Supplementary file

Novel quinoxaline derivatives as dual EGFR and COX-2 inhibitors; Synthesis, molecular docking and biological evaluation as potential anticancer and anti-inflammatory agents

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1. Experimental

1.1. Chemistry

All commercially available reagents were purchased from Merck, Aldrich and Fluka and were used without further purification. All reactions were monitored by thin layer chromatography (TLC) using precoated plates of silica gel G/UV-254 of 0.25 mm thickness (Merck 60F254) using UV light (254 nm/365 nm) for visualization. Melting points were detected with a Kofler melting points apparatus and uncorrected. Infrared spectra were recorded with a FT-IR-ALPHBROKER-Platinum-ATR spectrometer and are given as cm-1using the attenuated total reflection (ATR) method. 1H NMR and 13C NMR spectra for all compounds were recorded in DMSO-d6 on a Bruker Bio Spin AG spectrometer at 400 MHz and 100 MHz, respectively. For 1H NMR, chemical shifts (δ) were given in parts per

million (ppm) with reference to tetramethylsilane (TMS) as an internal standard (δ=0); For 13CNMR, TMS (δ=0) or DMSO (δ=39.51) was used as internal standard and spectra were obtained with complete proton decoupling. Elemental analyses were obtained on a Perkin-Elmer CHN-analyze rmodel.

2. Biological evaluation

2.1. Assay for anticancer effect

To explore the antiproliferative potential of compounds MTT assay was performed [1] using different cell lines such as Hepatocellular carcinoma (HepG2), Mammary gland (MCF-7) and Colorectal carcinoma (HCT-116), respectively. The cytotoxic activity was performed using MTT (3-(4.5-dimethylthiazol-2-yl)-2,0.5-diphenyltetrazolium bromide) assay according to the described method [2]. Cells were plated in 96-multiwell plate in DMEM containing 10% FBS (fetal bovine serum) for 24 h. Then, cells, test compounds and reference drugs were treated with vehicle DMSO for 48 h. After that, the media was replaced with 200 lL DMEM containing 0.5 mg/ml of MTT and were incubated for 2 h. Remove the supernatant and dissolve formazans precipitate using 200 lL of DMSO. A micro plate reader was used to determine the absorbance at 570 nm.

2.2. *In vitro* cyclooxygenase (COX) inhibition assay

The ability of the test compounds listed in the Table 1 to inhibit ovine COX-1 and COX-2 (IC50 values, μM) was determined using an enzyme immuno assay (EIA) kit (catalog number 560101, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Cyclooxygenase catalyzes the first step in the biosynthesis of arachidonic acid (AA) to PGH2. PGF2α, produced from PGH2 by reduction with stannous chloride, is measured by enzyme immunoassay (ACE™ competitive EIA). Stock solutions of test compounds

were dissolved in a minimum volume of DMSO. Briefly, to a series of supplied reaction buffer solutions (960 µL, 0.1 M Tris–HCl pH 8.0 containing 5 mM EDTA and 2 mM phenol) with either COX-1 or COX-2 (10 µL) enzyme in the presence of heme (10 µL) were added 10 µL of various concentrations of test drug solutions (0.01, 0.1, 1, 10, 50, and 100 µM in a final volume of 1 mL). These solutions were incubated for a period of 5 min at 37 °C after which 10 µL of AA (100 µM) solution were added and the COX reaction was stopped by the addition of 50 µL of 1 M HCl after 2 min. PGF2α, produced from PGH2 by reduction with stannous chloride was measured by enzyme immunoassay. This assay is based on the competition between PGs and a PG-acetylcholinesterase conjugate (PG tracer) for a limited amount of PG antiserum. The amount of PG tracer that is able to bind to the PG antiserum is inversely proportional to the concentration of PGs in the wells since the concentration of PG tracer is held constant while the concentration of PGs varies. This antibody–PG complex binds to a mouse anti-rabbit monoclonal antibody that had been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent, which contains the substrate to acetylcholine esterase, is added to the well. The product of this enzymatic reaction produces a distinct yellow color that absorbs at 405 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of PG tracer bound to the well, which is inversely proportional to the amount of PGs present in the well during the incubation: Absorbance α [Bound PG Tracer] α1/PGs. Percent inhibition was calculated by the comparison of compound treated to various control incubations. The concentration of the test compound causing 50% inhibition (IC_{50}, µM) was calculated from the concentration–inhibition response curve (duplicate determinations) [3, 4].

2.3. EGFR inhibitory assay

A cell-free assay was used to investigate the mechanism of inhibition of EGFR kinase according to the reported method [2, 5]. Kit used for immune-assay was cloud clone SEA757Hu 96 Tests. 200 lM (EGFR) was used. From the following equation: E (%) = E max/(1 + [I]/ID50), where E (%) is the fraction of the enzyme activity measured in the presence of the inhibitor, E max is the activity in the absence of the inhibitor, [I] is the inhibitor concentration and ID50 is the inhibitor concentration at which E (%) = 0.5 E max, a dose–response curve was generated. Mean values of two independent replicates for each experiment were used for the interpolation.

3. **Docking methodology**

The 3.5A°3D crystal structures of EGFR (PDB ID: 1M17) [2, 5] and COX-2 (PDB ID: 3LN1) [6, 7] were retrieved from protein data bank. All molecular modeling calculations and docking studies were carried out using Discovery Studio software 2016 client v16.1.0.15350 (San Diego, CA)with CDOCKER program. Automatic protein preparation module was used applying CHARMm force field. The binding site sphere has been defined automatically by the software. The docked compounds were built using Chem. 3D ultra 12.0 software [Chemical Structure Drawing Standard; Cambridge Soft corporation, USA (2010)], and copied to Discovery Studio 2016 client v16.1.0.15350. Ligands were prepared using “Prepare Ligands” protocol in Discovery Studio where hydrogen atoms were added at their standard geometry, optical isomers and 3D conformations were automatically generated.Docking was performed using CDOCKER protocol in Discovery Studio keeping the parameters at default. Each compound would retain 10 poses and the best scoring pose of the docked compounds was recognized. Receptor–ligand interactions of the complexes were examined in 2D and 3D styles.

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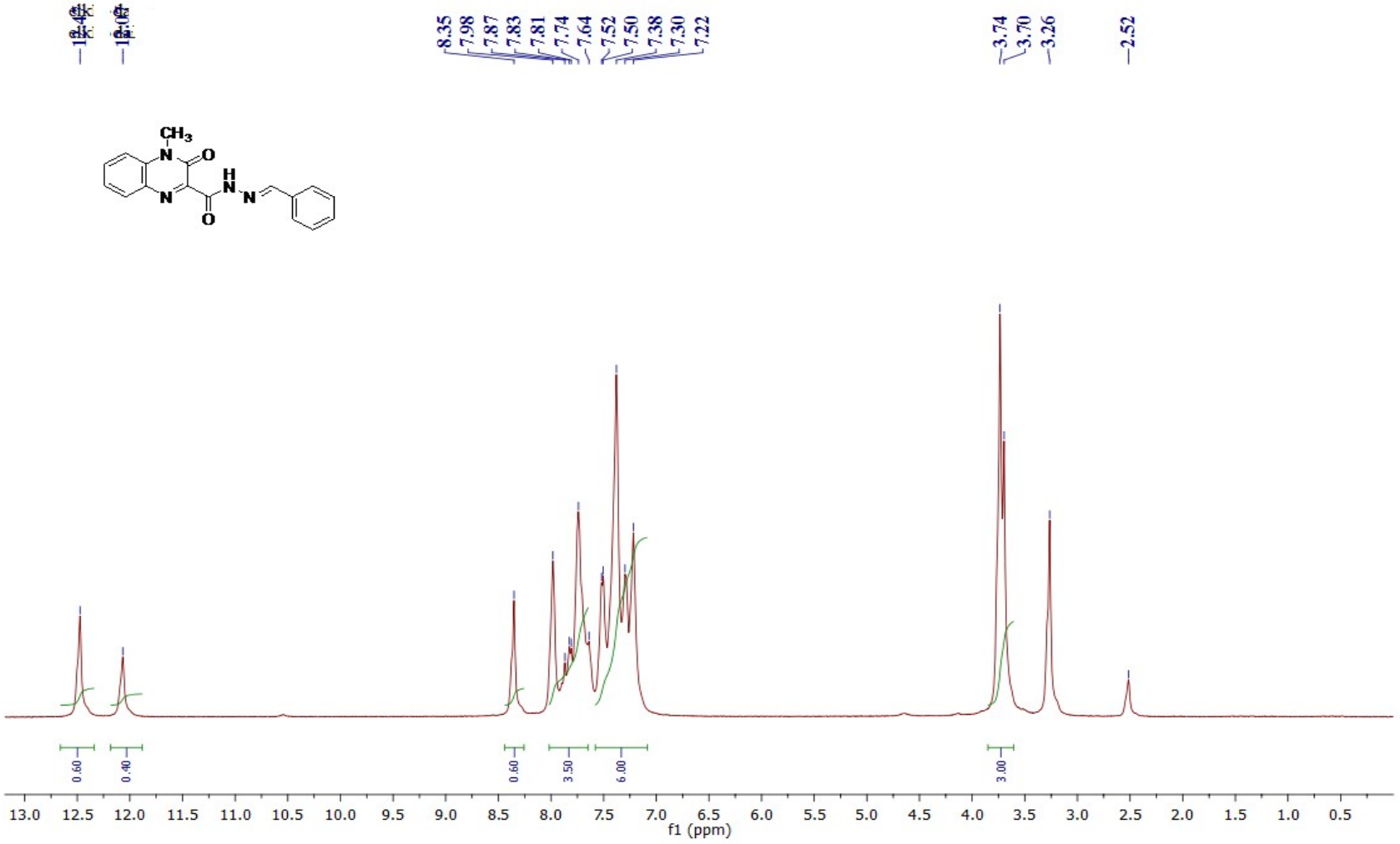

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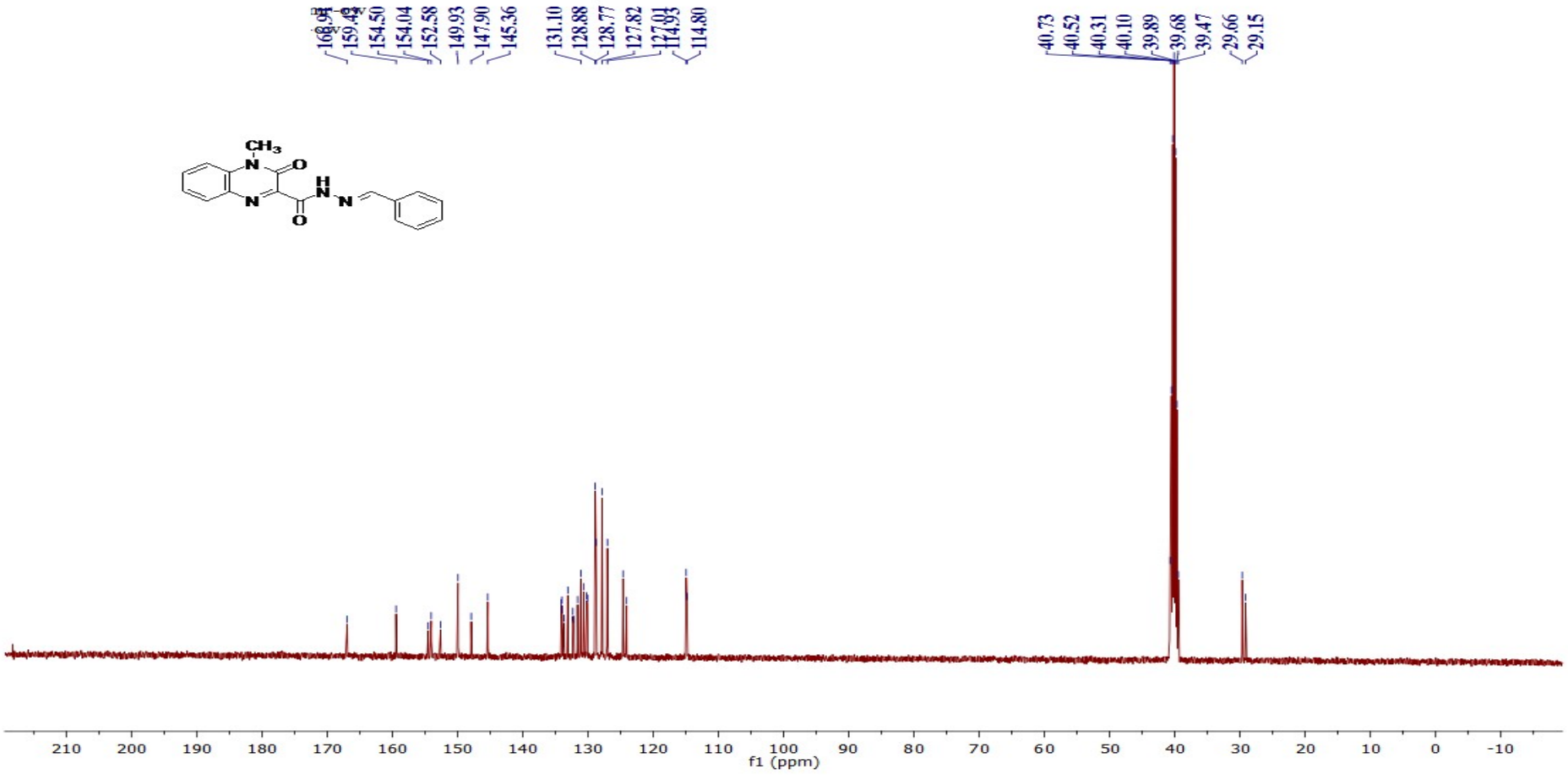

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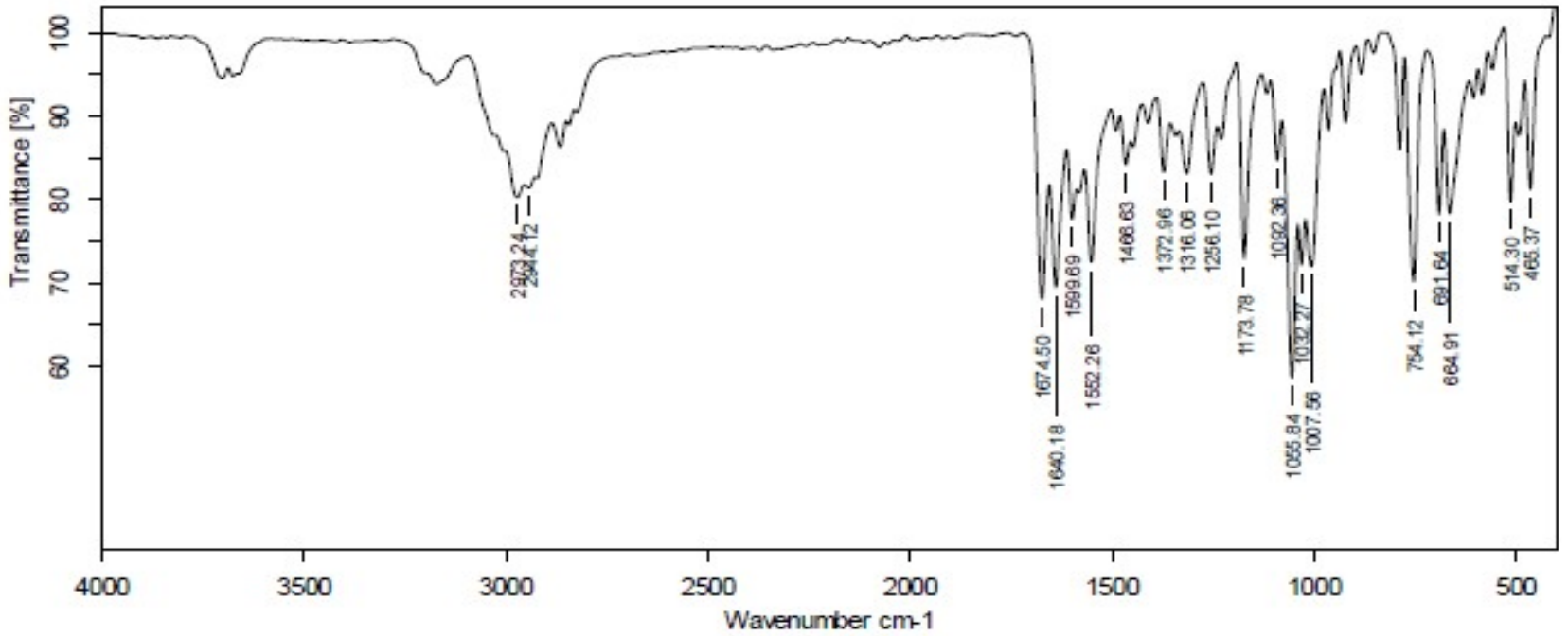
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1007.56
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Wavenumber cm-1

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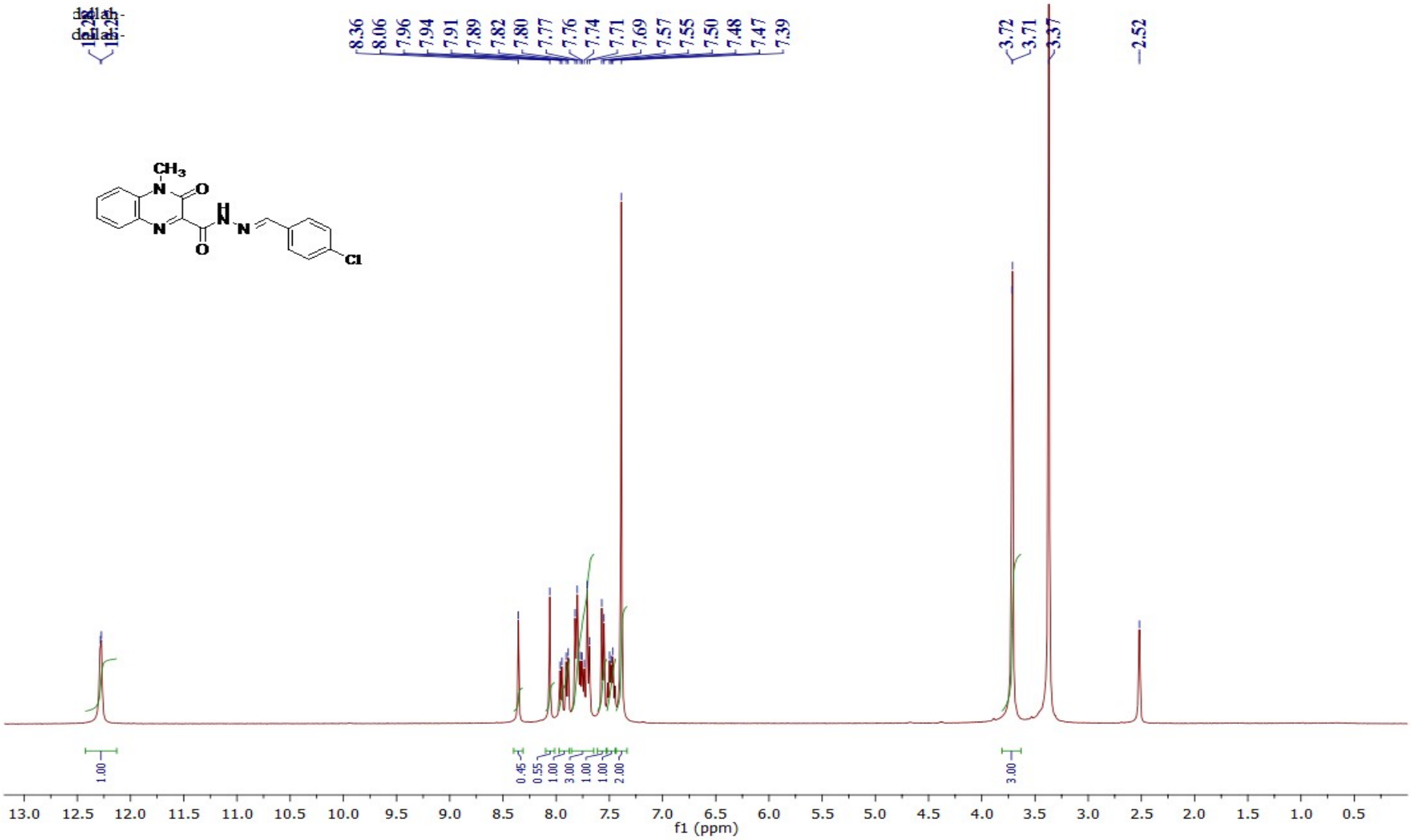
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3.37
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H
N
N
O
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f1 (ppm)

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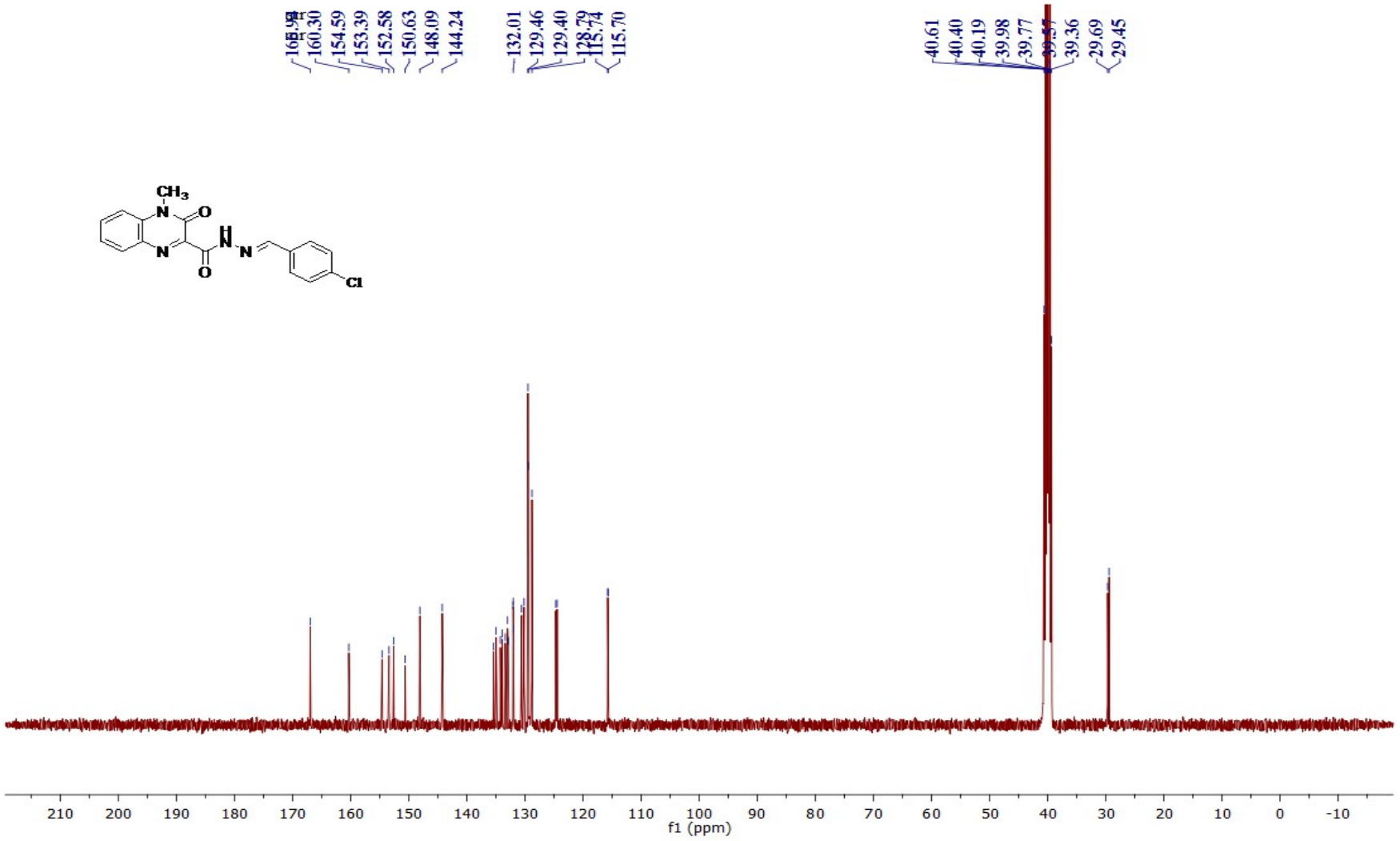

