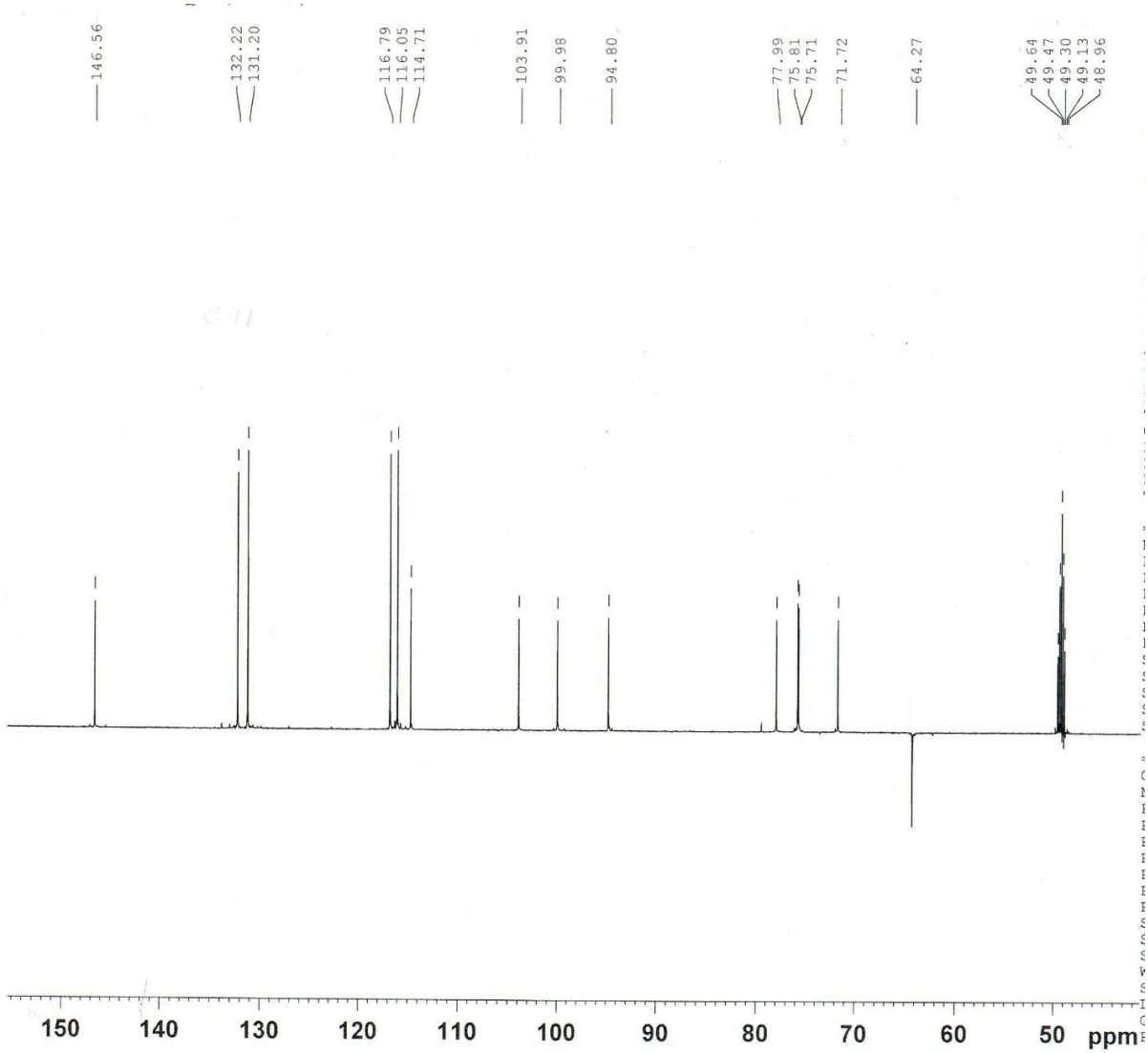
4 ^{13}C-NMR spectrum of compound 1



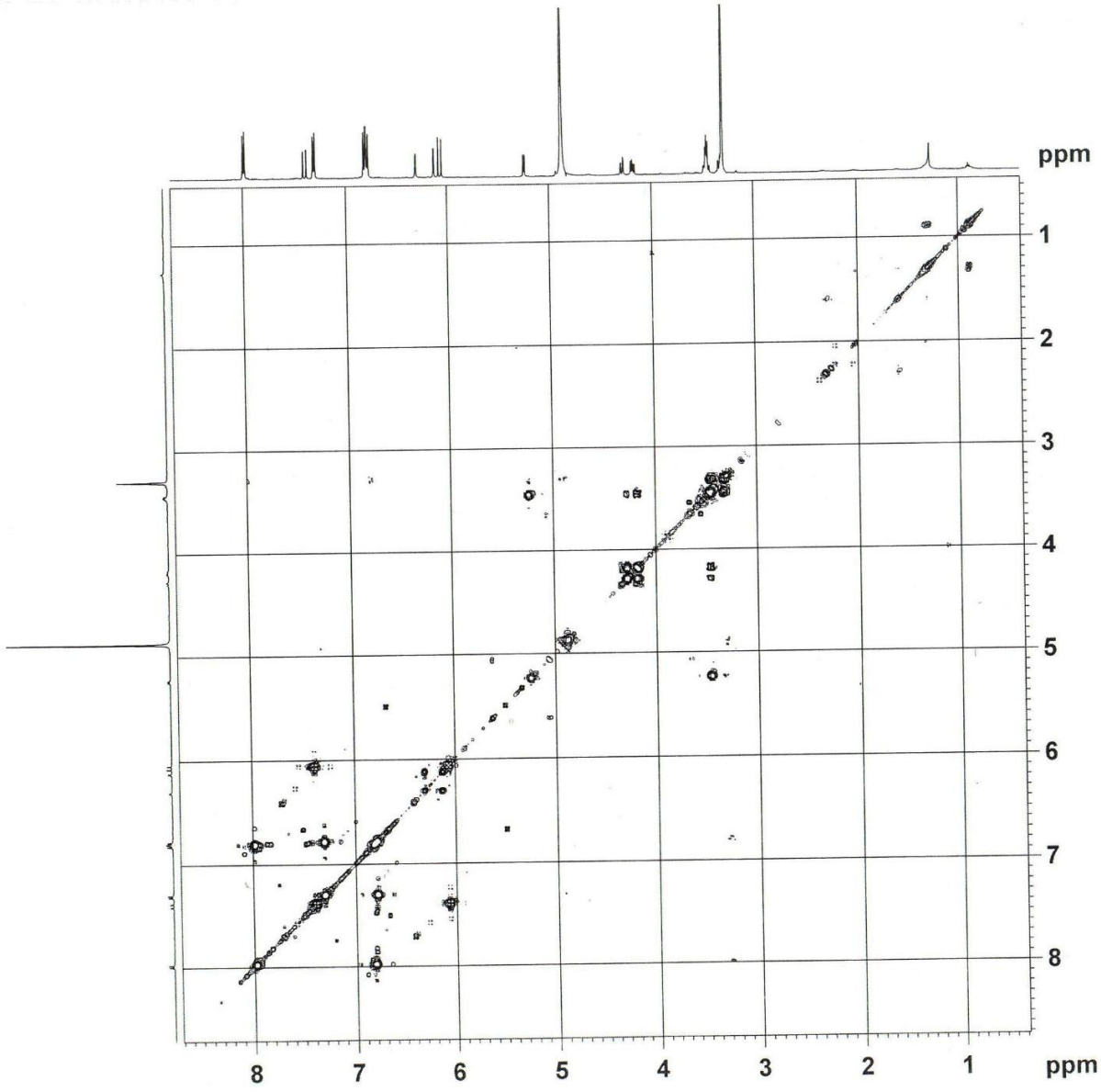
5 DEPT-90 spectrum of compound 1



