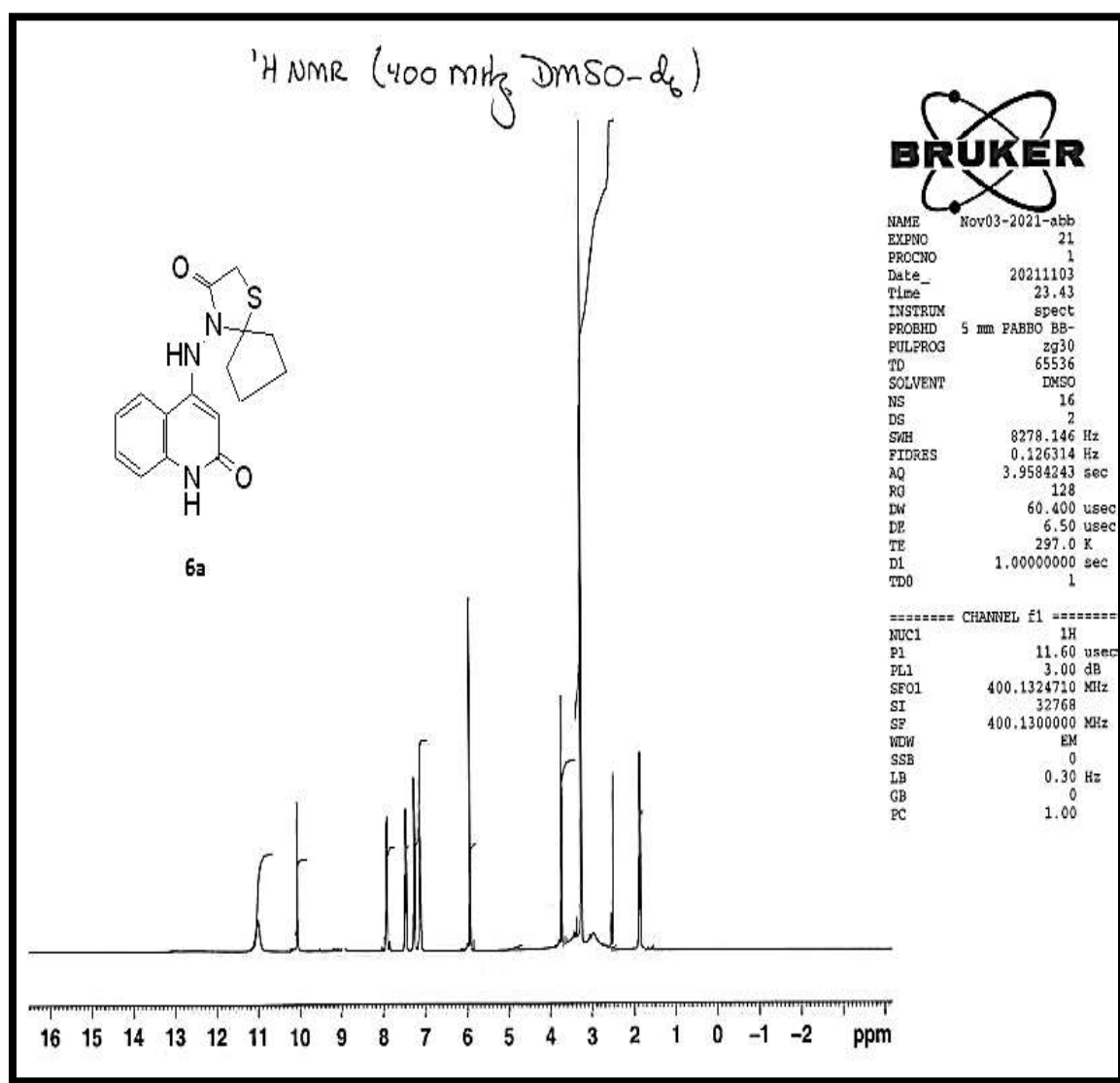


One-pot synthesis of 1-thia-4-azaspiro[4.4]alkan-3-ones via Schiff base: Design, synthesis, and apoptotic antiproliferative properties as dual EGFR/BRAF^{V600E} inhibitors

Lamya H. Al-Wahaibi¹, Essmat M. El-Sheref², Mohamed M. Hammouda^{3,4}, Bahaa G. M. Youssif^{5*}

Spectroscopic data.