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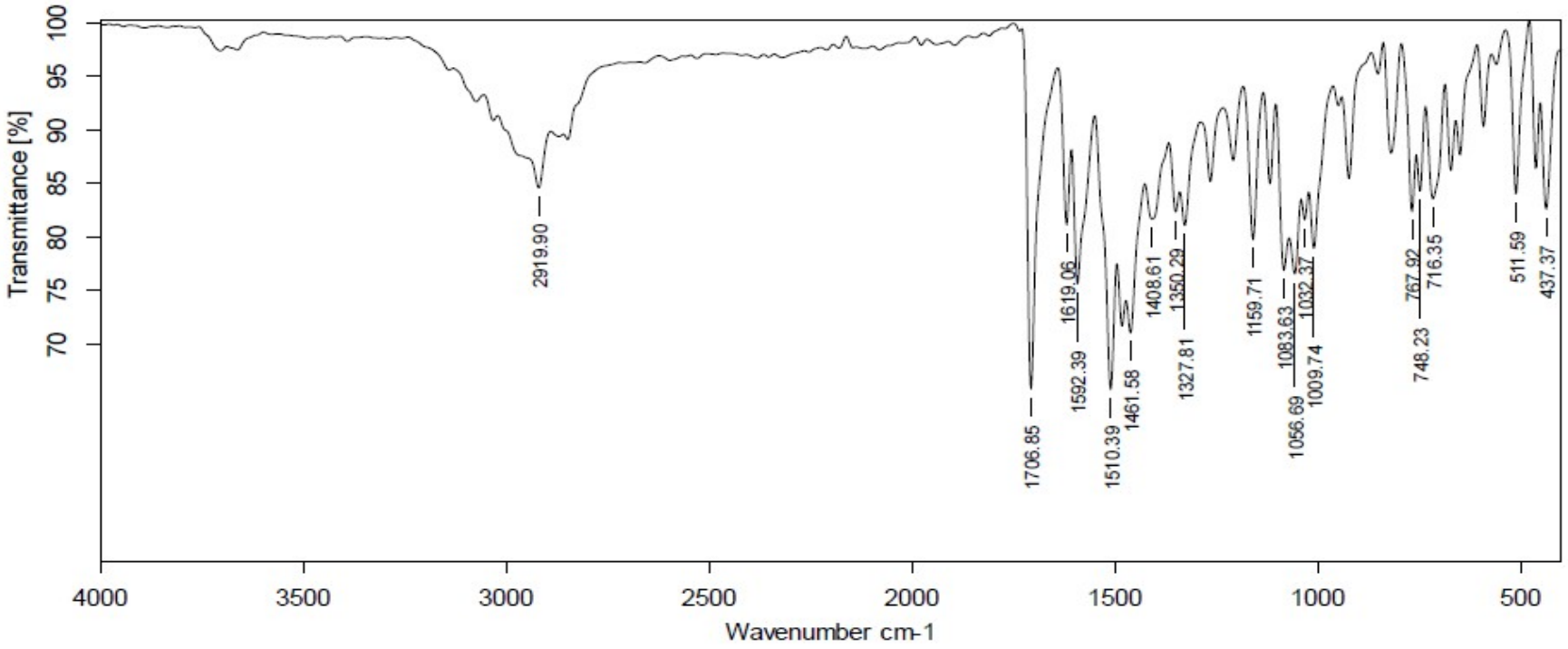

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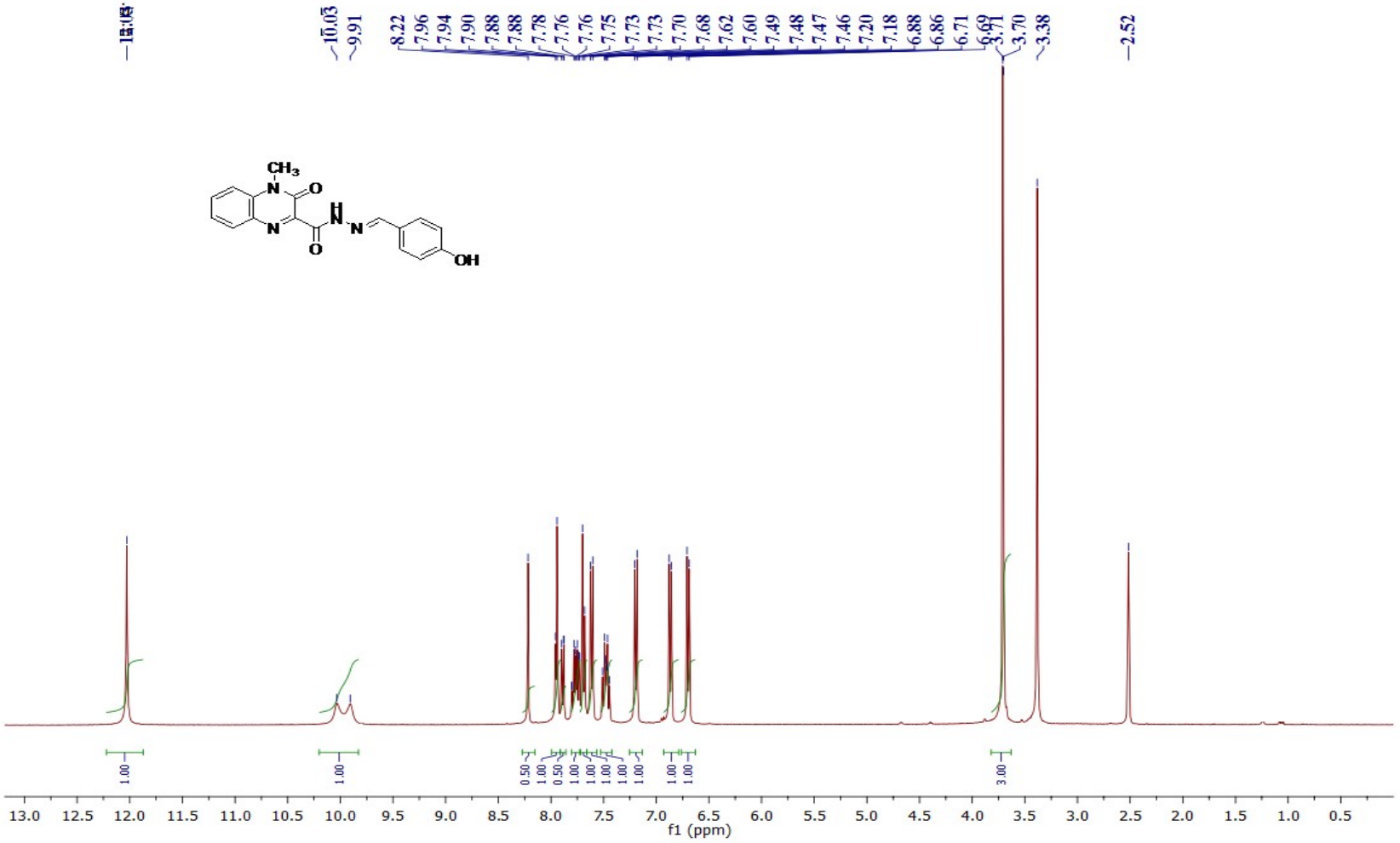

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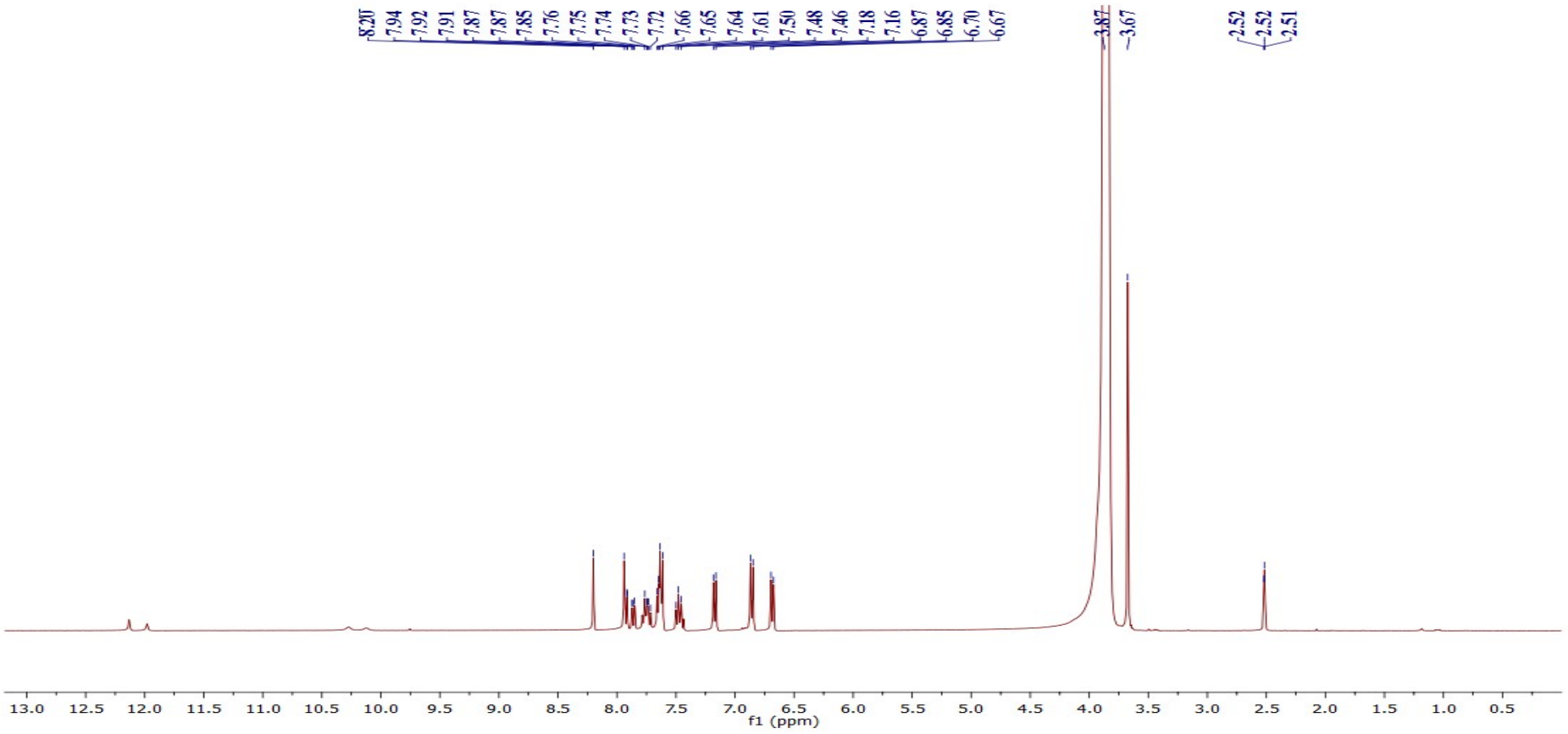

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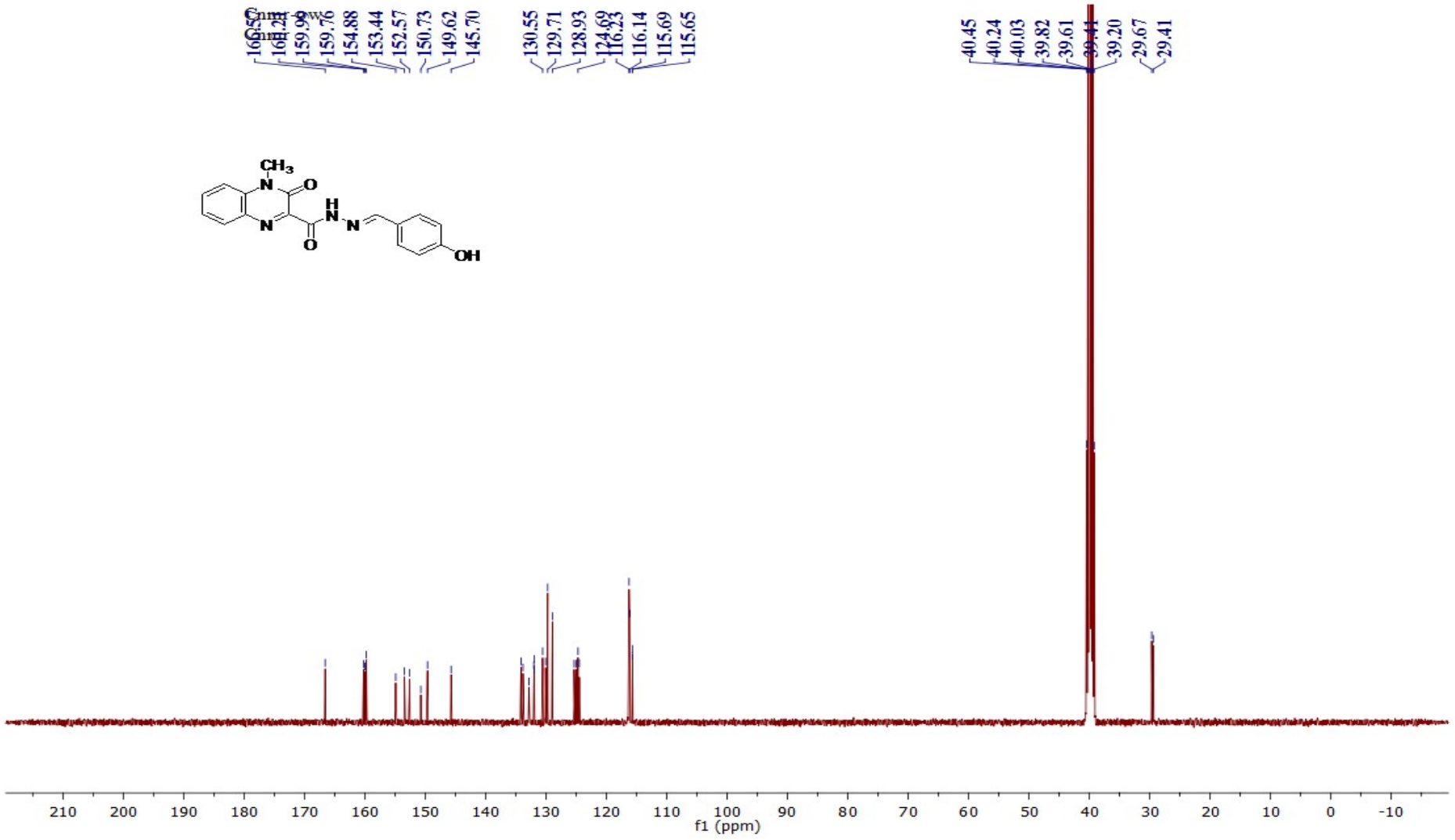

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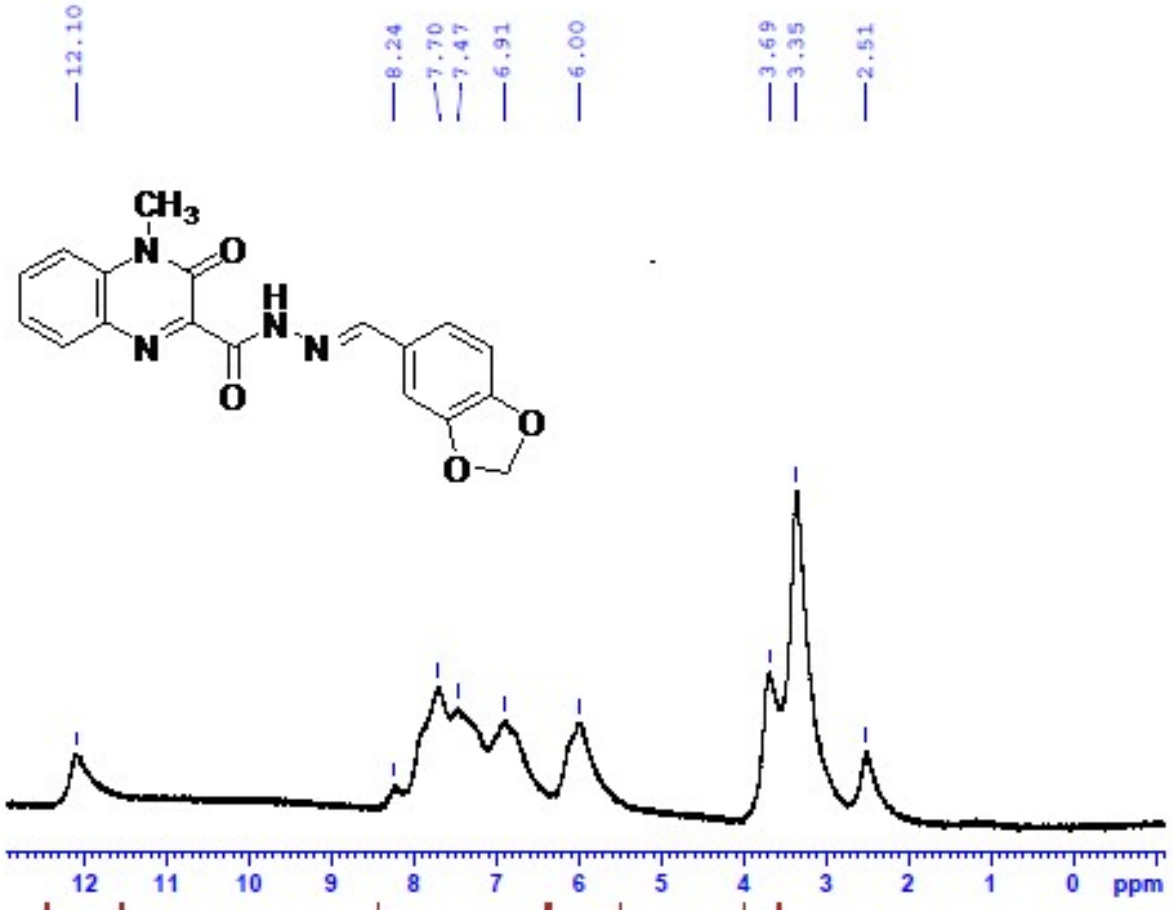


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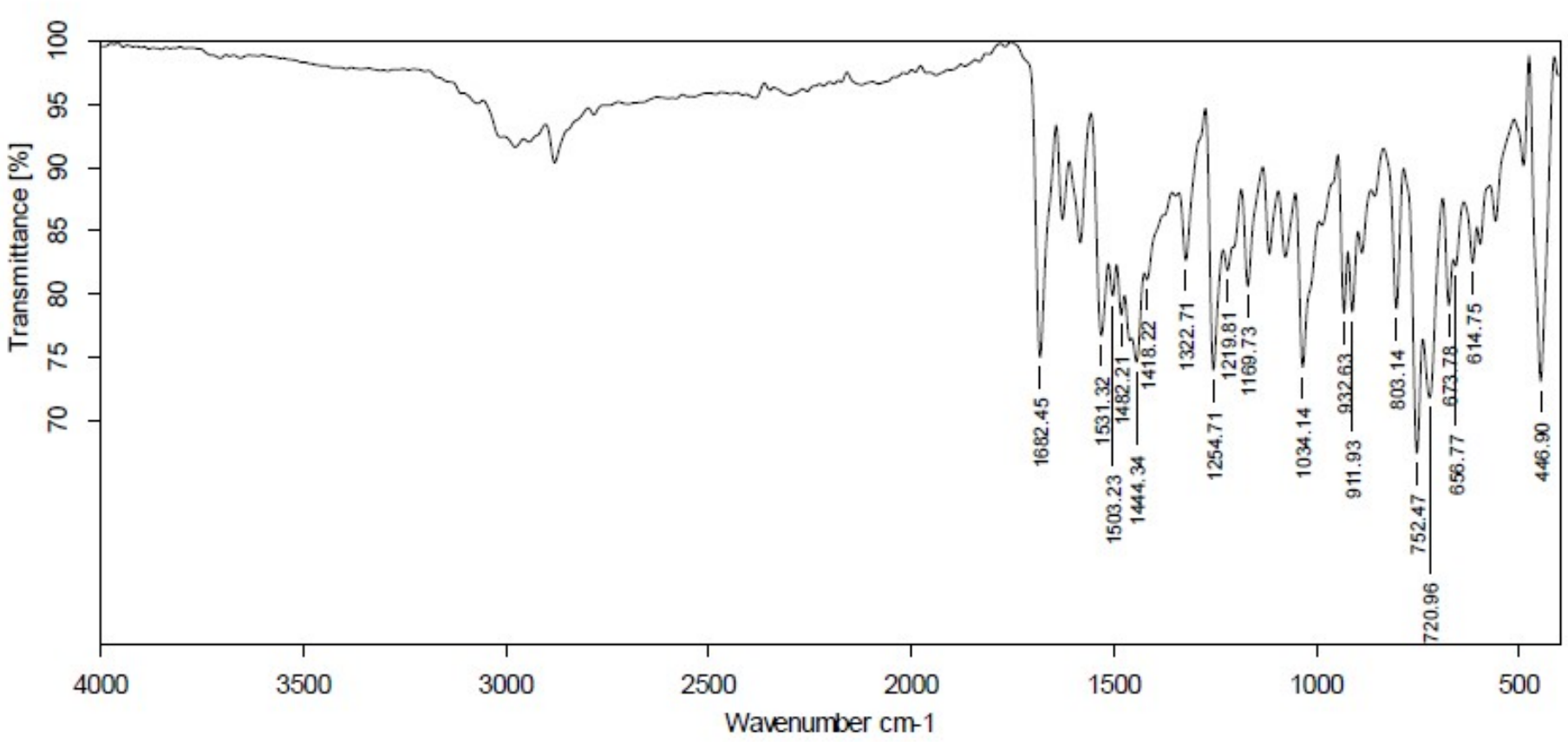

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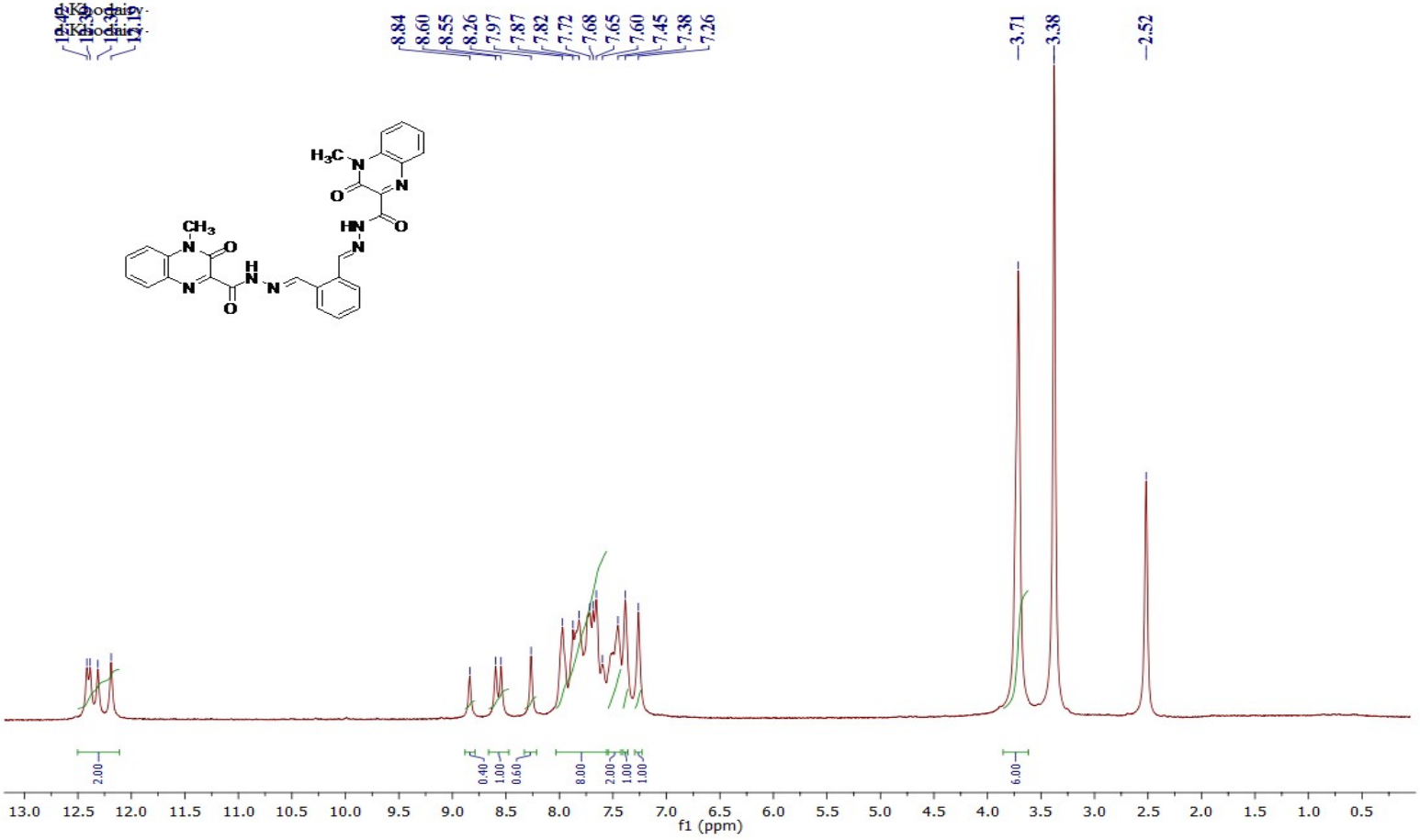