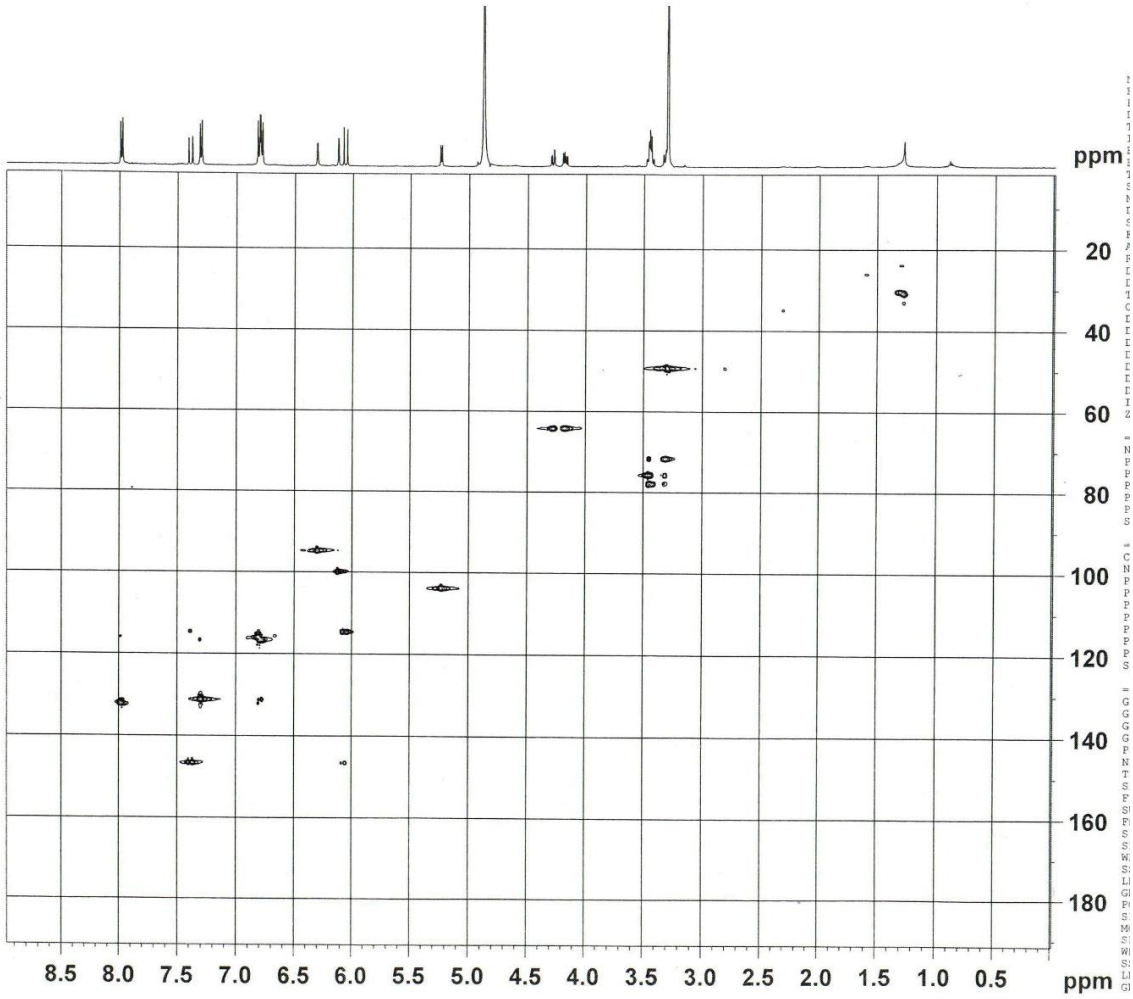
6 DEPT-135 spectrum of compound 1



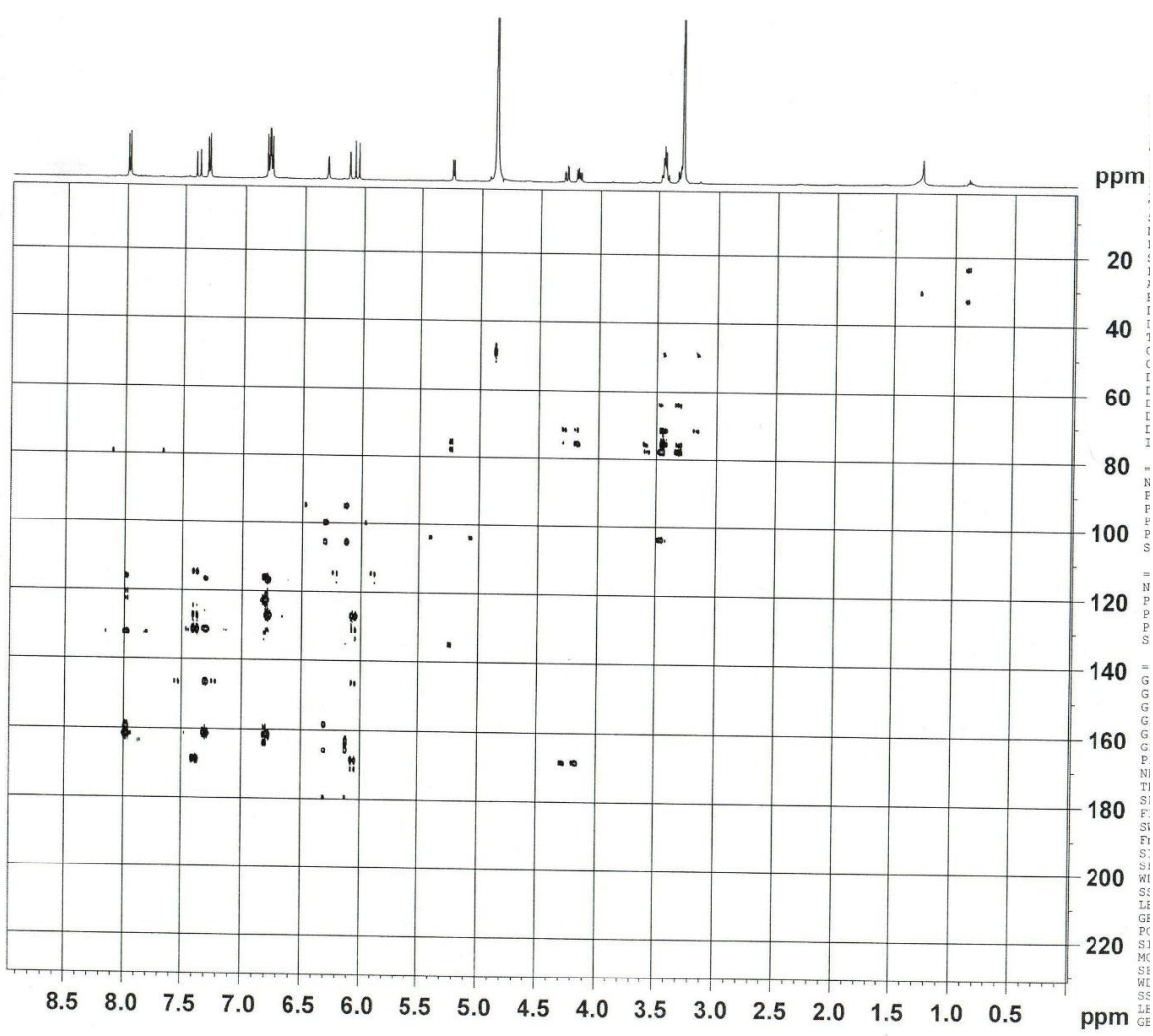
7 ^{1}H-^{1}H COSY spectrum of compound 1