SI Fig. 1. ¹H NMR spectrum (DMSO-*d*₆) for compound 6a.



1H NMR spectrum of compound 10a in CDCl₃. The spectrum shows peaks at 11.0120, 10.0748, 7.8761, 7.8285, 7.8082, 7.7617, 7.6882, 7.6610, 7.5590, 7.5178, 0.9355, 0.8847, 0.9810, 1.0002, 1.0367, and 1.0160 ppm. Integration values are shown below the baseline.

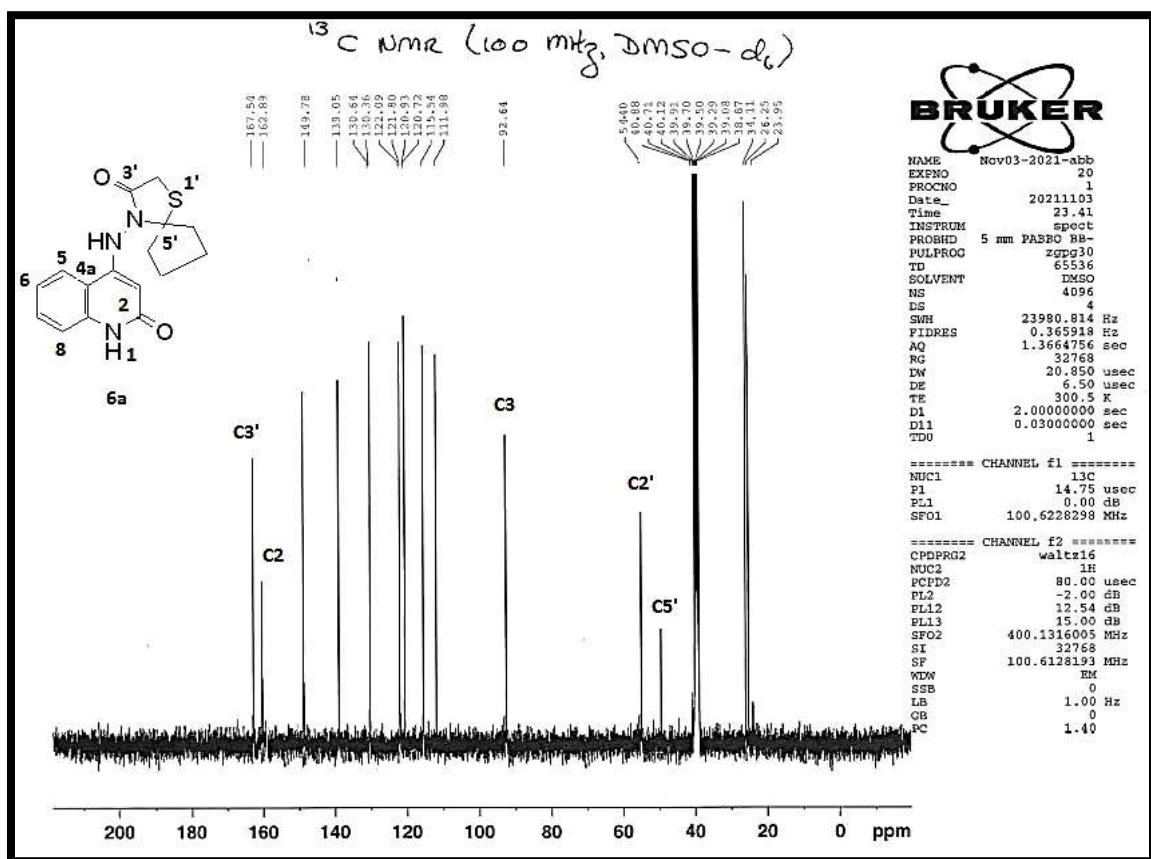
¹H-¹H COSY

NAME Nov03-2021-abb
EXPNO 23