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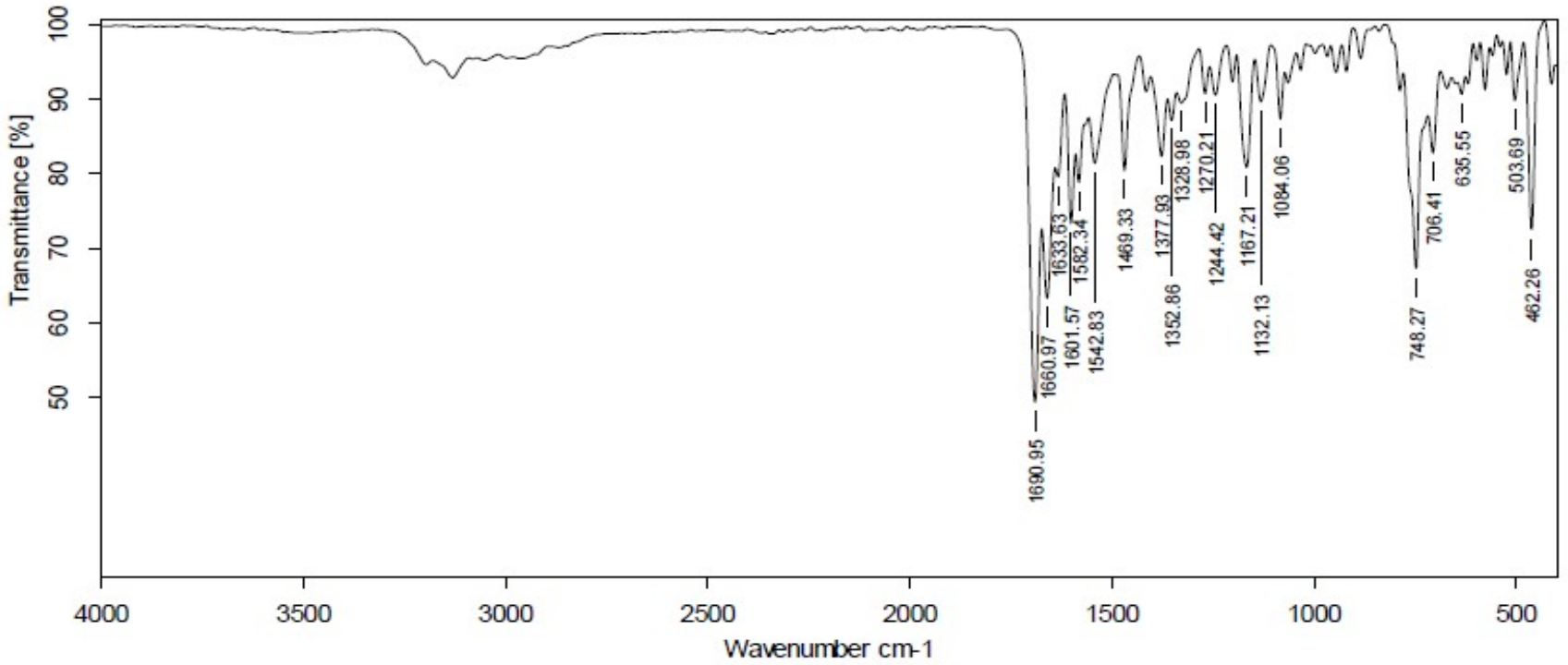

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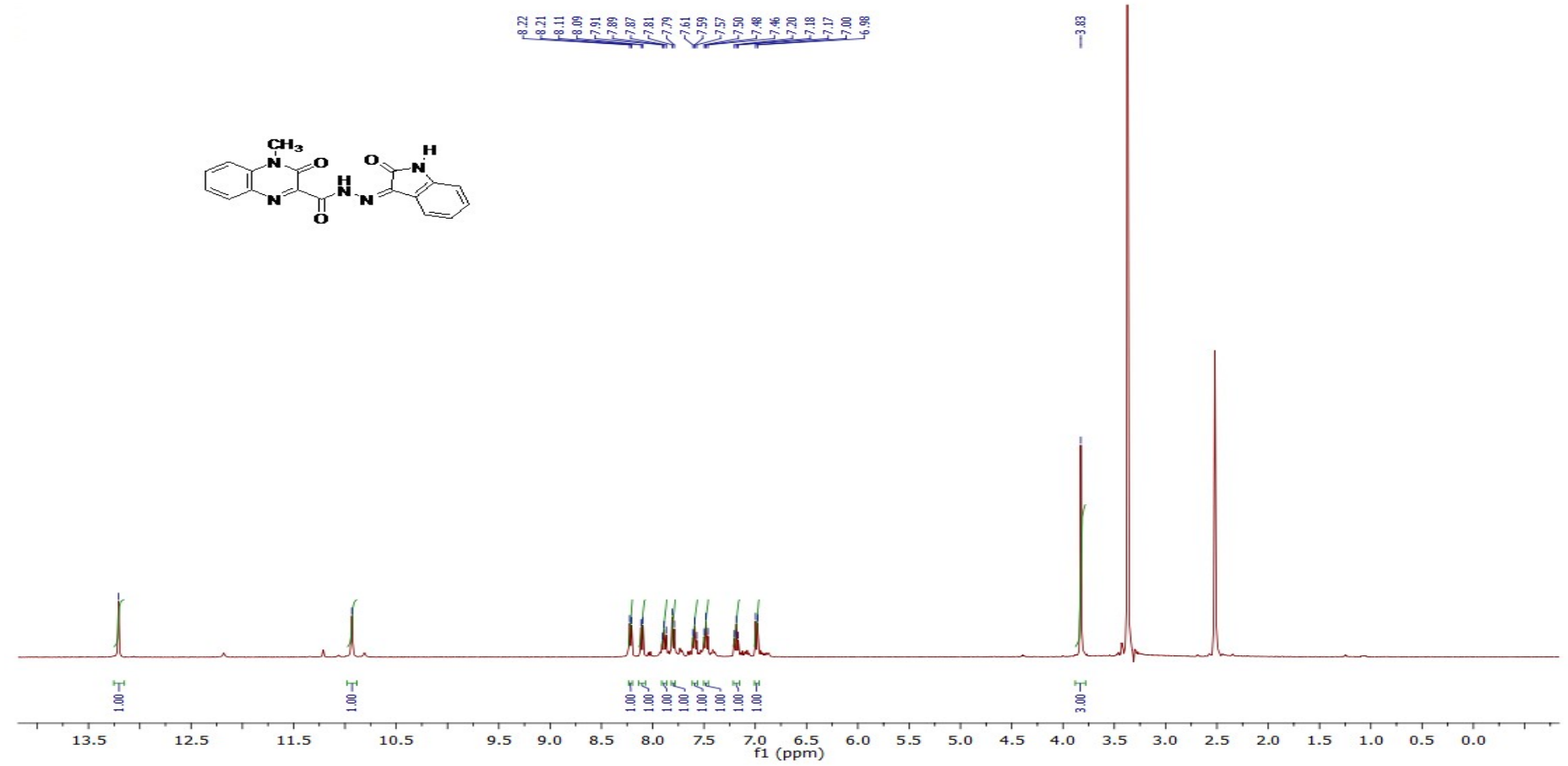

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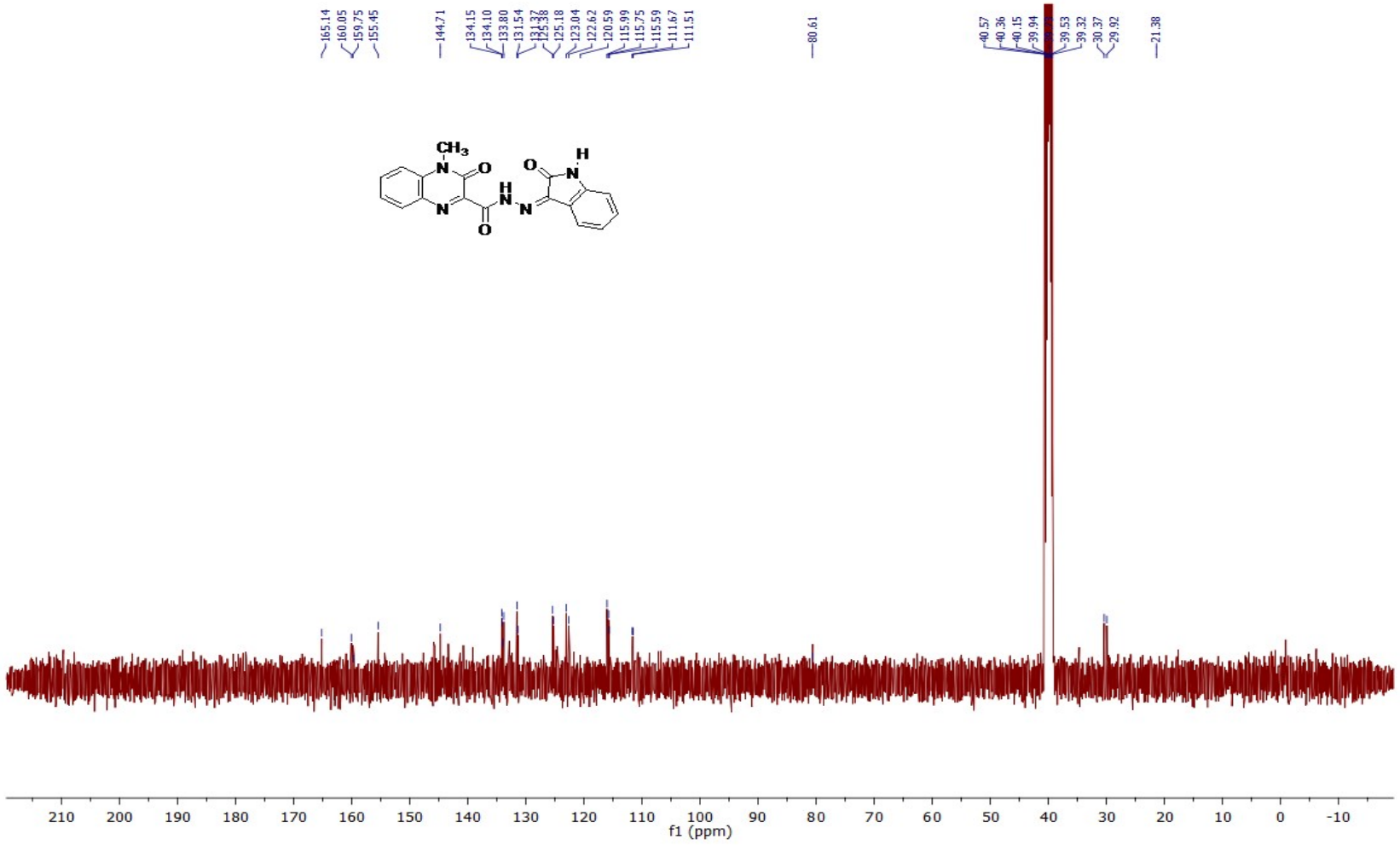

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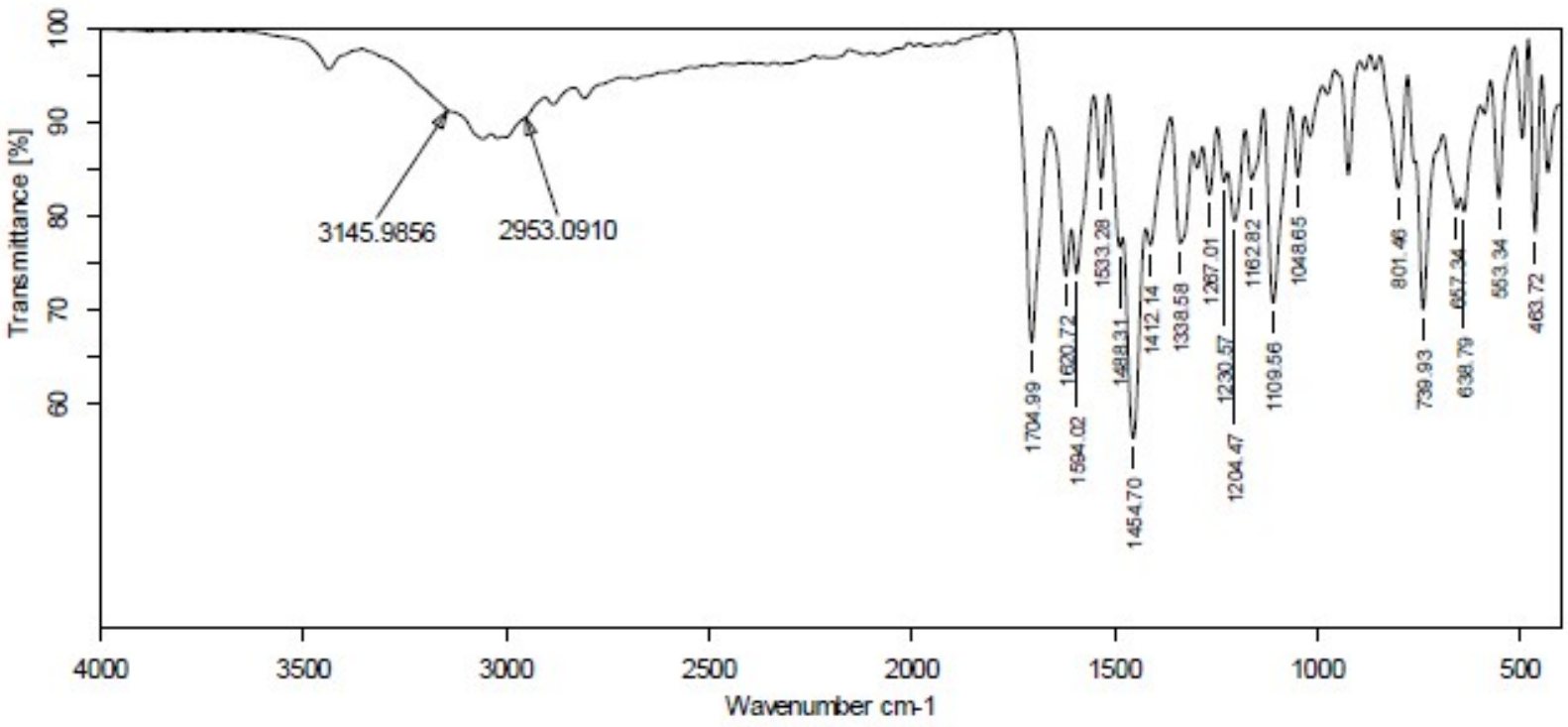

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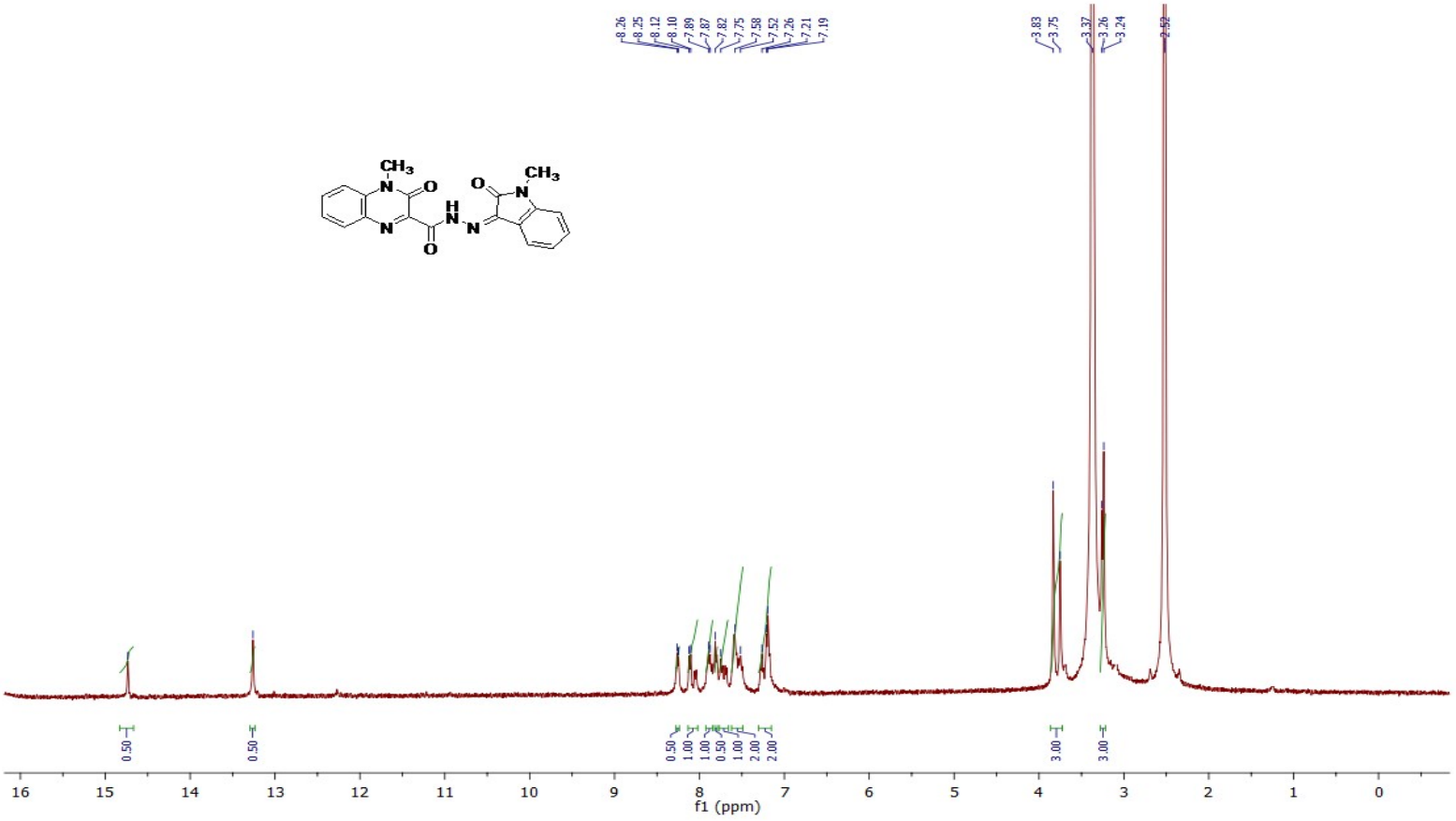

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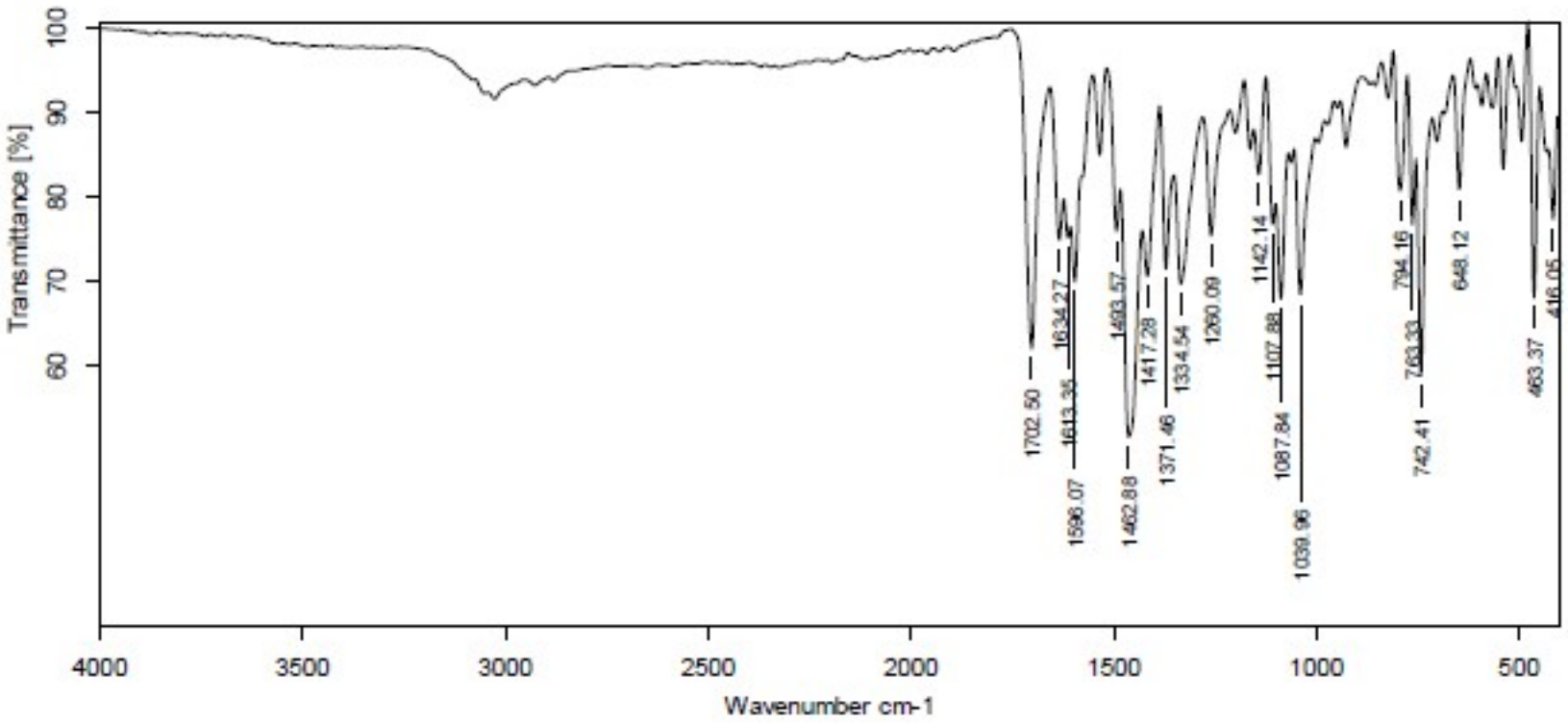