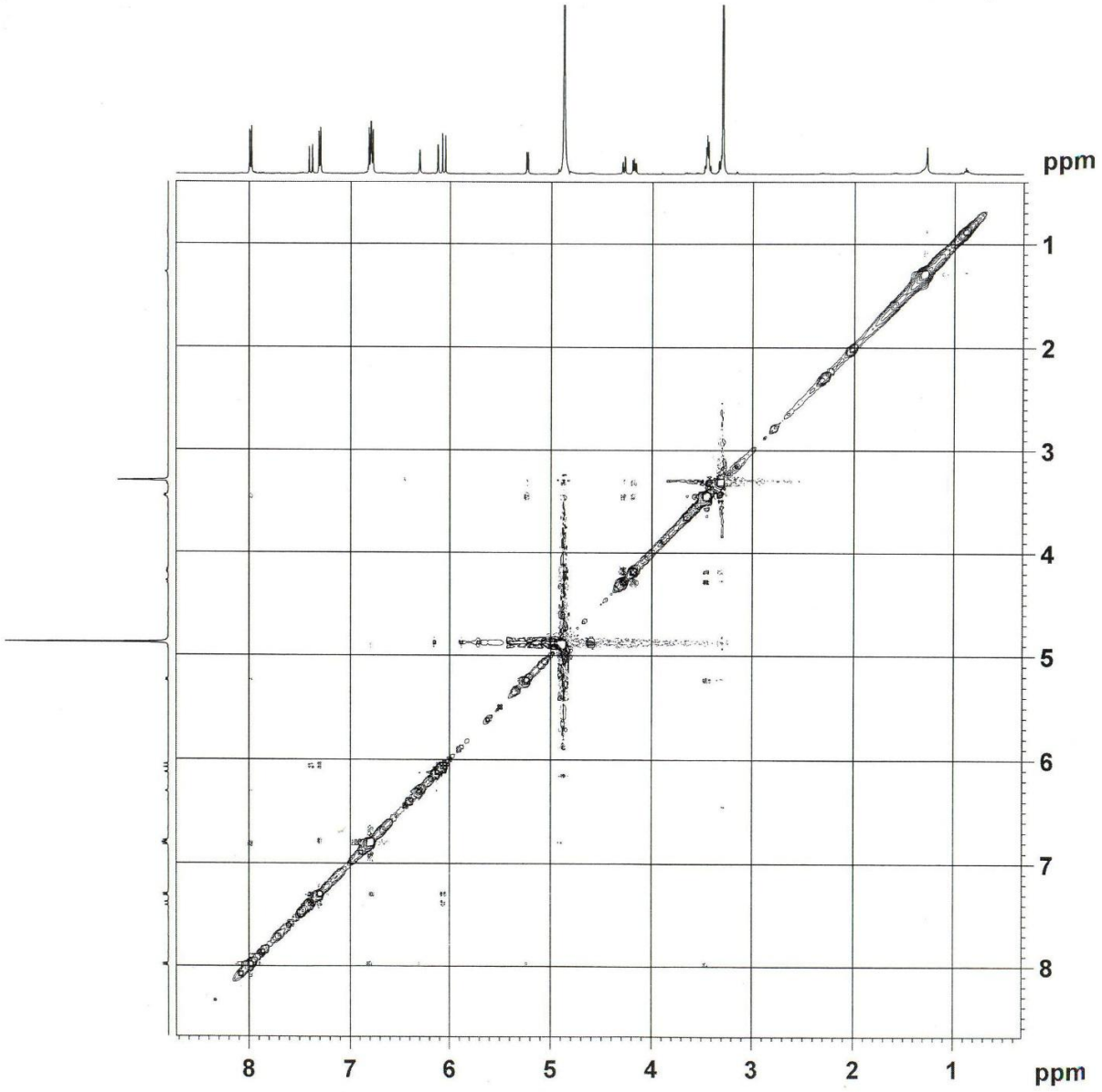
8 HSQC spectrum of compound 1



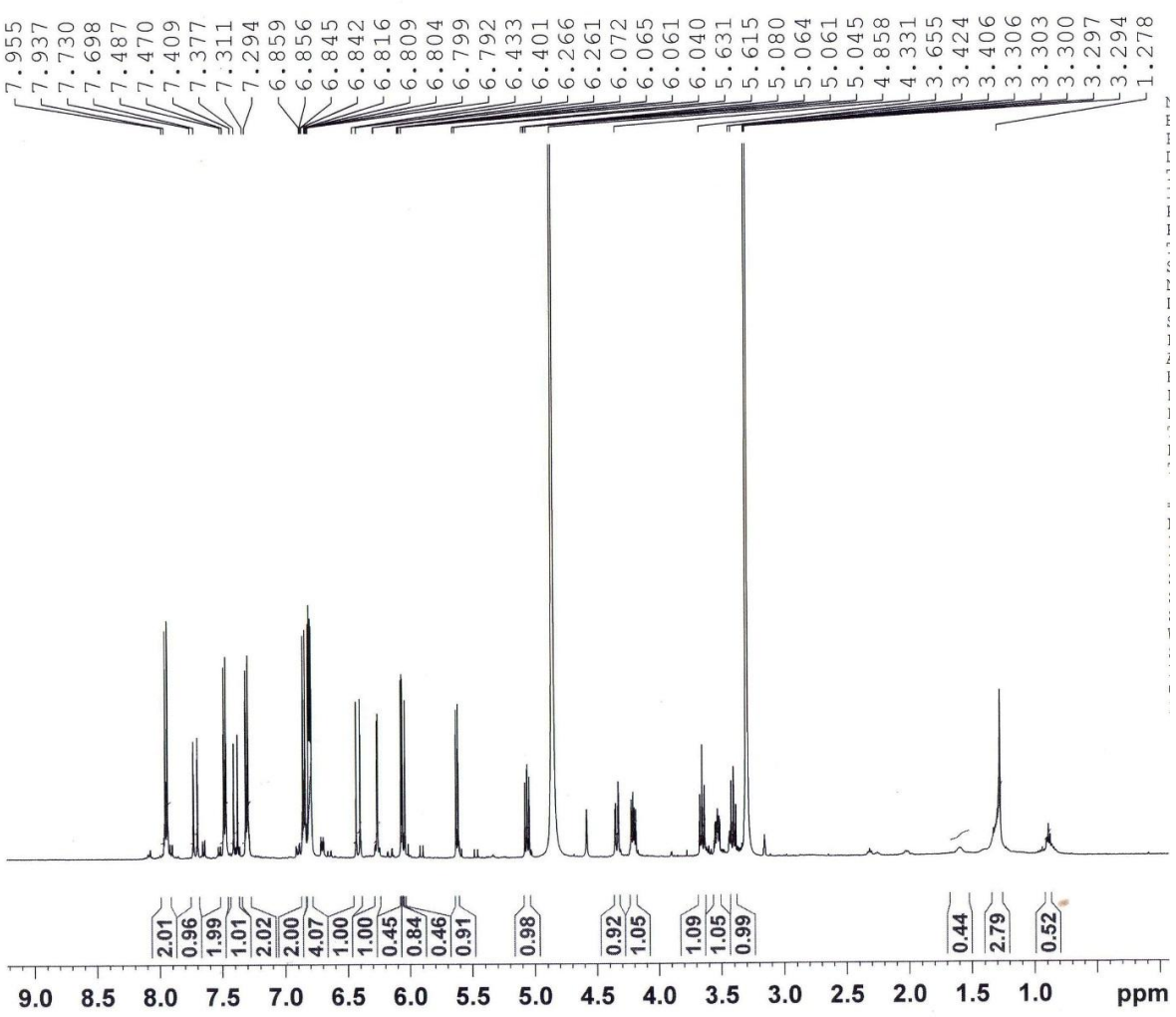
9 HMBC spectrum of compound 1



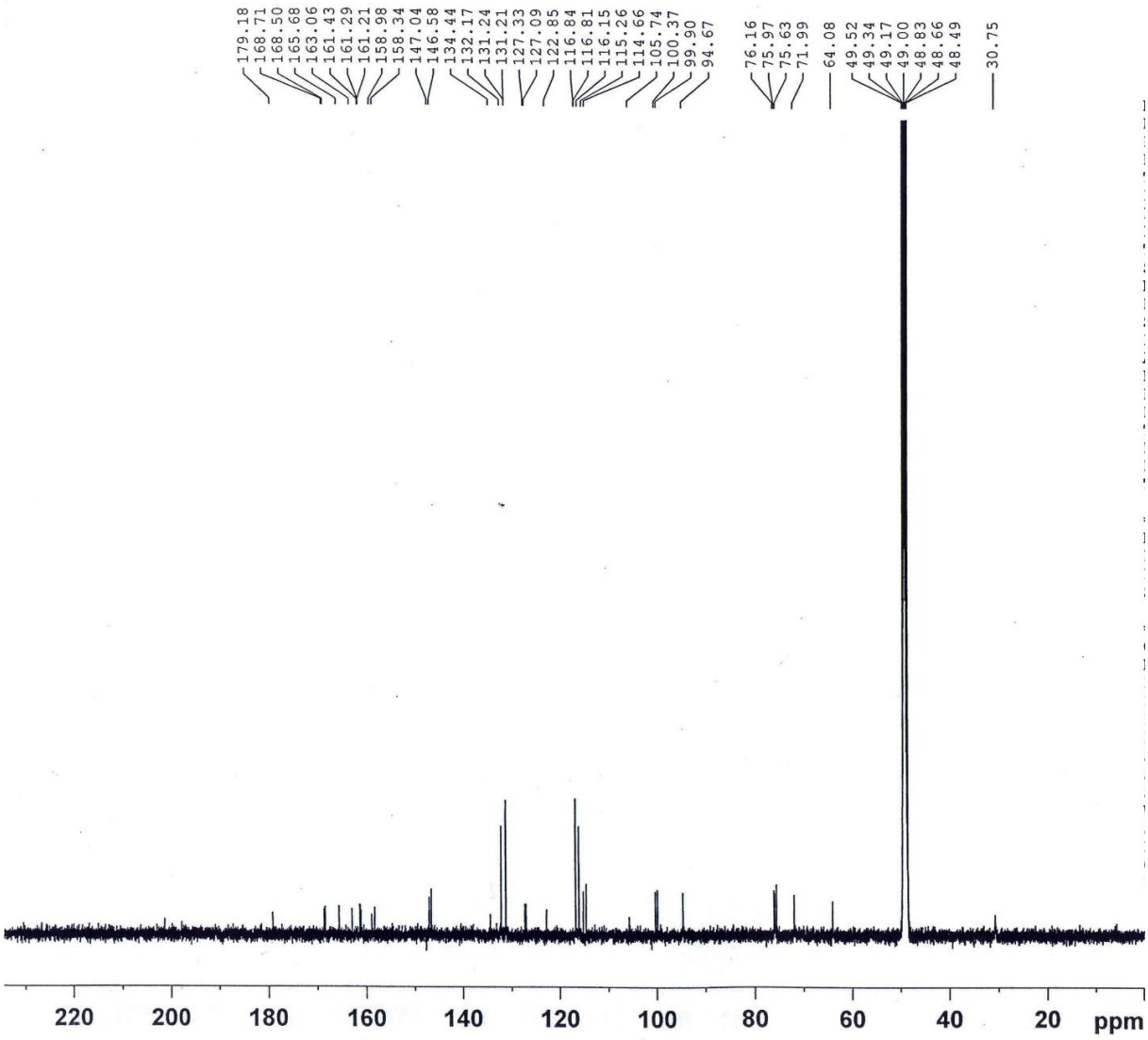
10 NOESY spectrum of compound 1



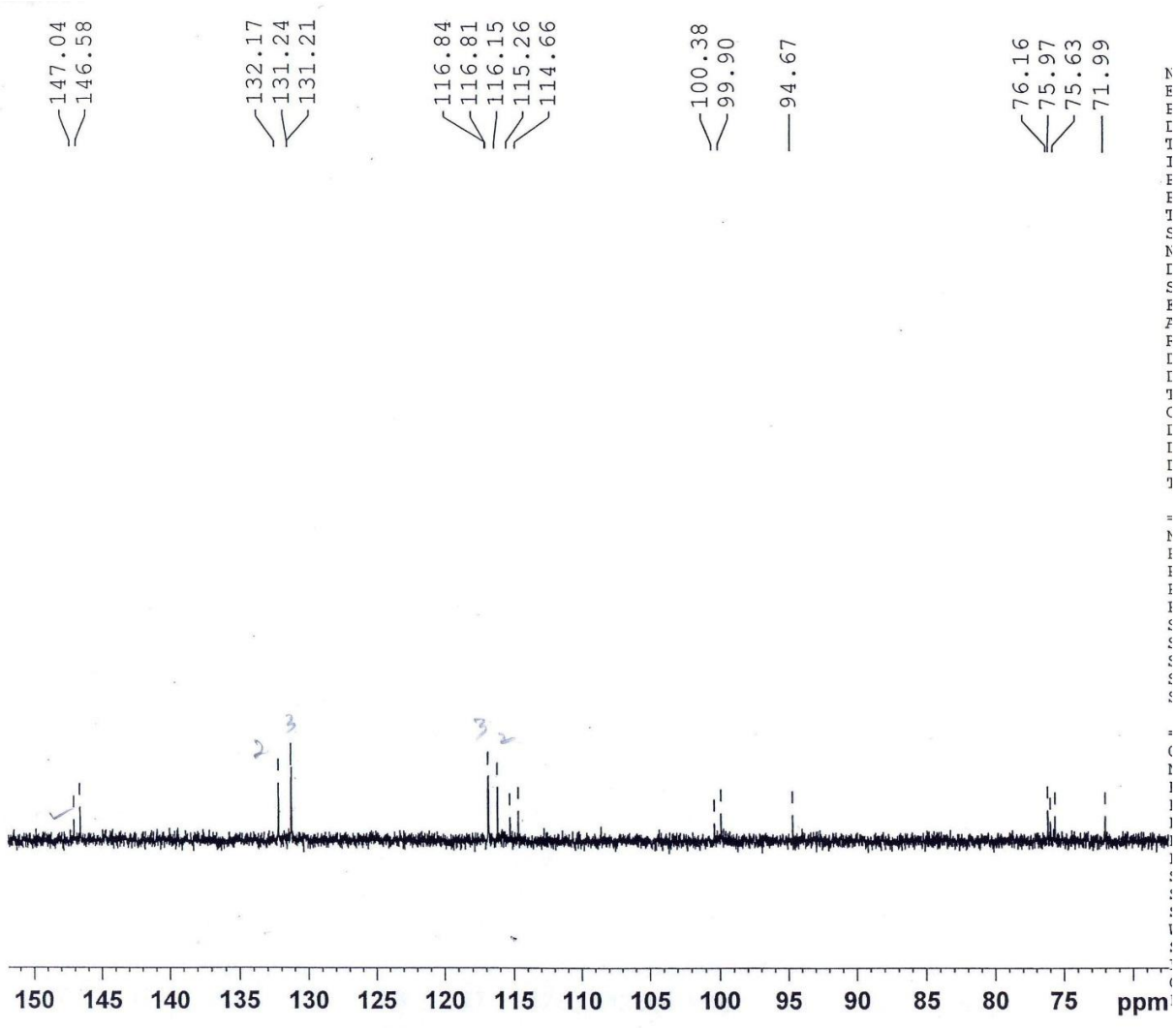