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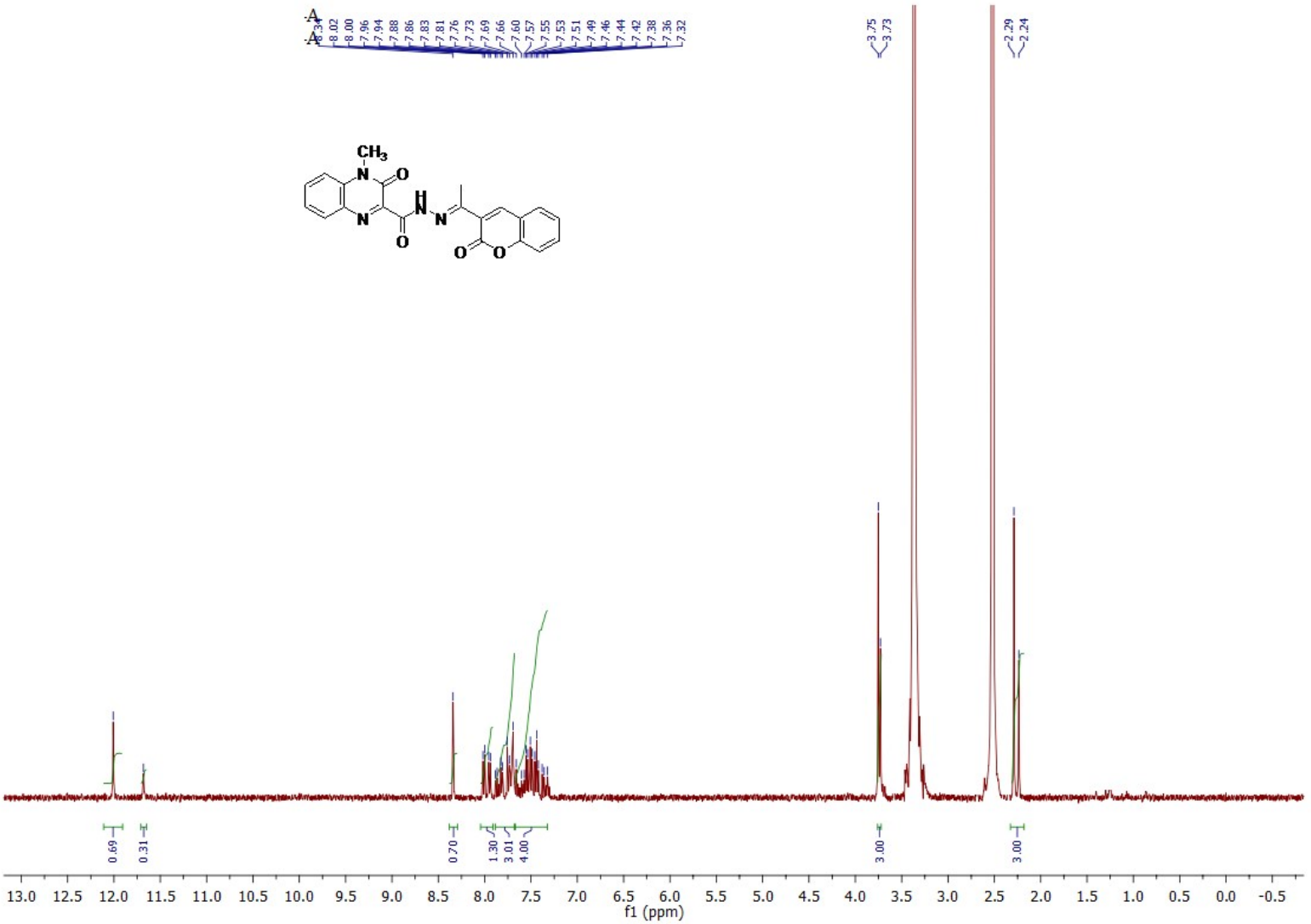
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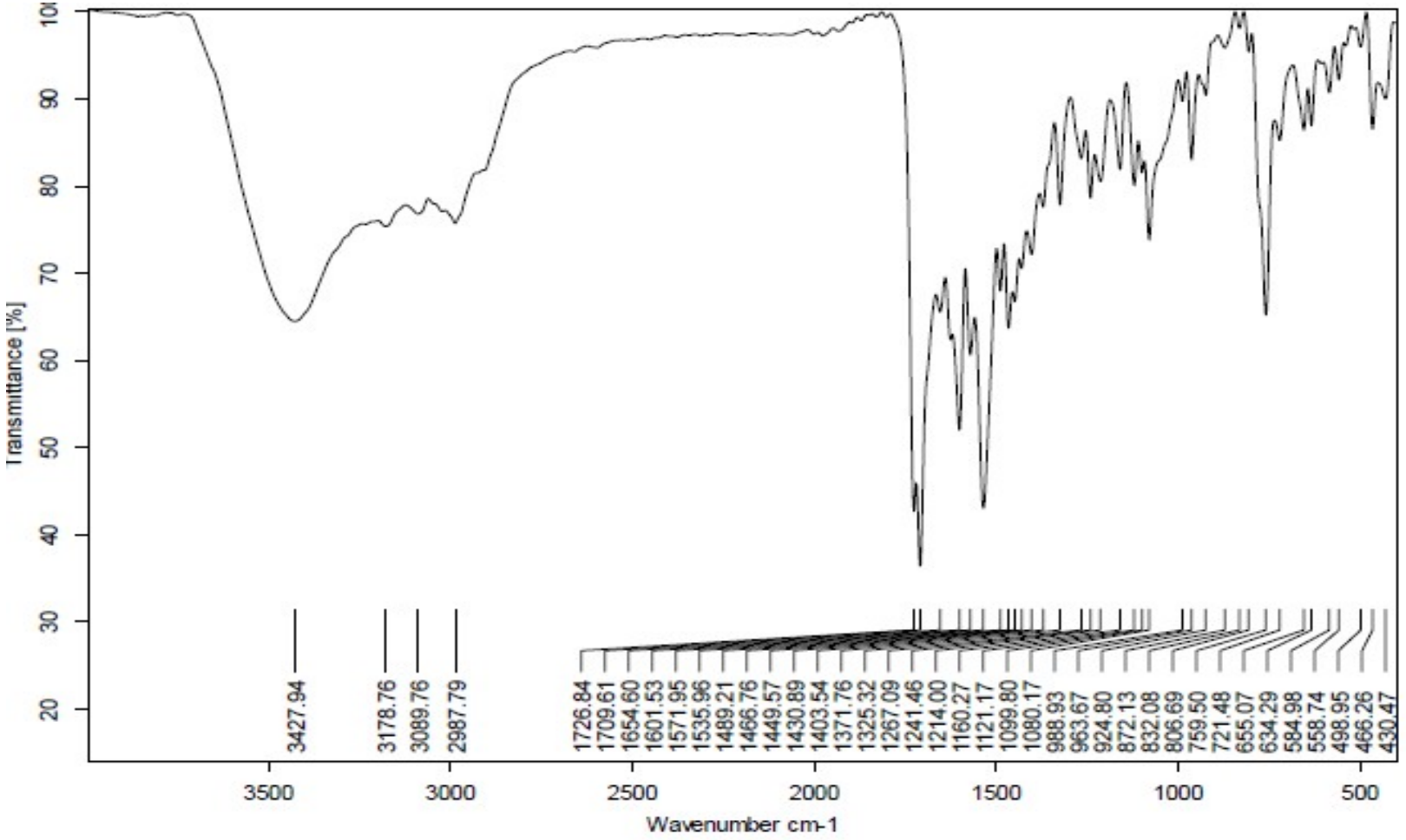

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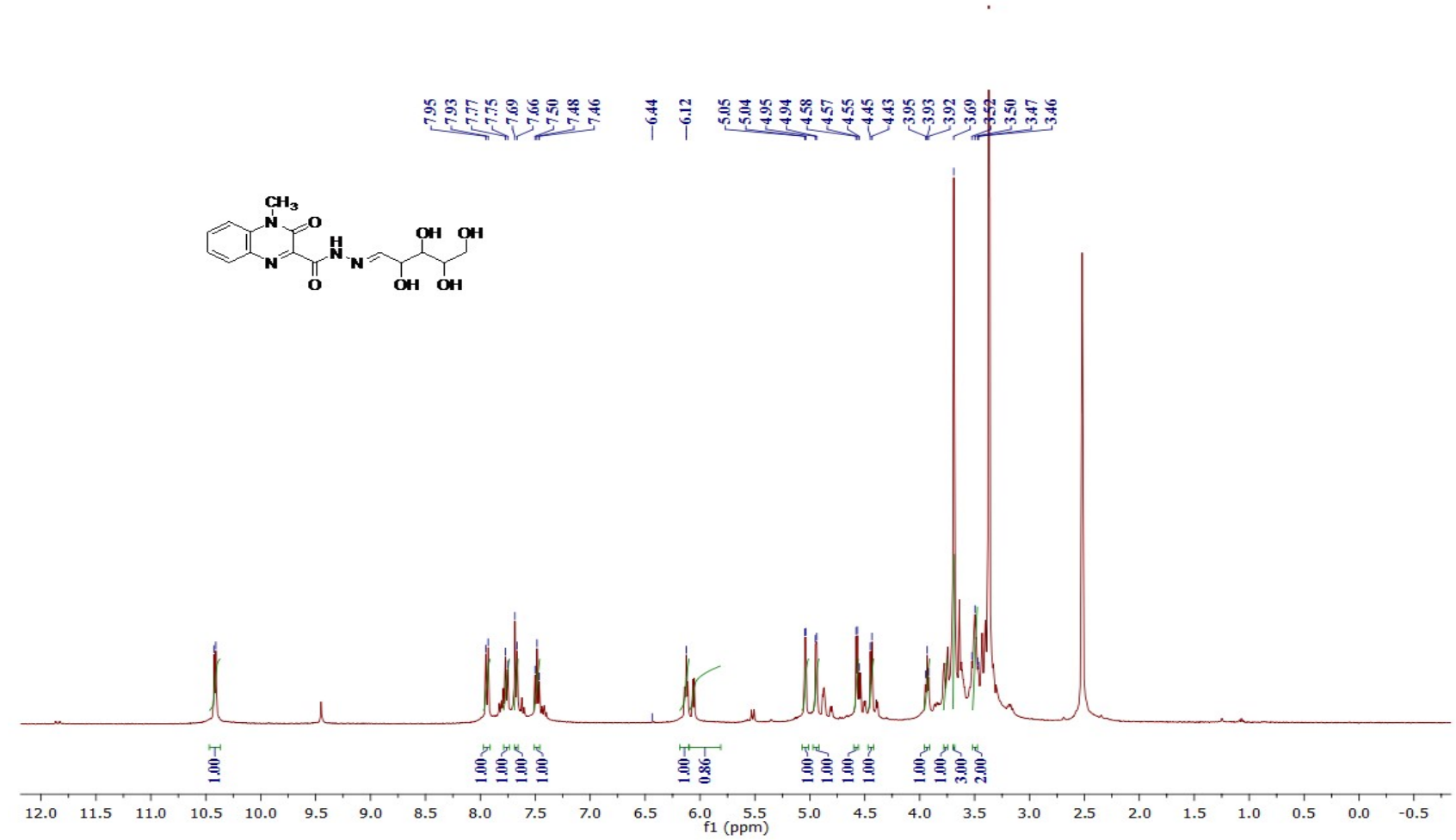

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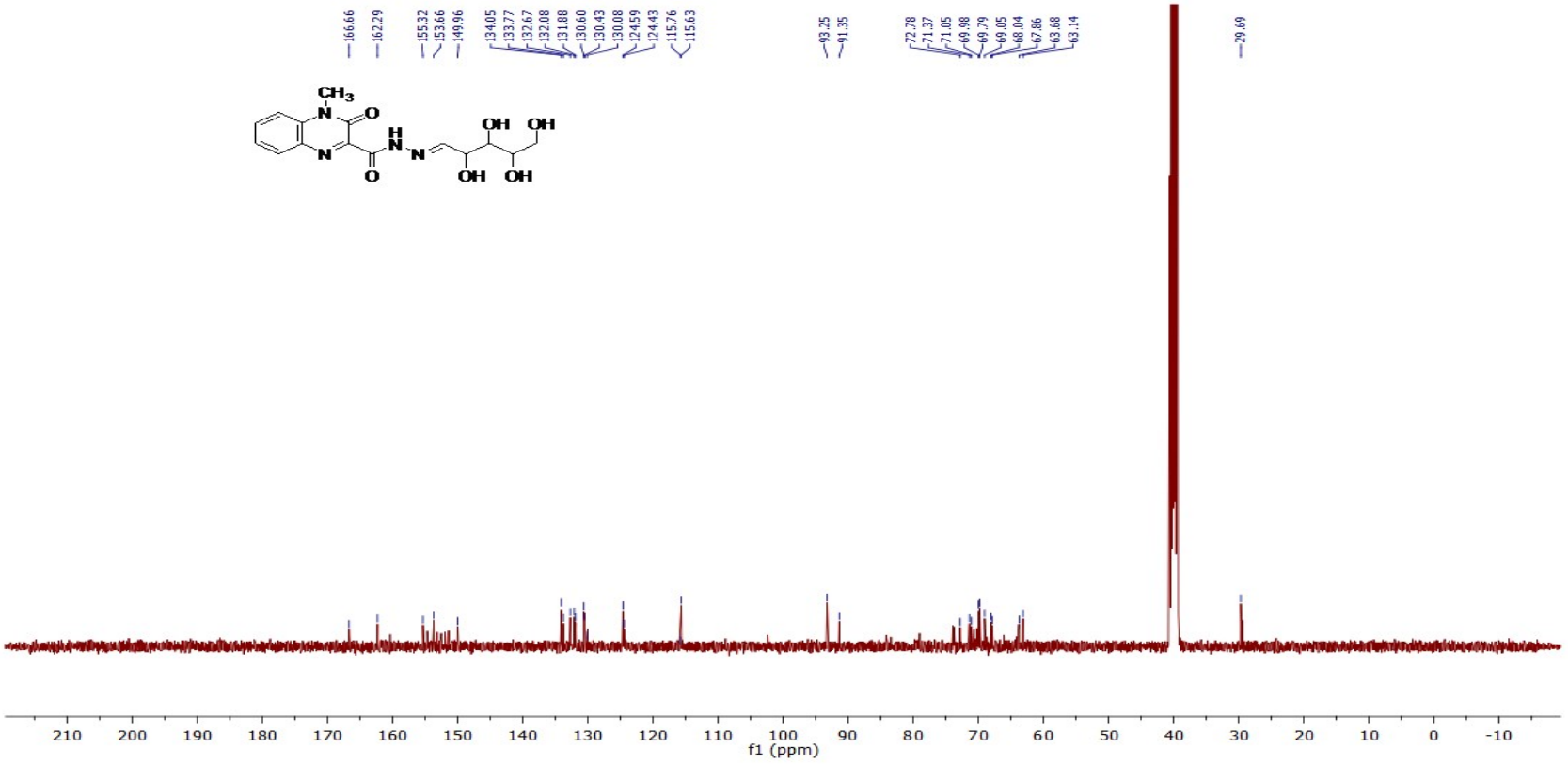

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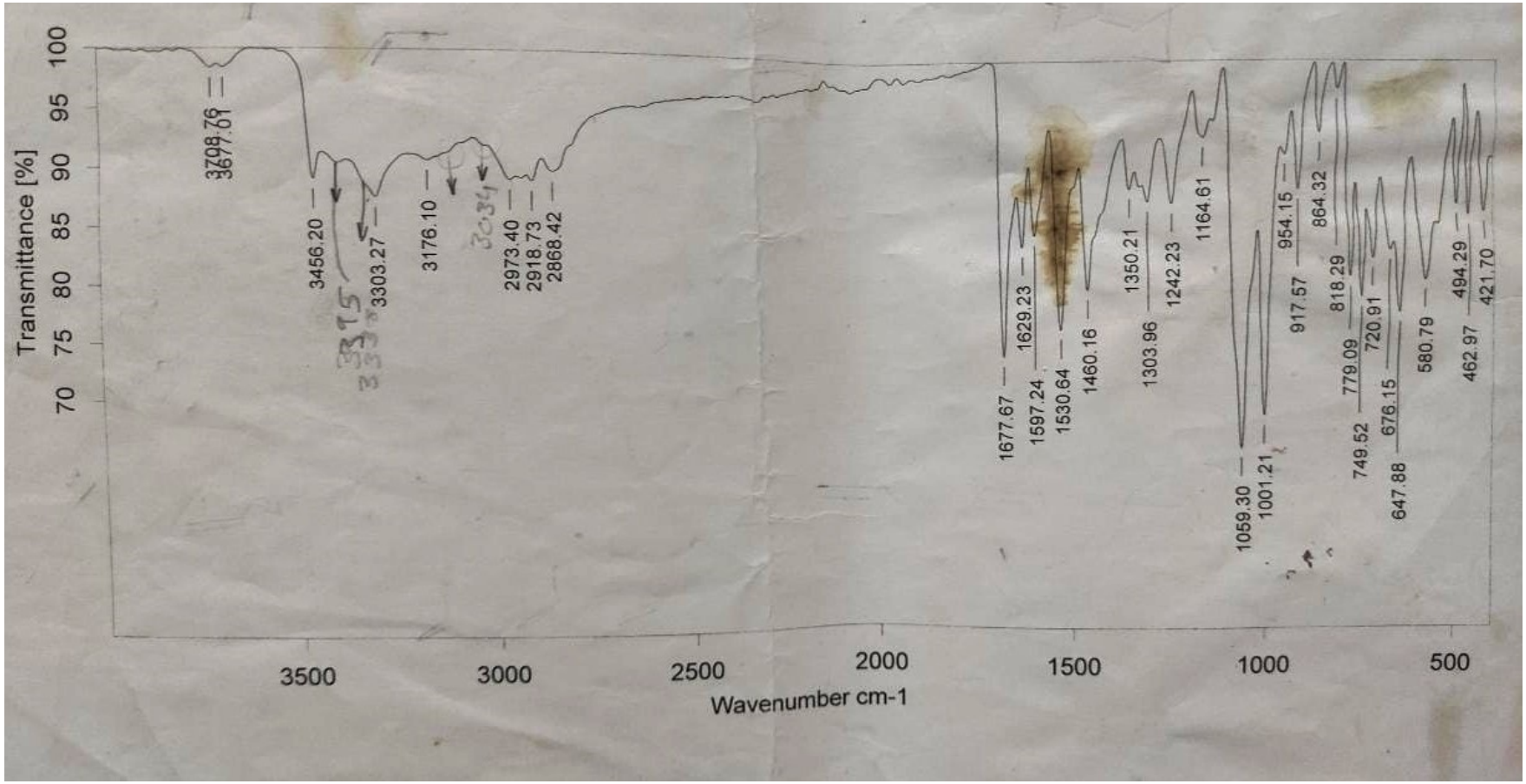

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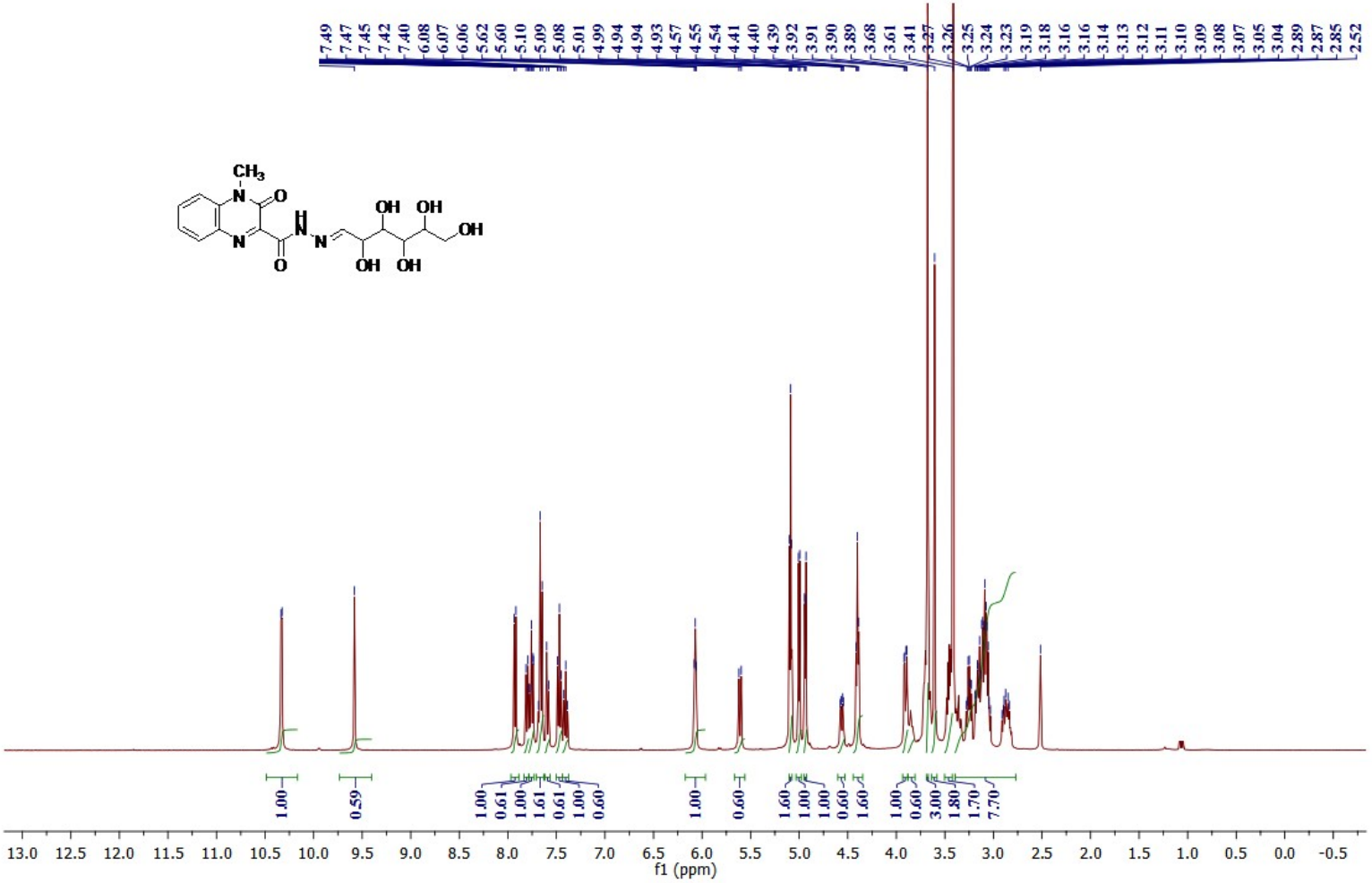

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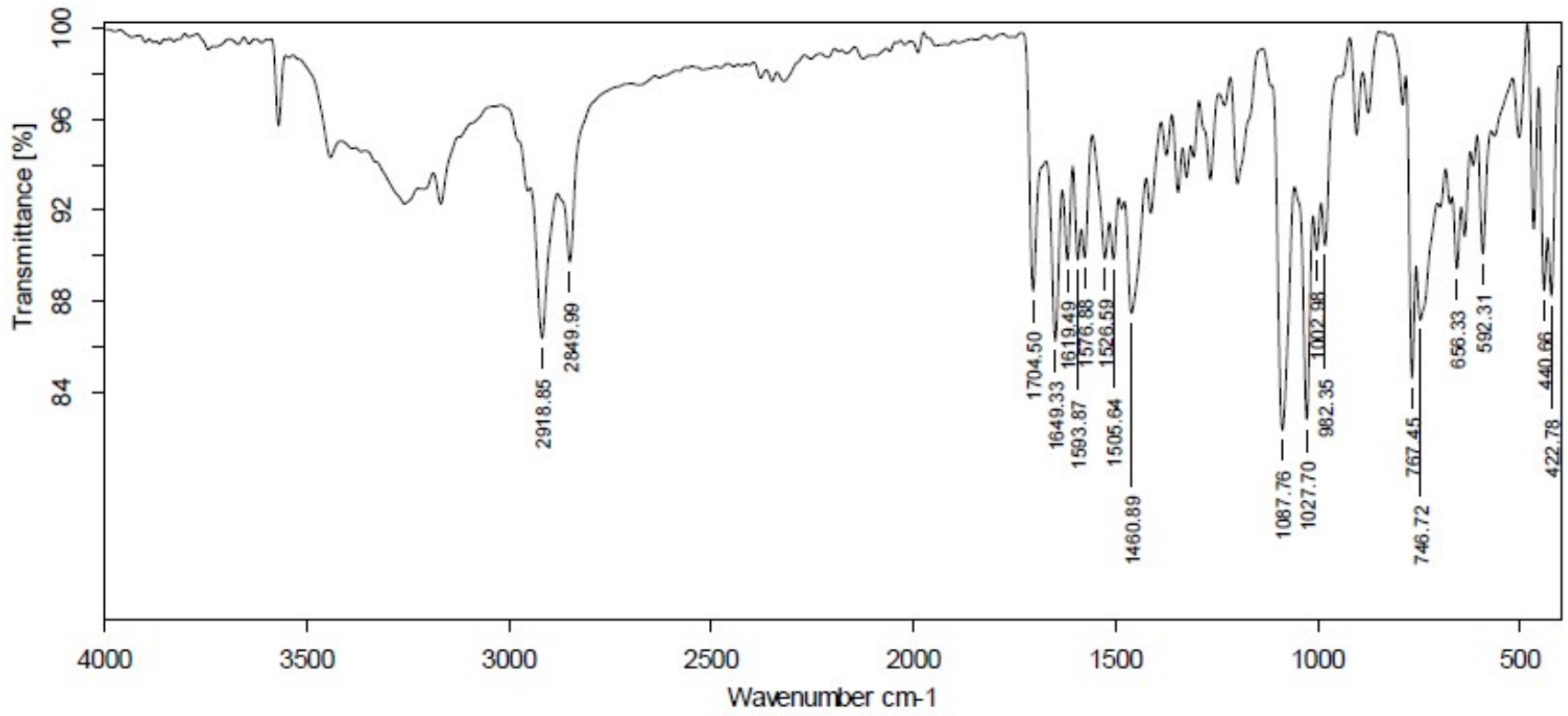