11 ^{1}H-NMR spectrum of compound 2



12 ^{13}C-NMR spectrum of compound 2



13 DEPT 90 spectrum of compound 2



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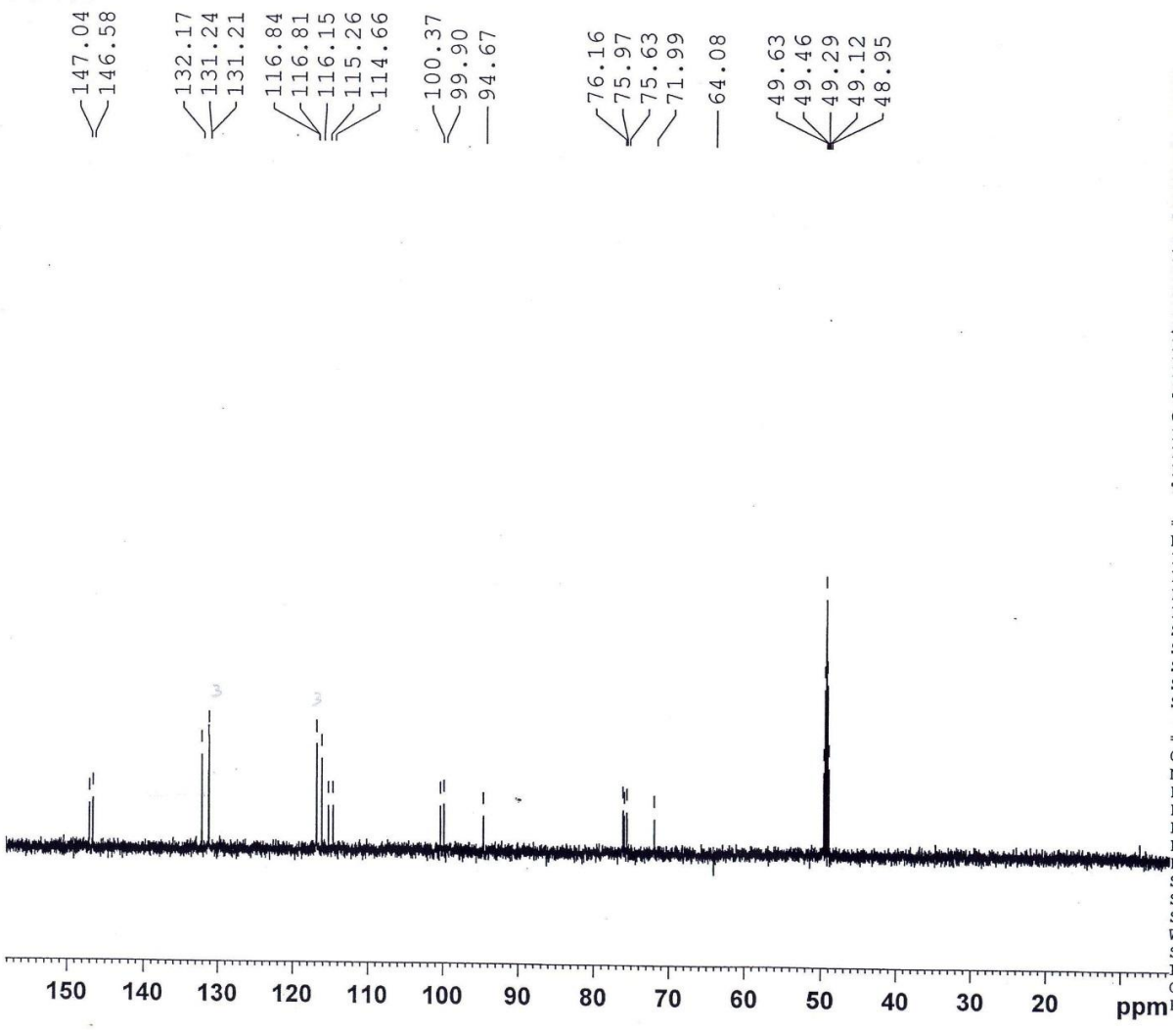
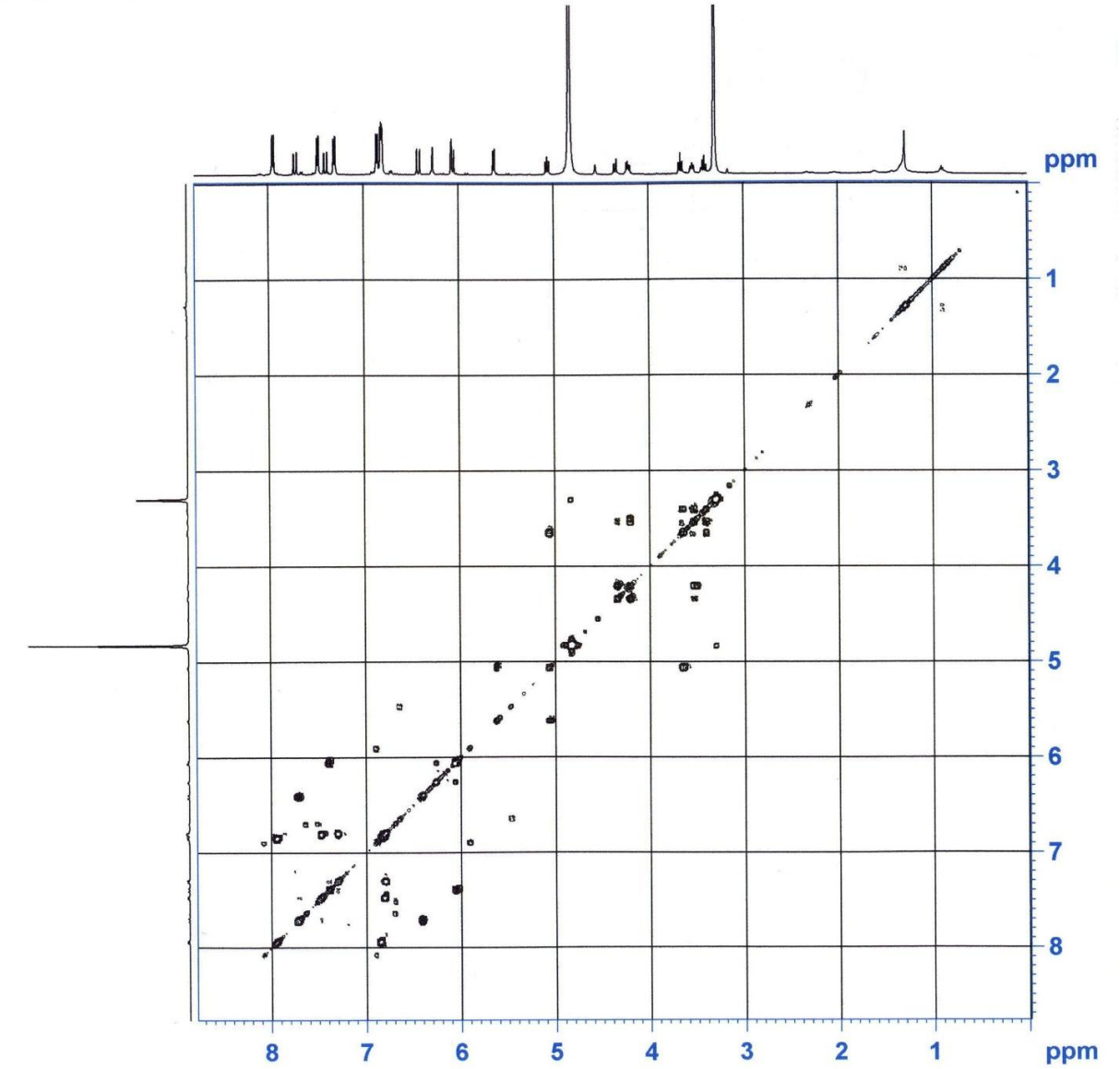
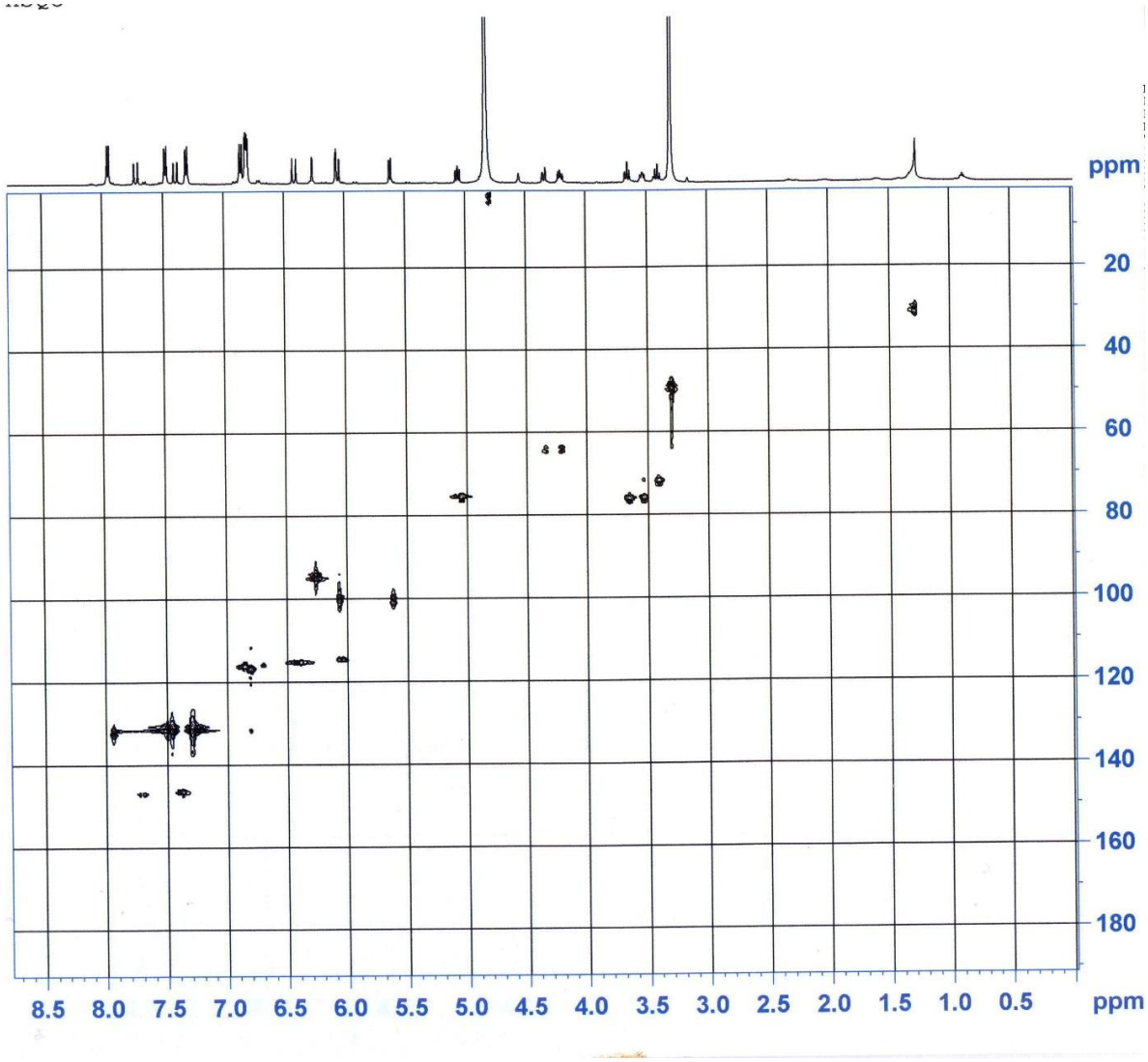