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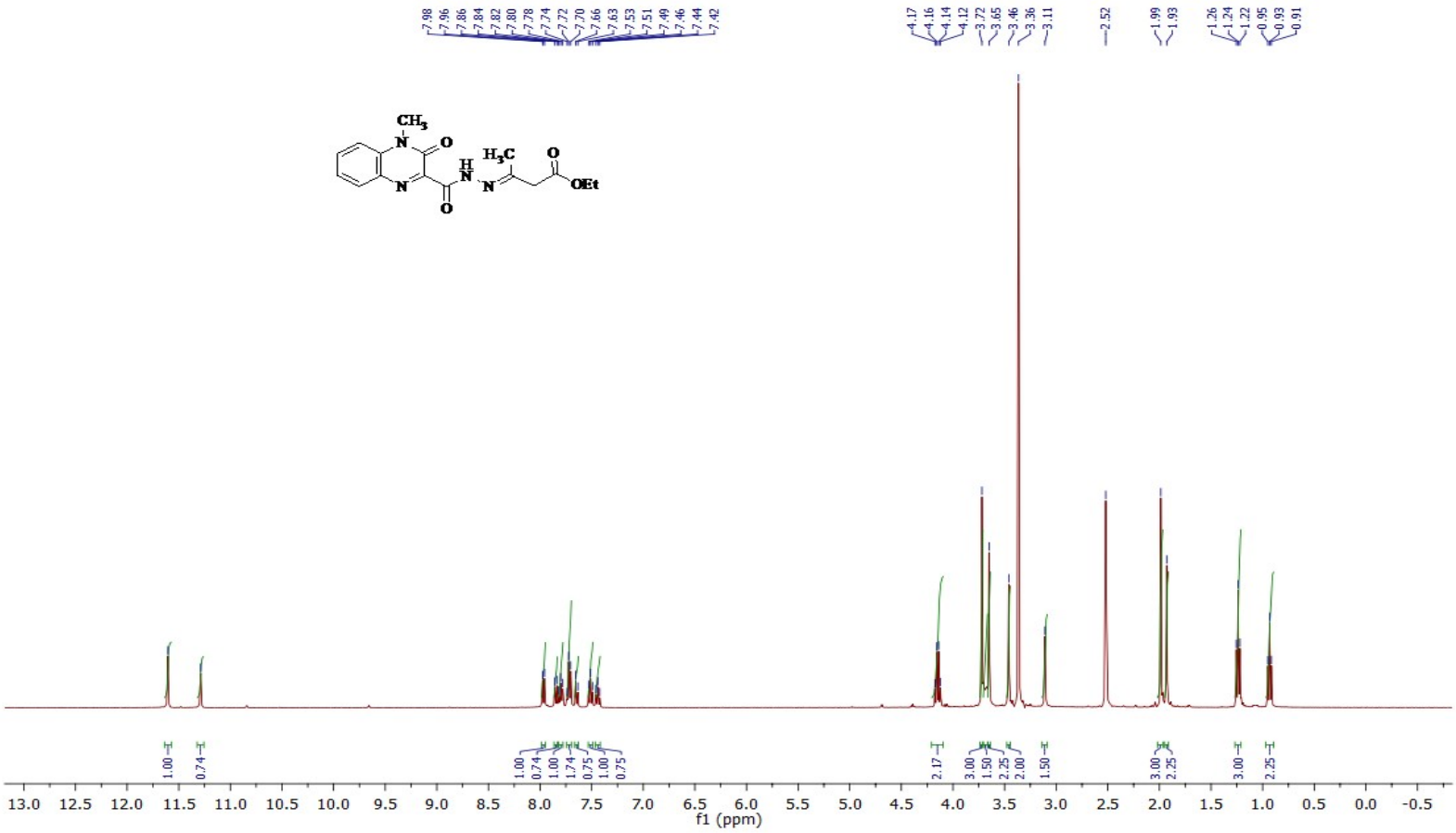

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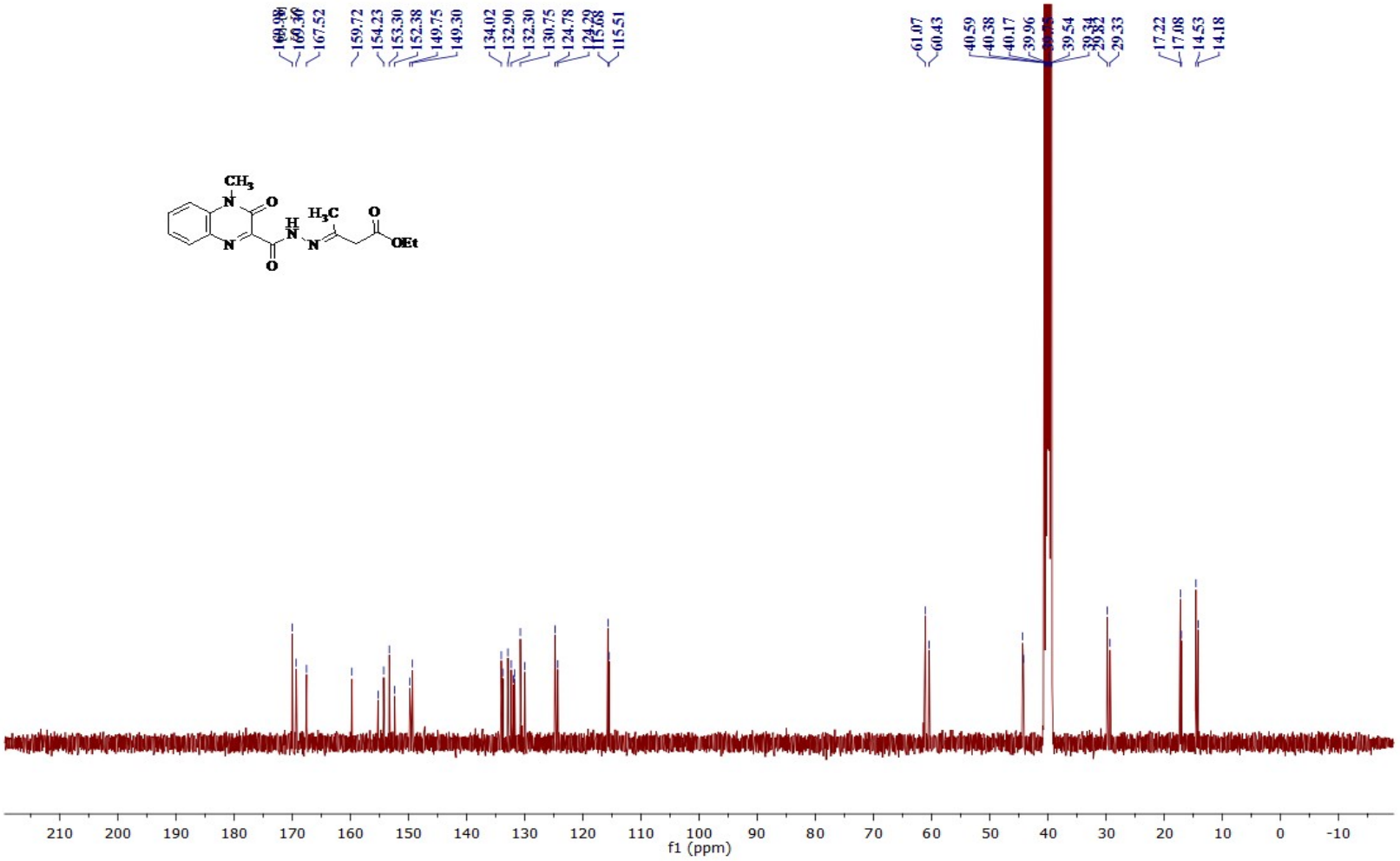

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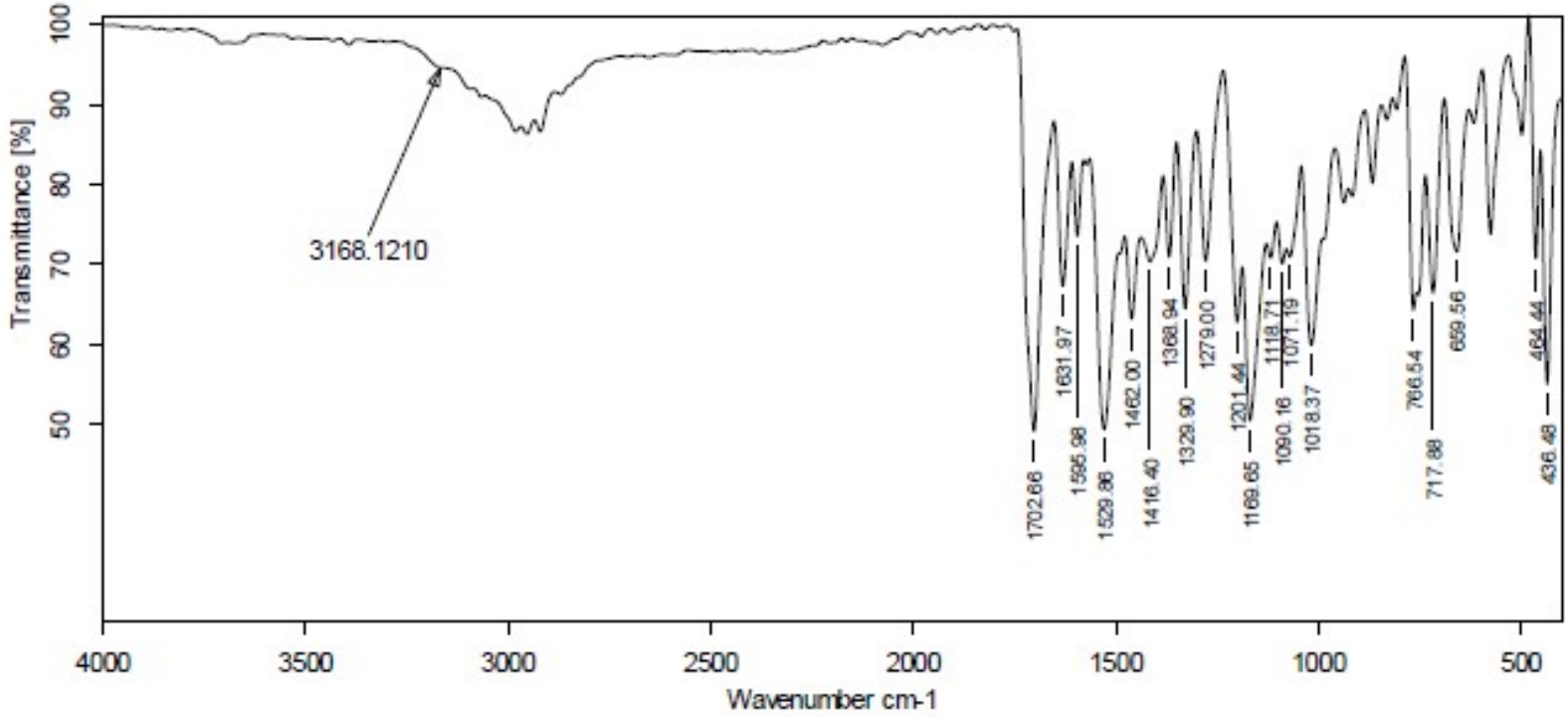

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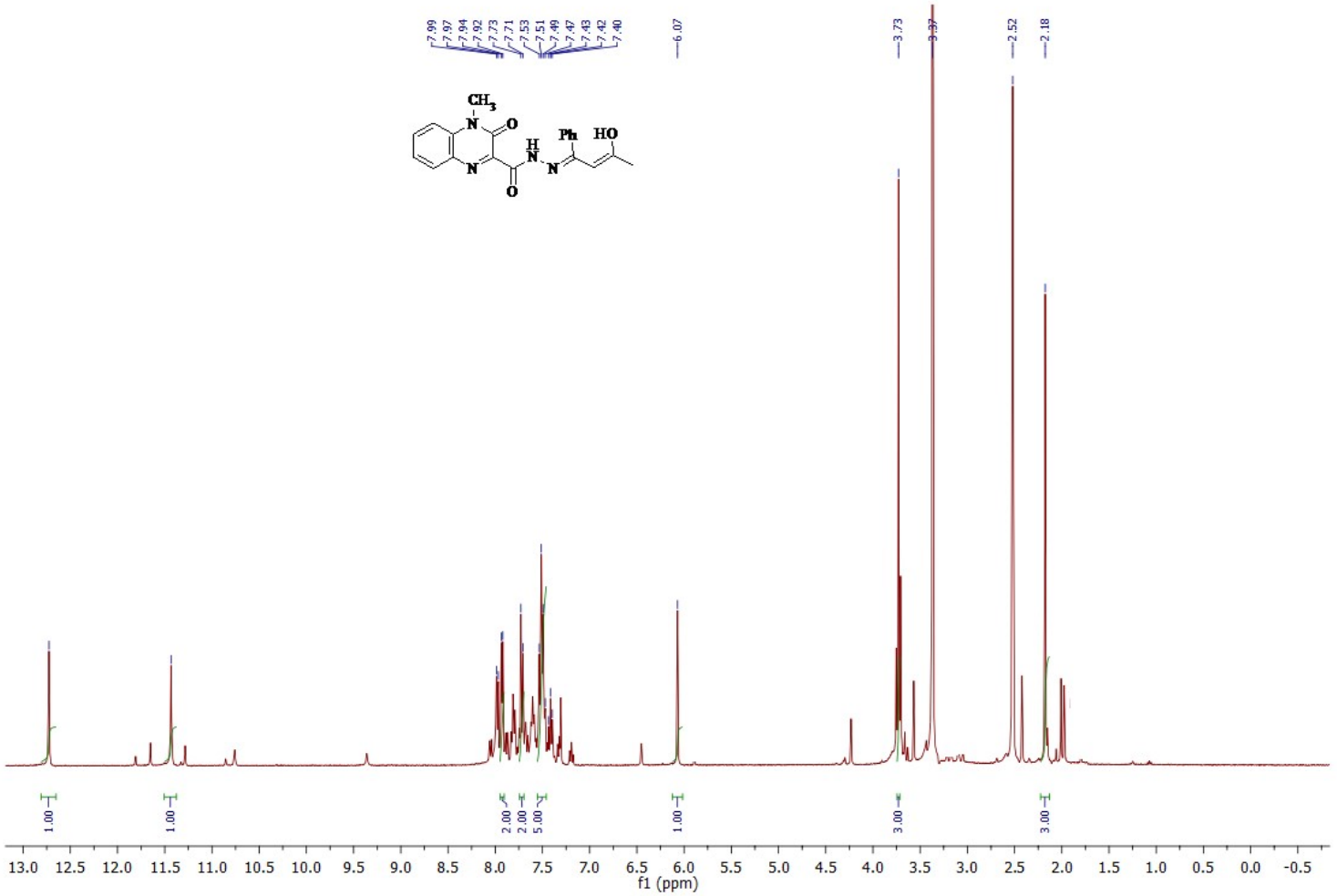

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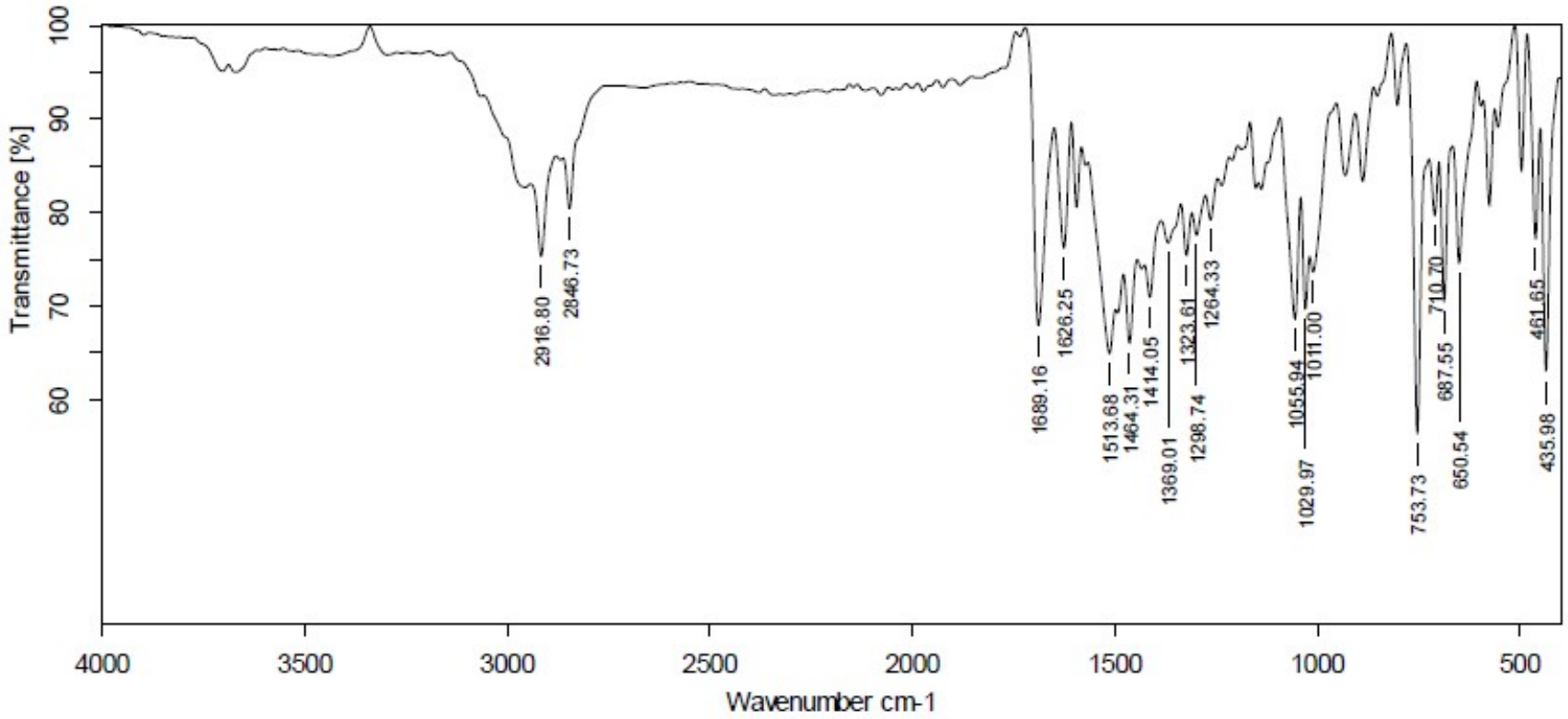

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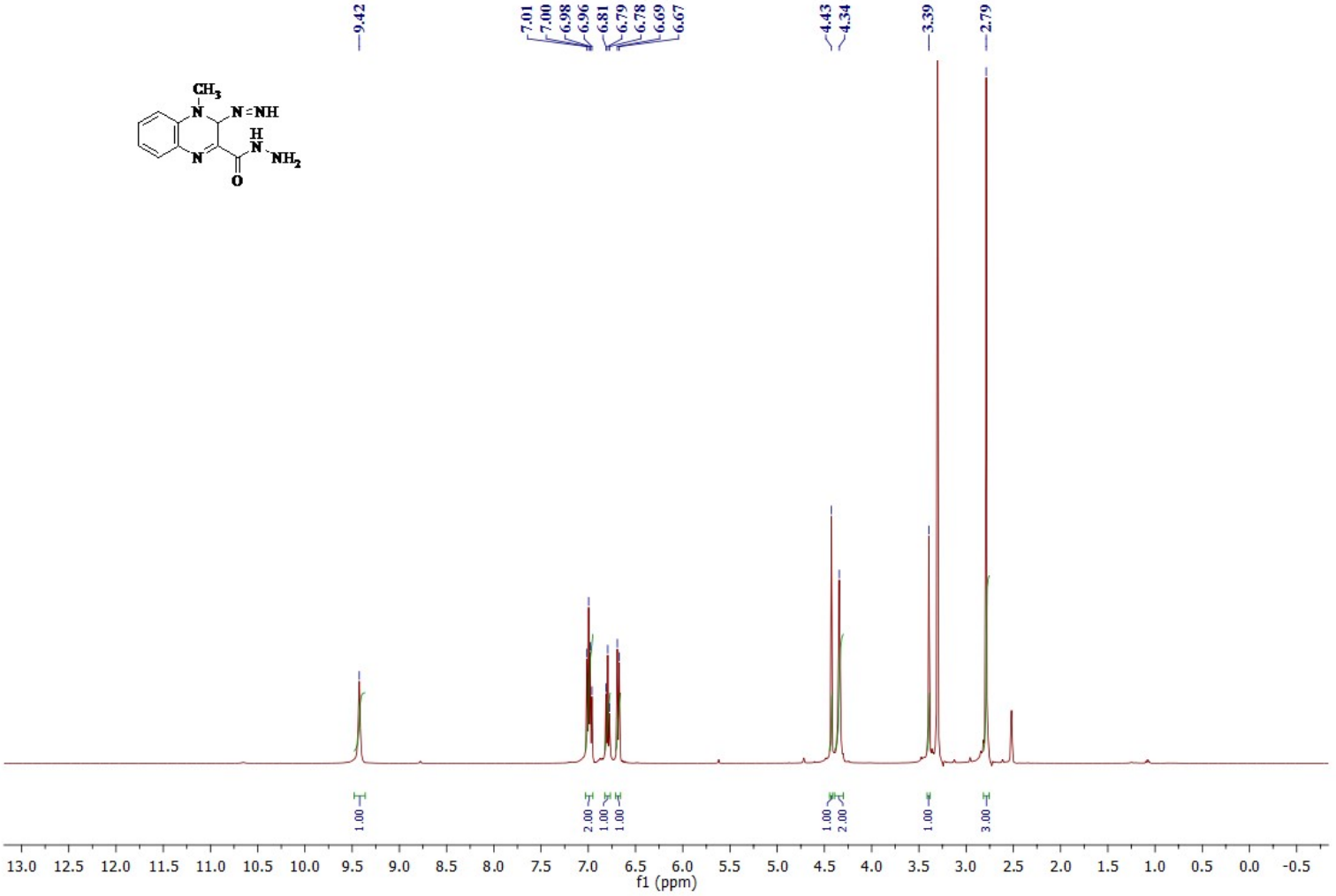