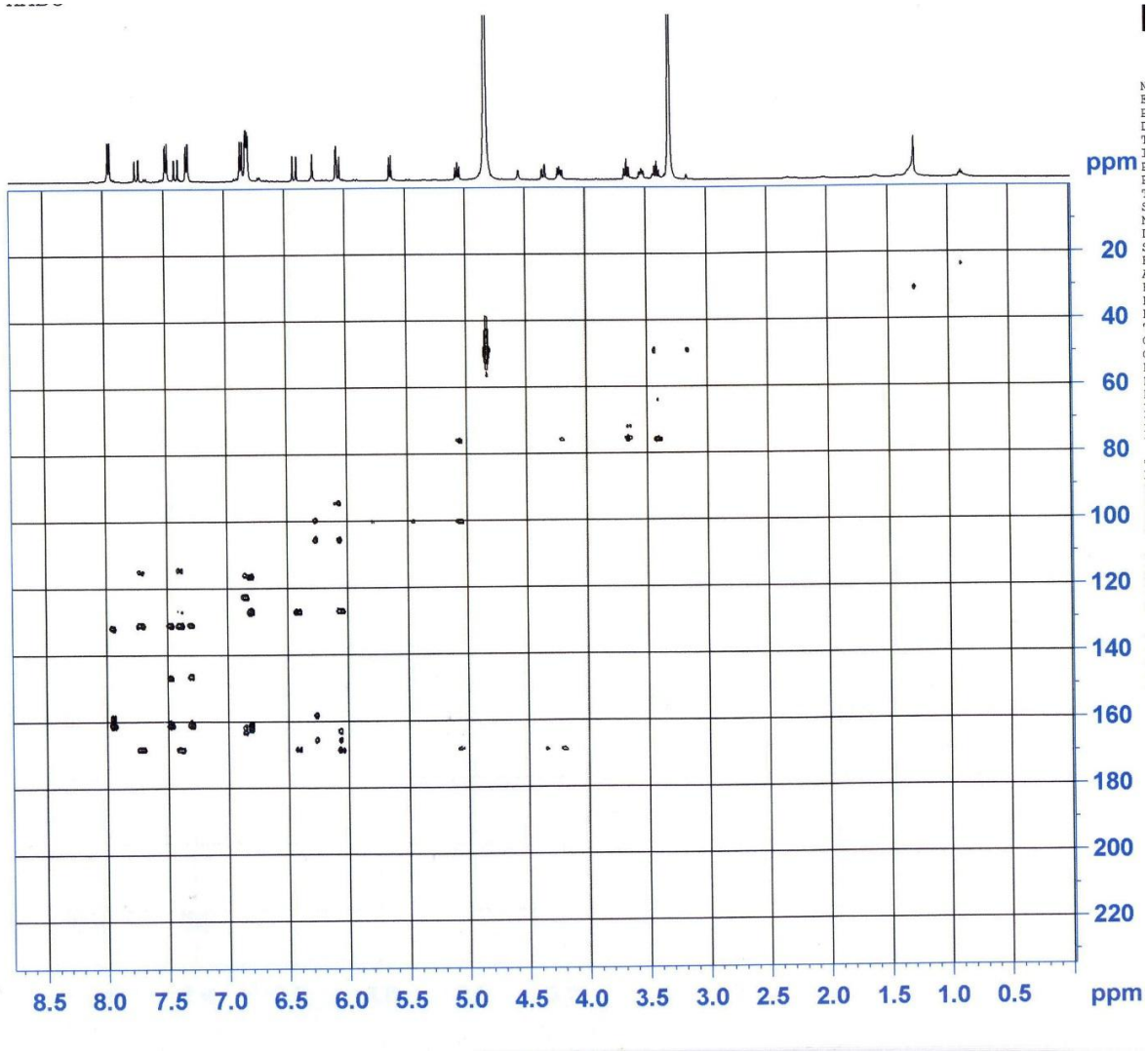
15 ^{1}H-^{1}H COSY spectrum of compound 2