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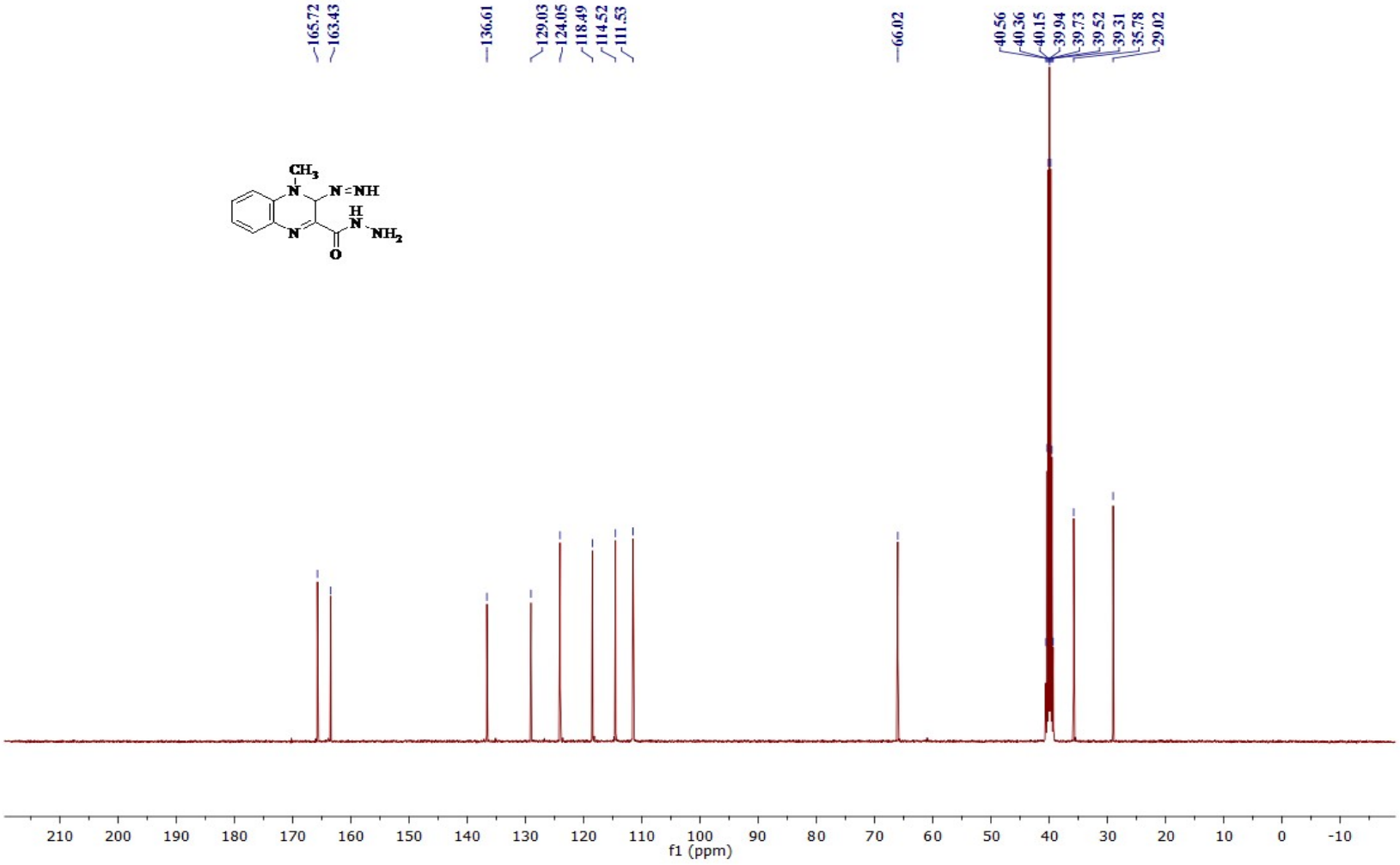

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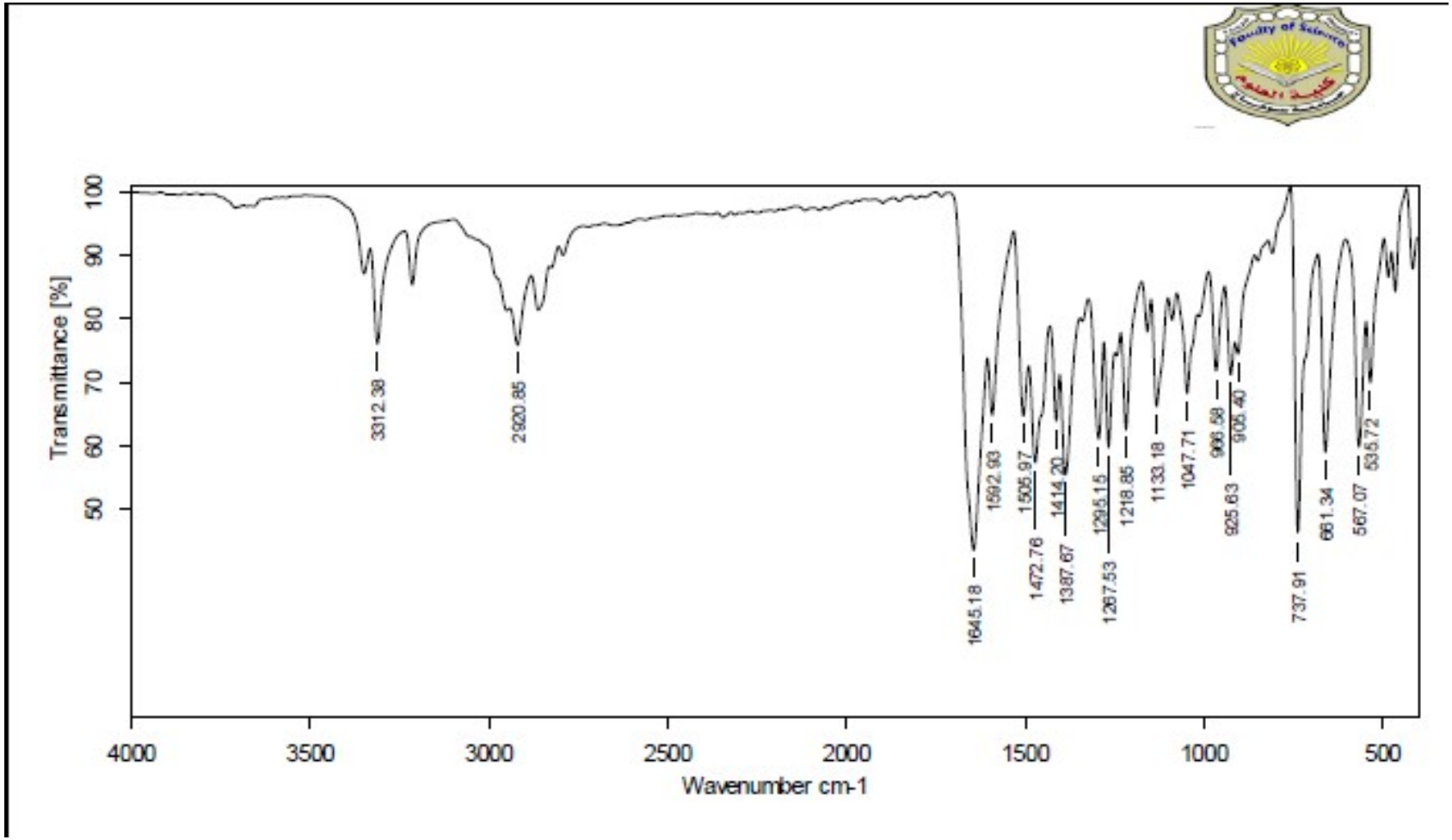
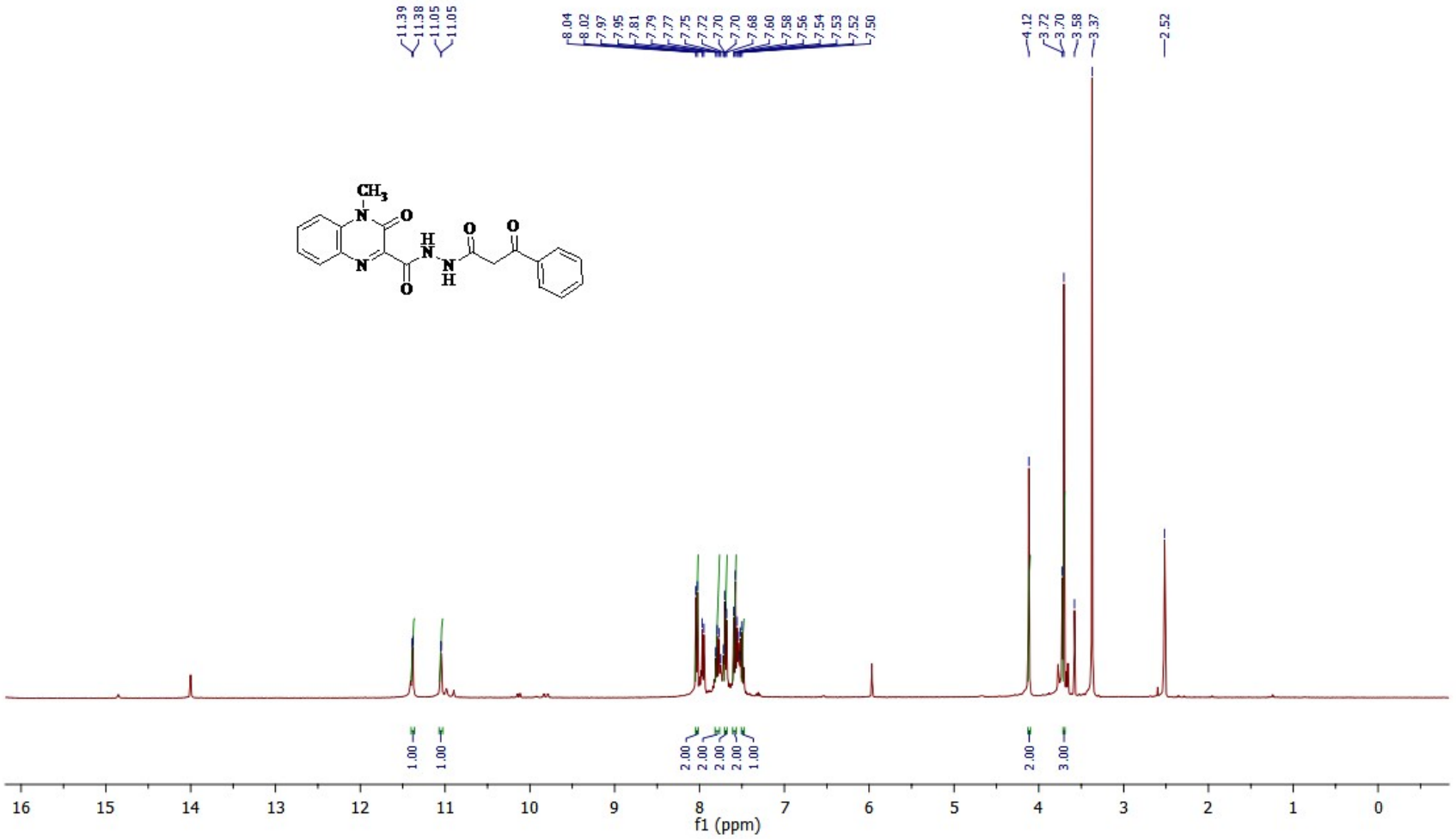
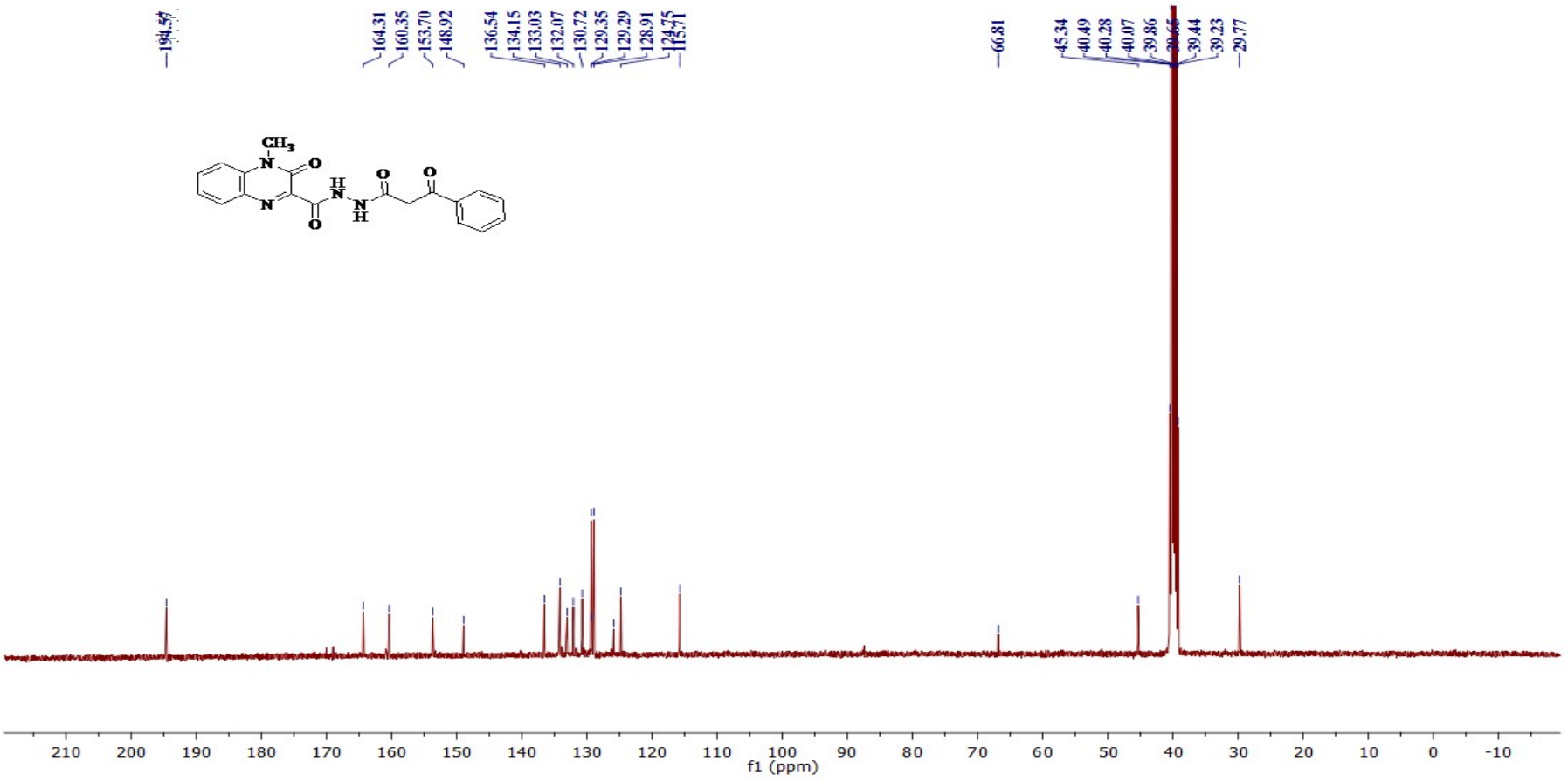


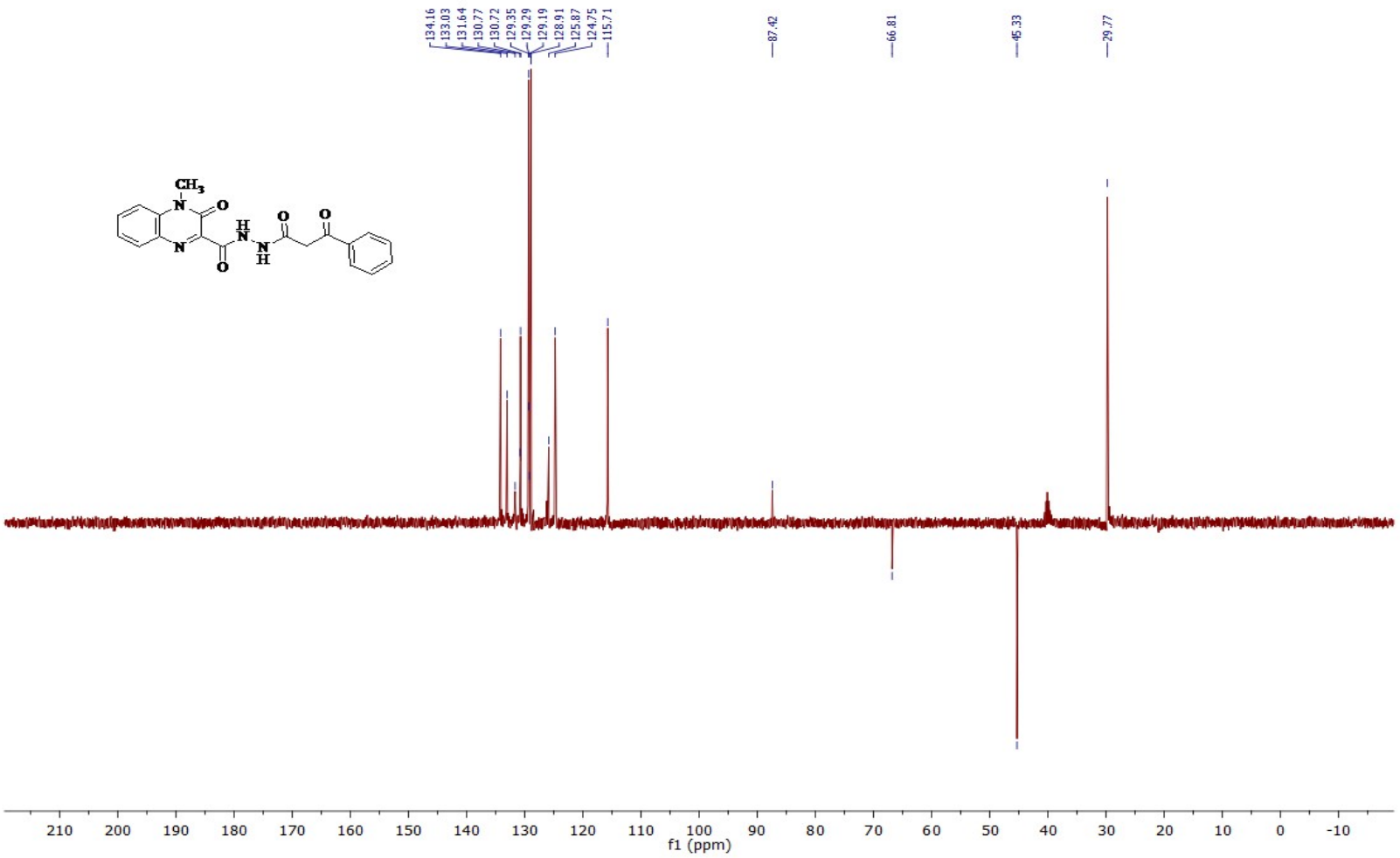
[1]Hnmr of compound 11:

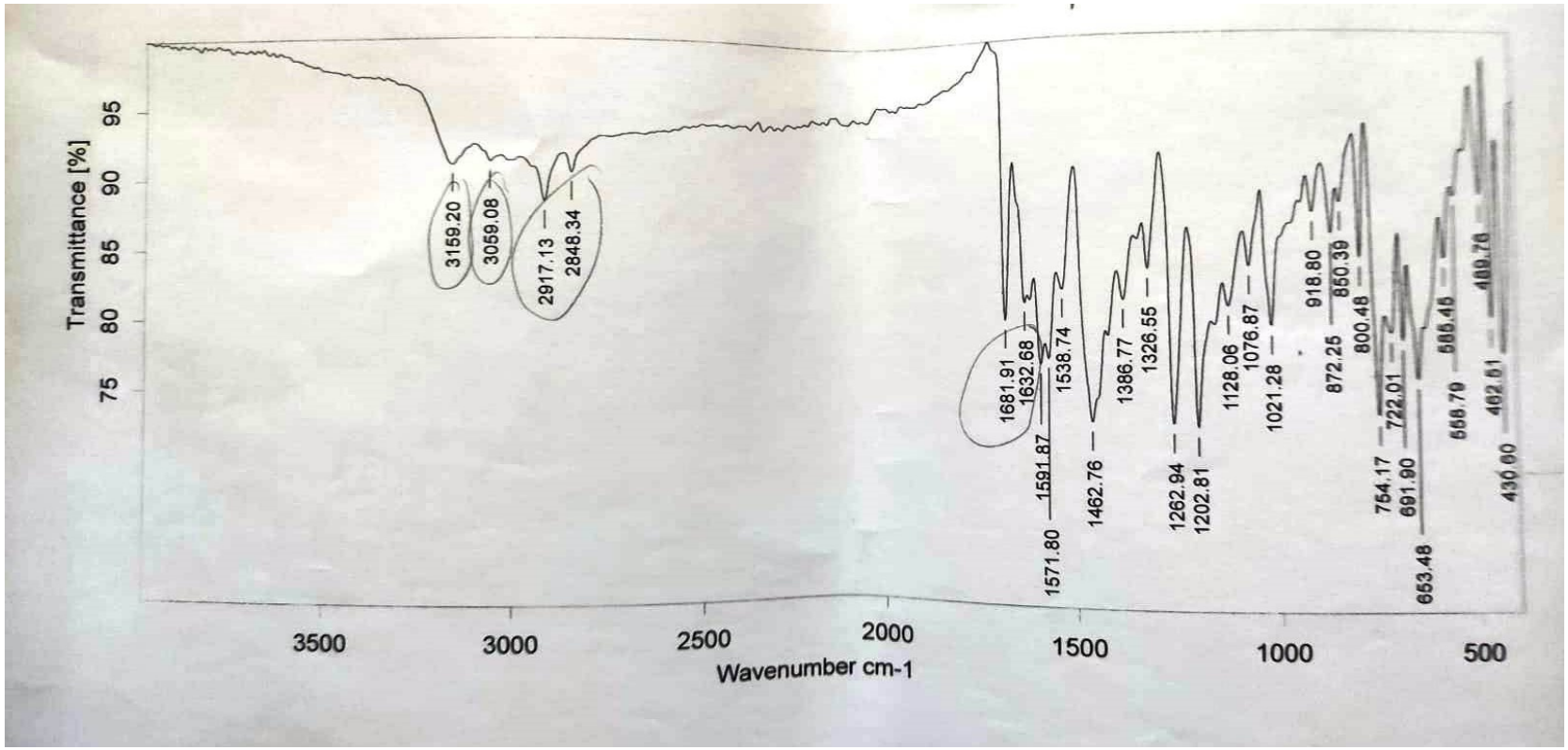


[13]Cnmr of compound 11:

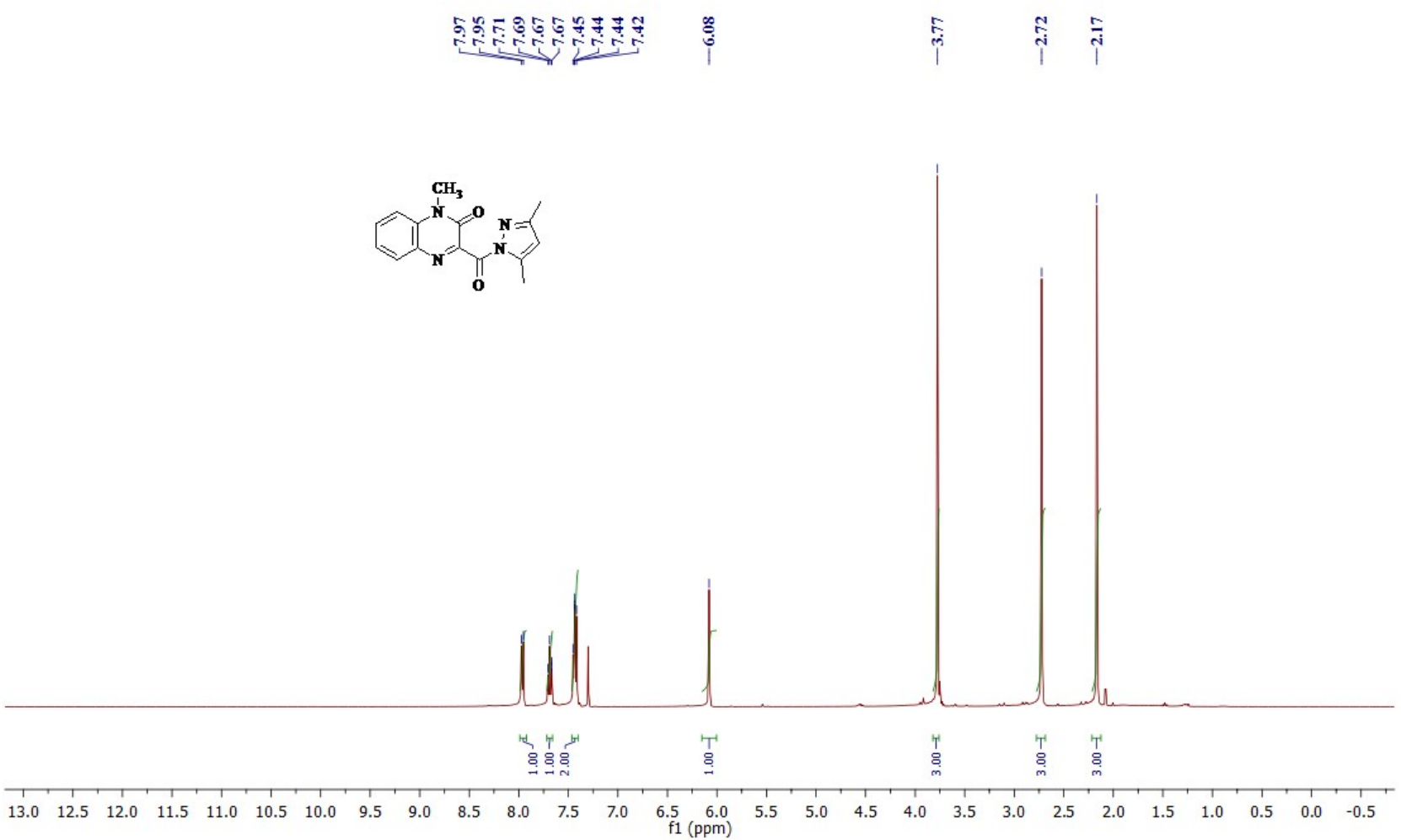


DEPT-135 of compound 11:

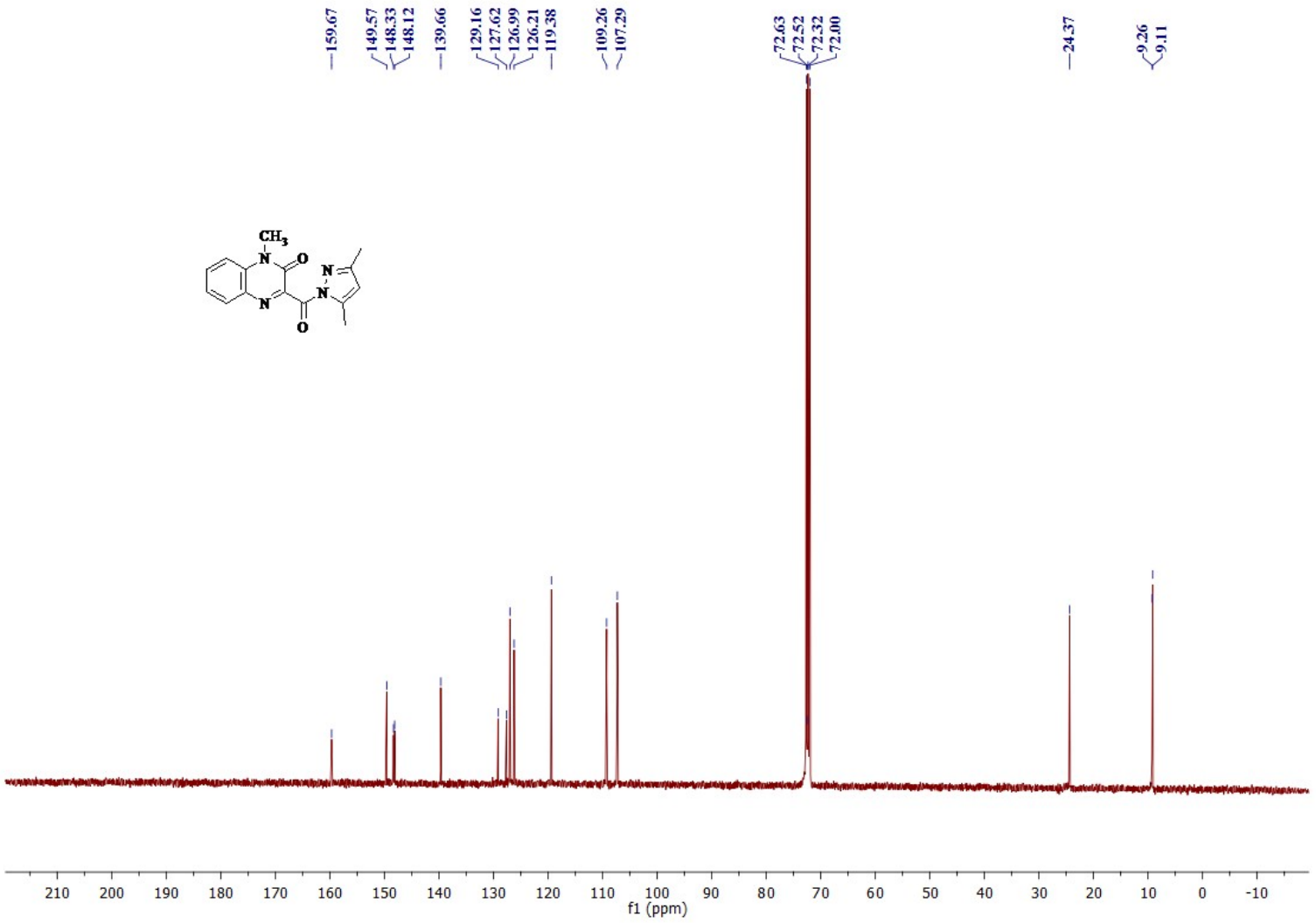



Transmittance [%]
95
90
85
80
75
3159.20
3059.08
2917.13
2848.34
1681.91
1632.68
1591.87
1571.80
1538.74
1462.76
1386.77
1326.55
1262.94
1202.81
1128.06
1076.87
1021.28
918.80
872.25
850.39
800.48
754.17
722.01
691.90
653.48
588.79
566.46
462.61
430.00
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1

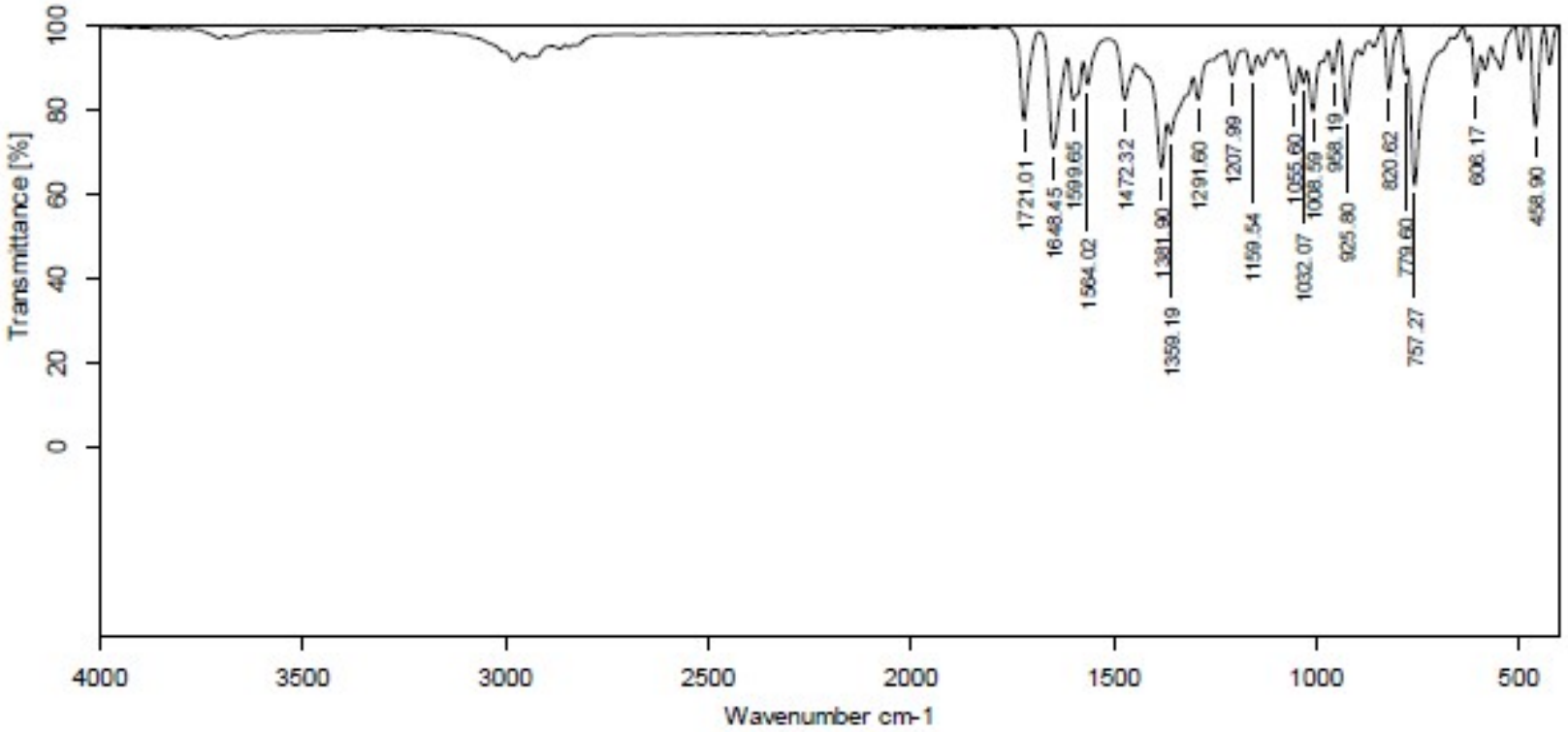
[1]Hnmr of compound 12:



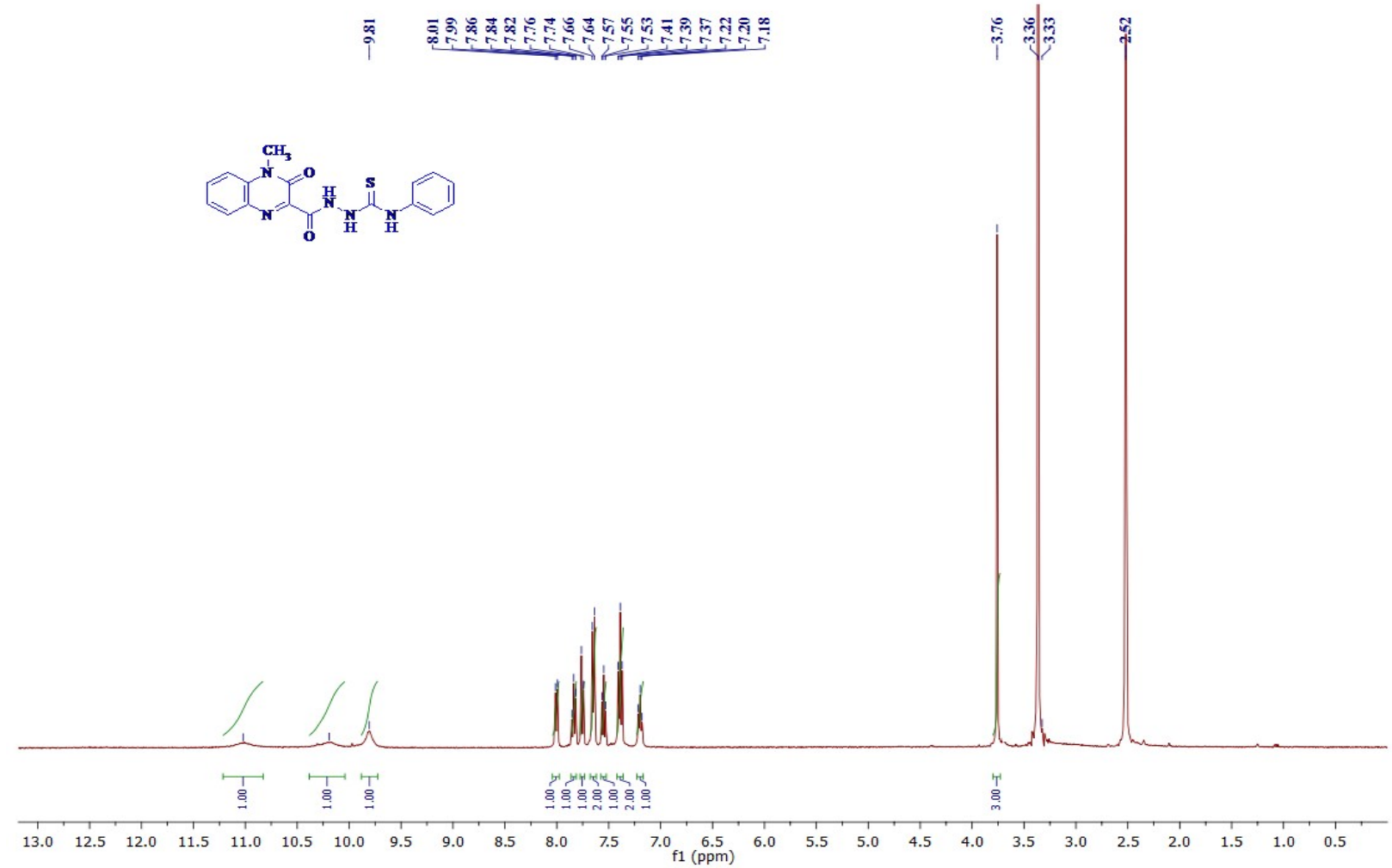
[13]Cnmr of compound 12:


159.67
149.57
148.33
148.12
139.66
129.16
127.62
126.99
126.21
119.38
109.26
107.29
72.63
72.52
72.32
72.00
24.37
9.26
9.11
CH3
N
O
N
N
N
O
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

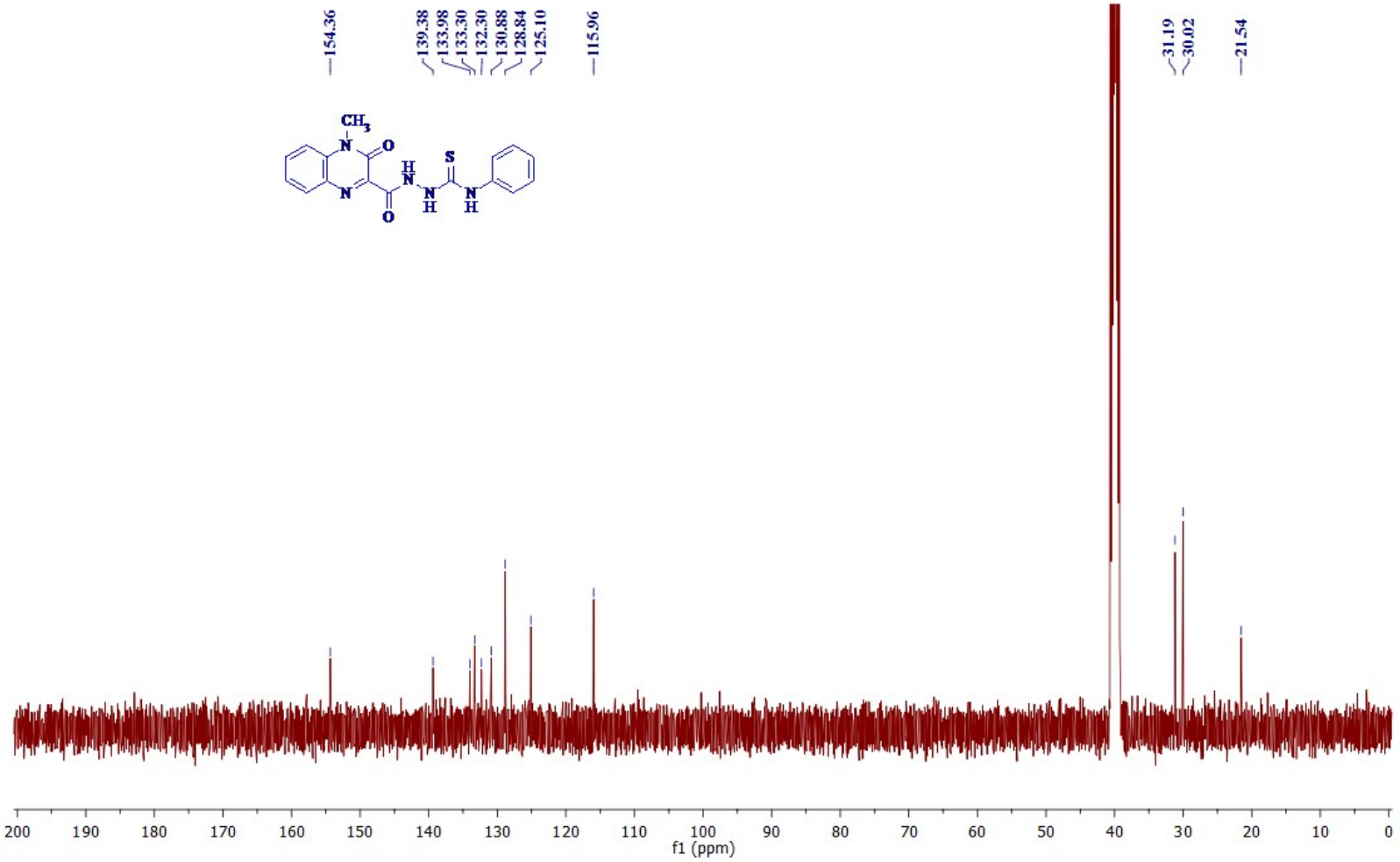
IR of compound 12:



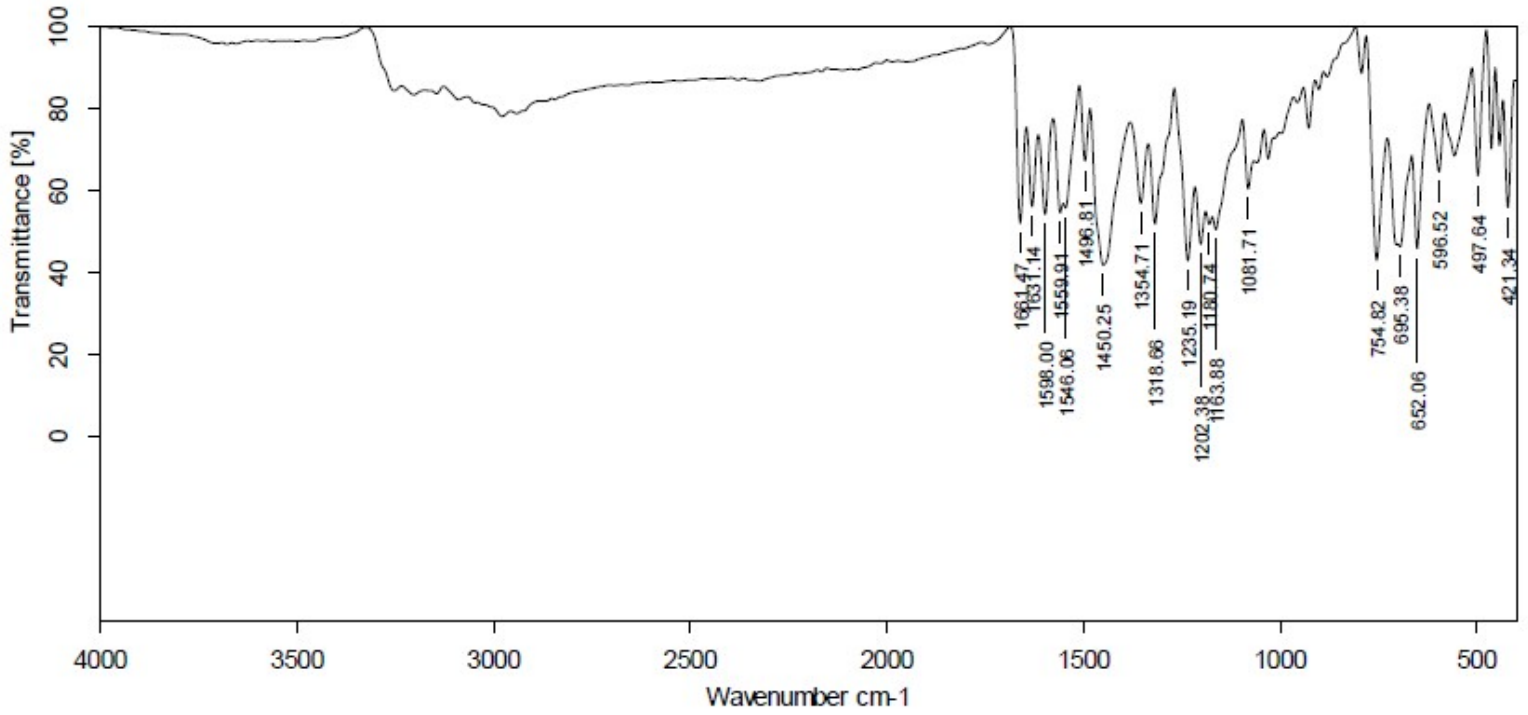
[1]Hnmr of compound 13:



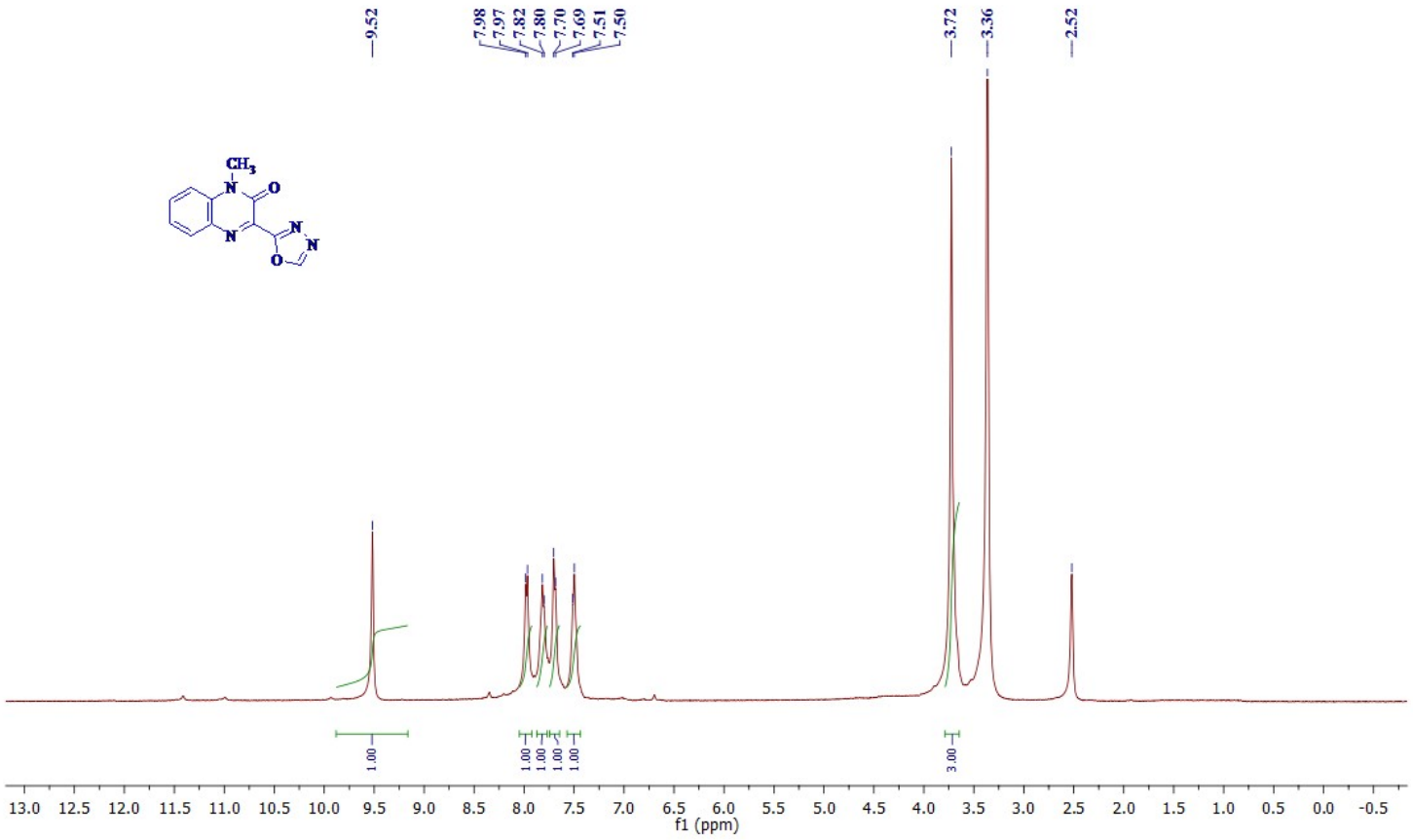
^{13}Cnmr of compound 13:



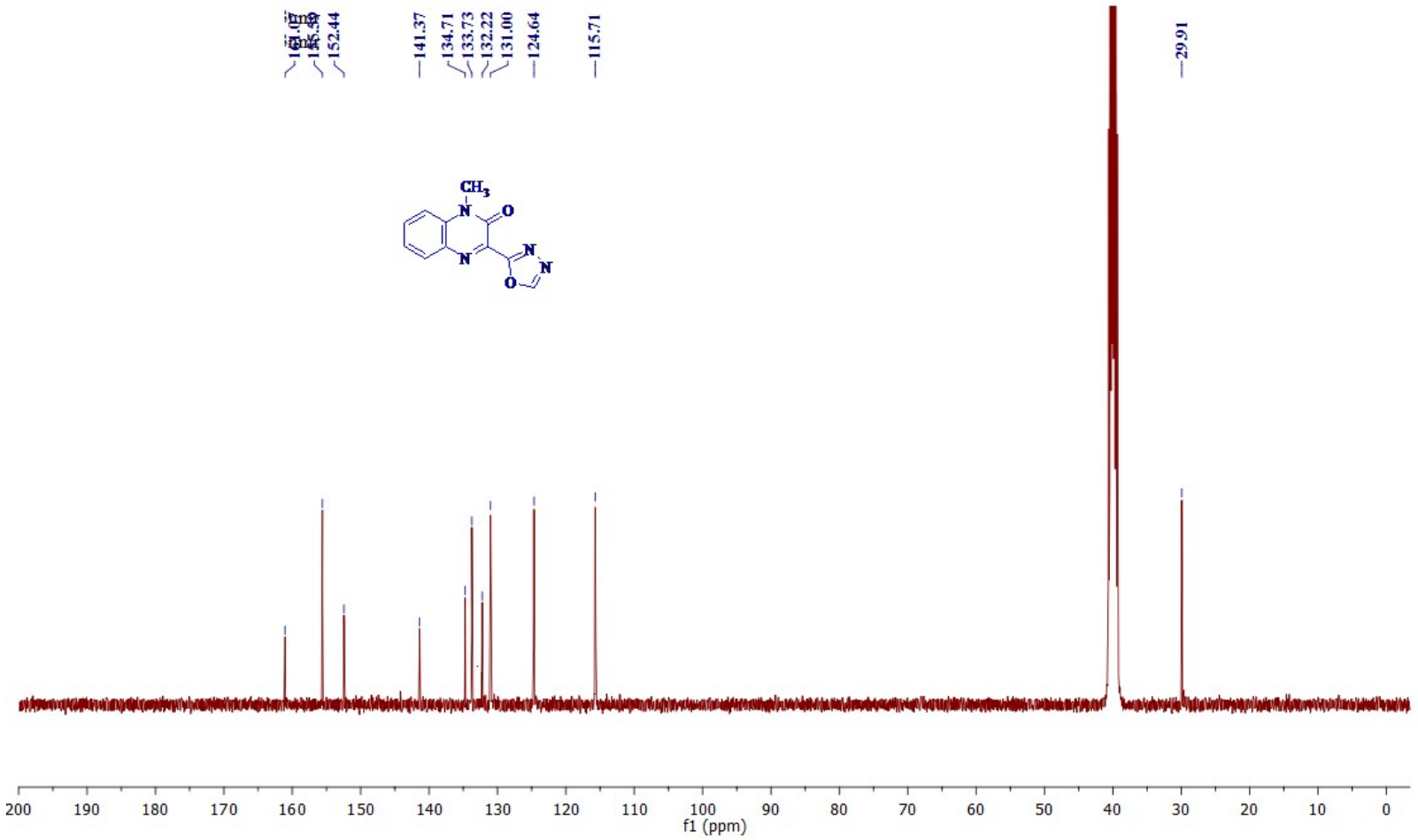
IR of compound 13:



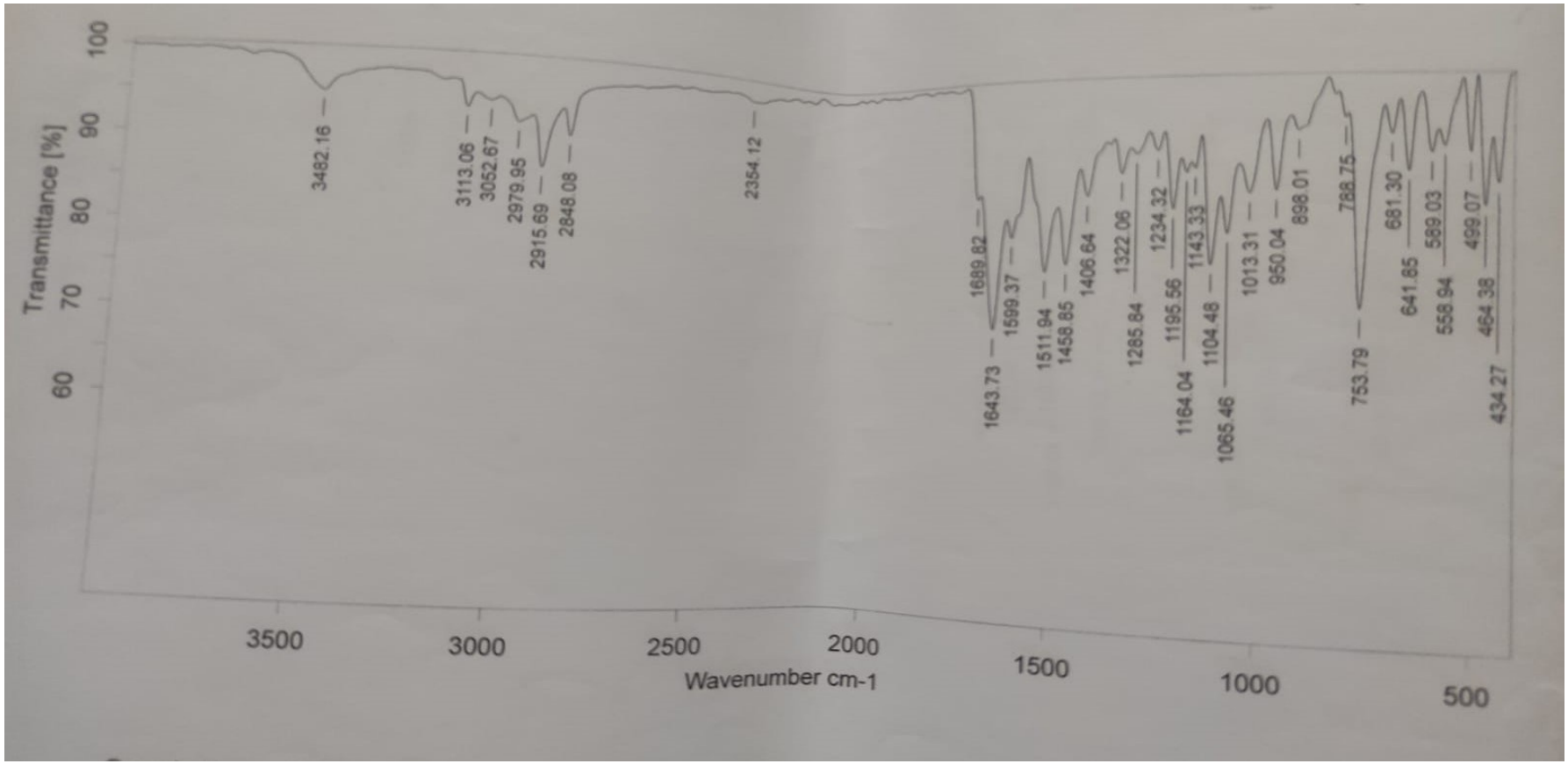
[1]Hnmr of compound 14:



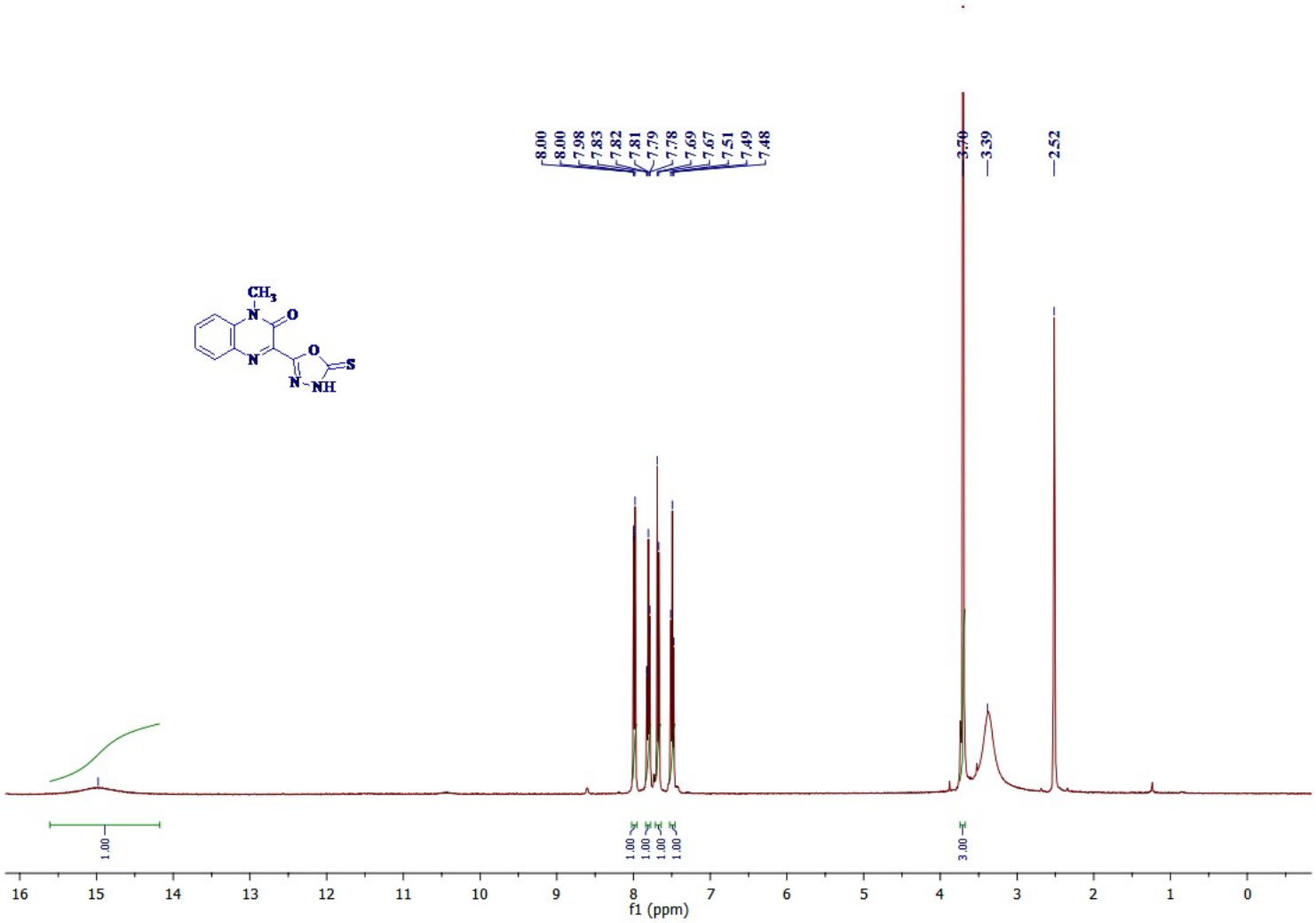
^{13}Cnmr of compound 14:

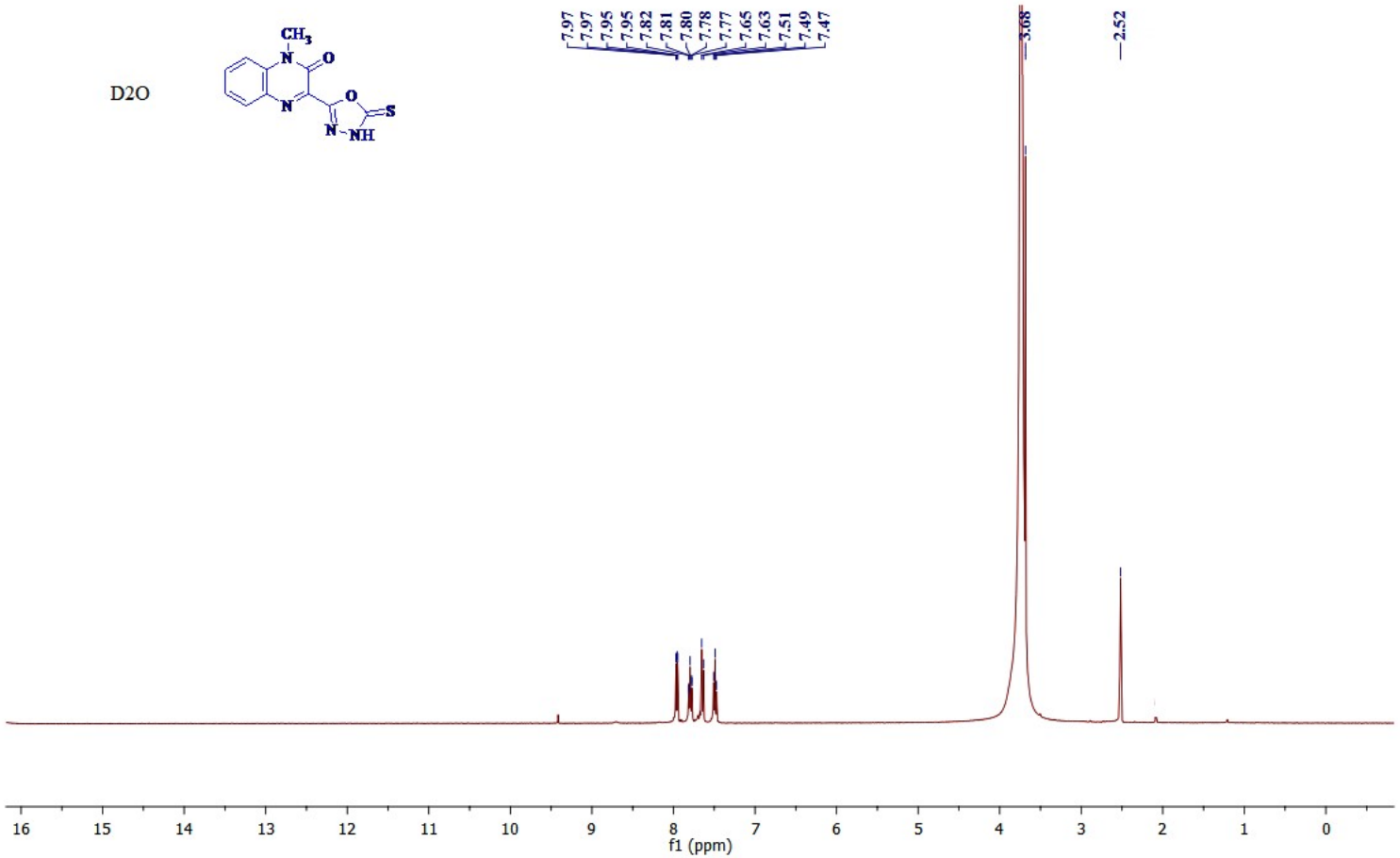


IR of compound 14:

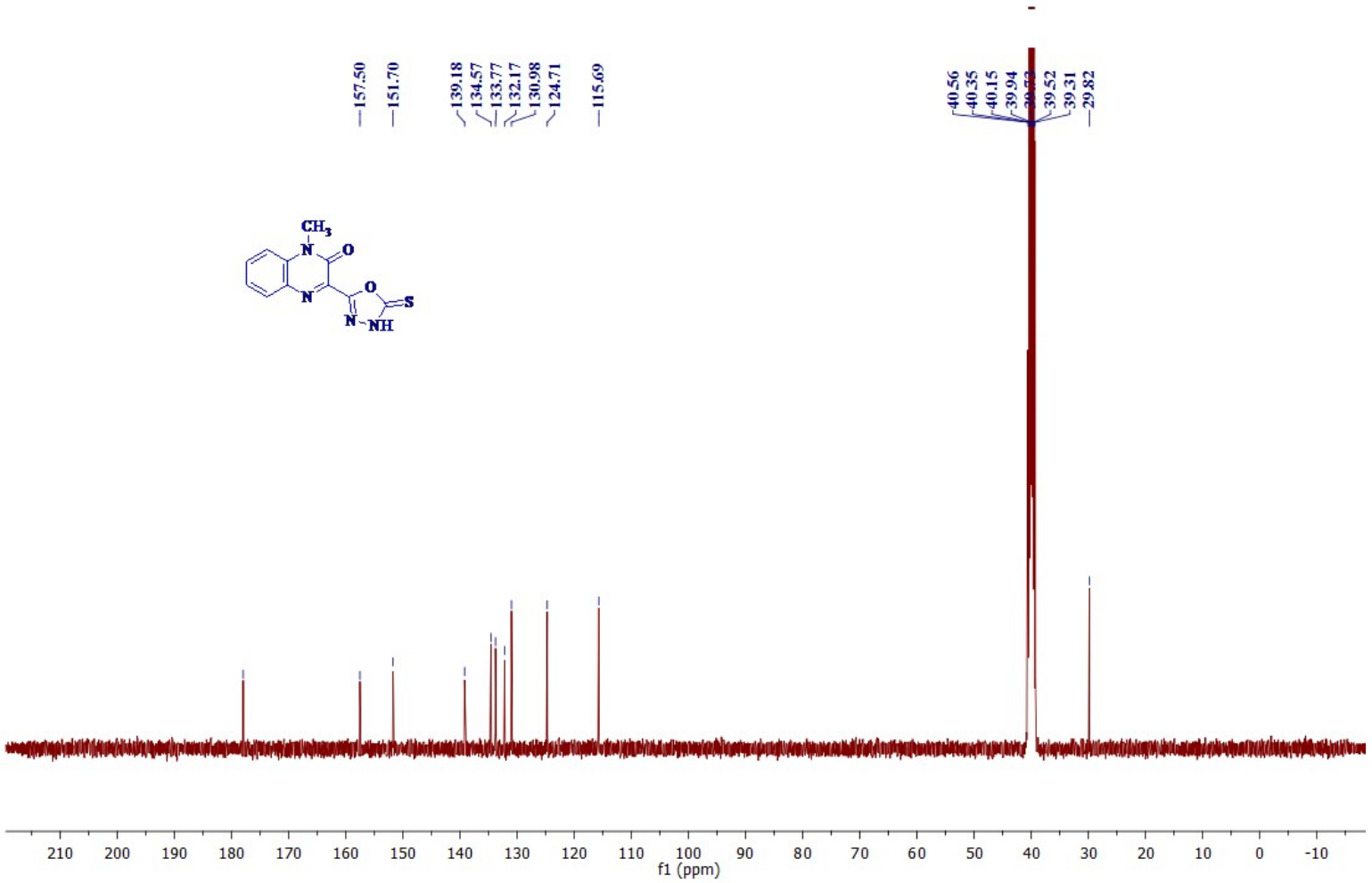


[1]Hnmr of compound 15:

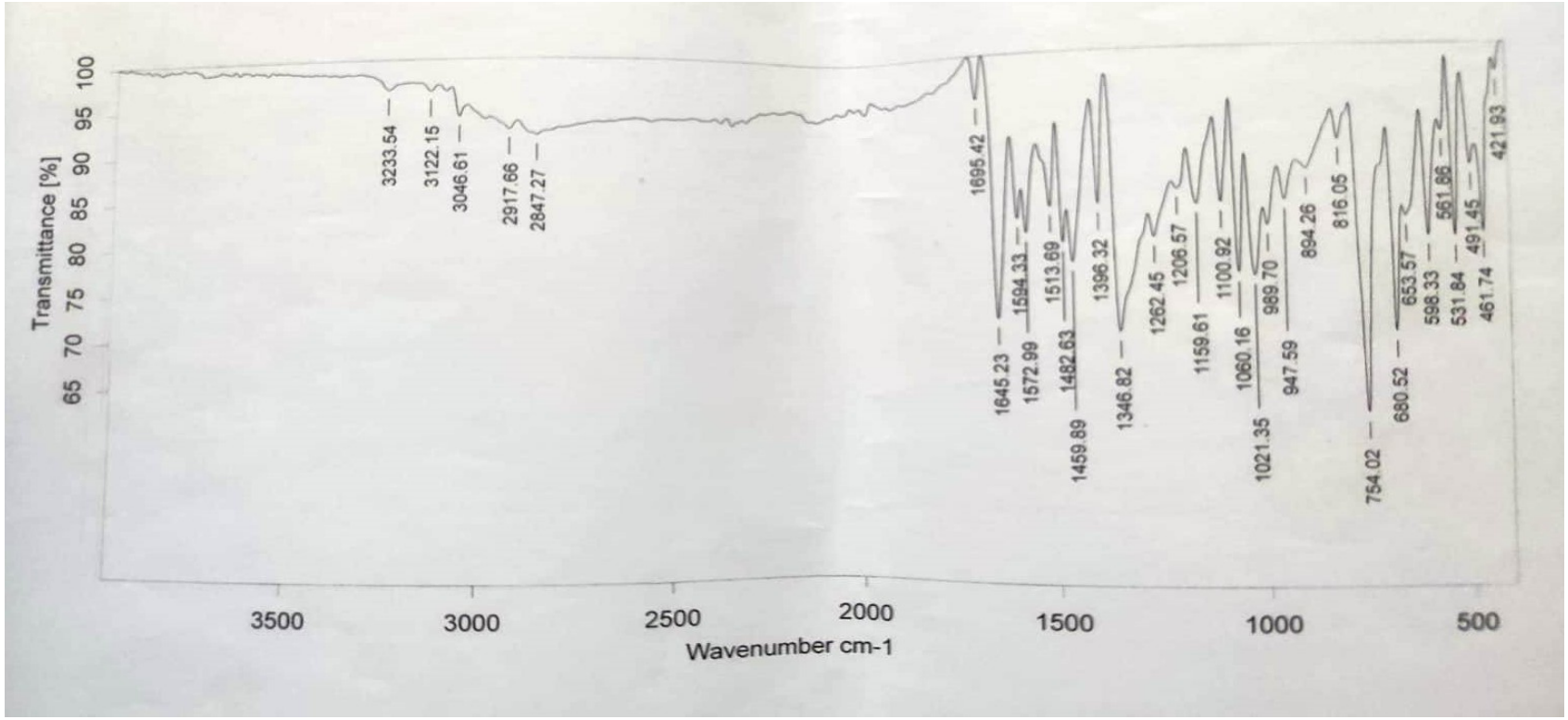



D2O
CH3
N
O
N
O
S
N
NH
7.97
7.97
7.95
7.95
7.82
7.81
7.80
7.78
7.77
7.65
7.63
7.51
7.49
7.47
3.68
2.52
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
f1 (ppm)

^{13}Cnmr of compound 15:



IR of compound 15:



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