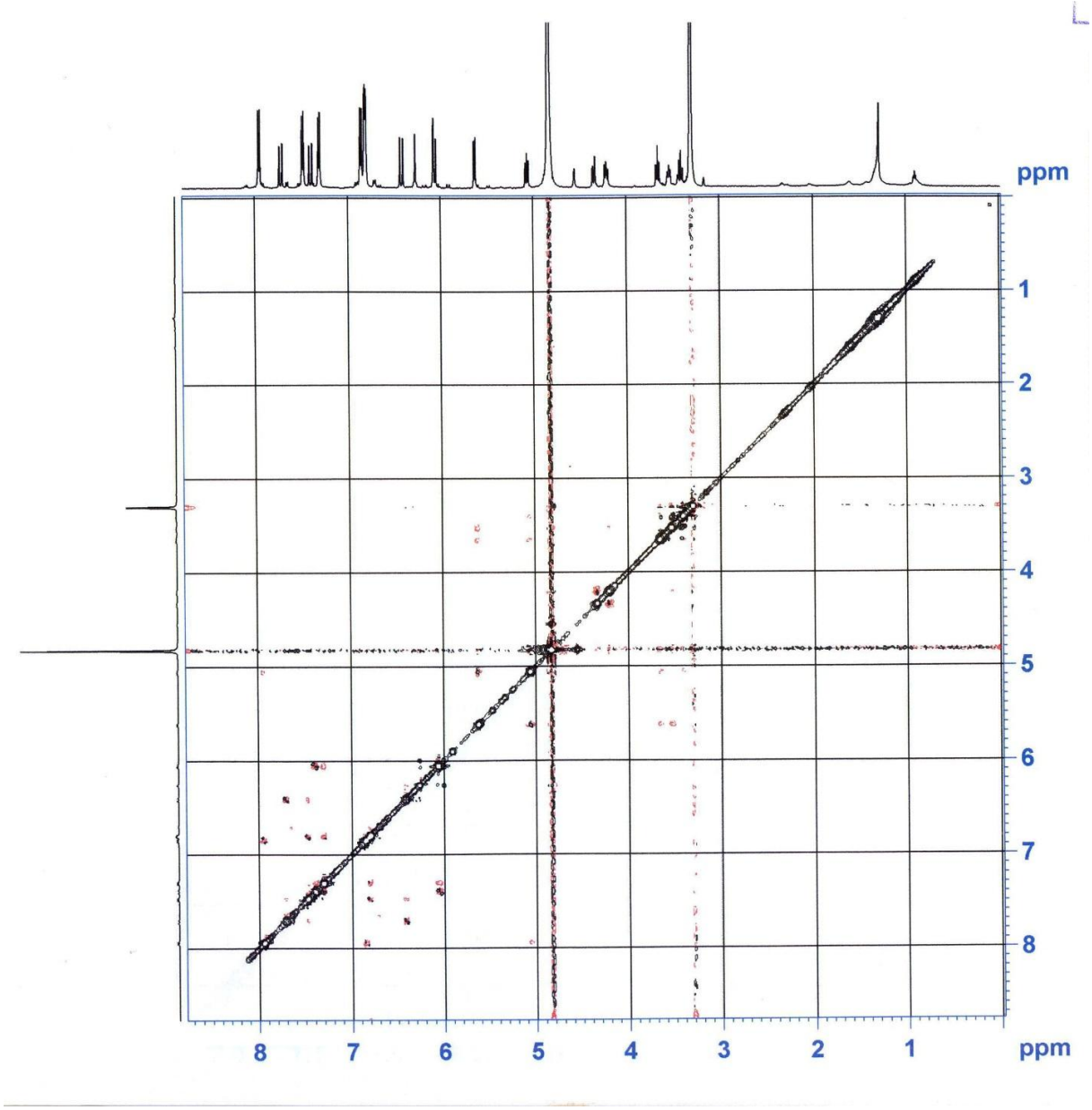
16 HSQC spectrum of compound 2



17 HMBC spectrum of compound 2



18 NOESY spectrum of compound 2



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