PROCNO 1
Date_ 20211103
Time 23.45
INSTRUM spect
PROBHD 5 mm PARDO DD-
PULPROG cosyprgf
TD 2048
SOLVENT DMSO
RS 1
DS 8
SWH 5112.475 Hz
FIDRES 0.496325 Hz
AQ 0.2003444 sec
RG 64
DW 97.600 usec
DE 6.50 usec
TE 296.7 K
EO 0.00000300 sec
D1 1.47788025 sec
D13 0.00000400 sec
D16 0.00020000 sec
IN0 0.00013560 sec

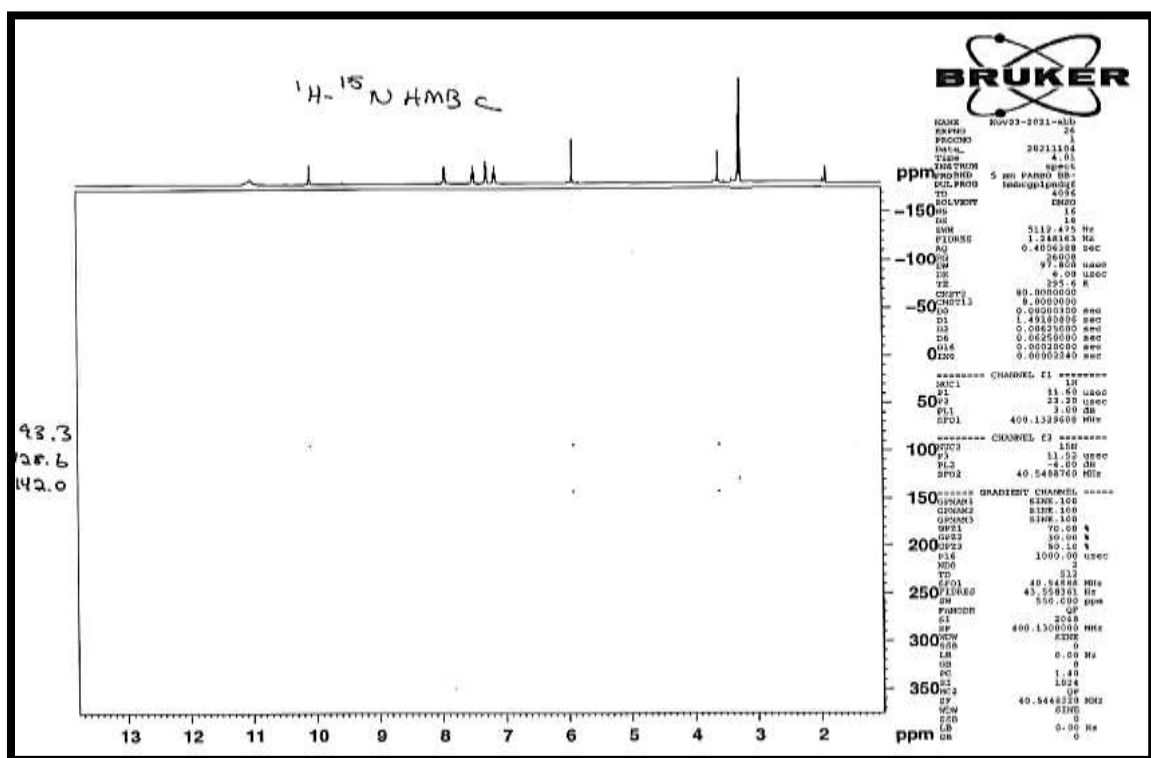
----- CHANNEL f1 -----
NUC1 1H
FO 11.00 usec
PL 11.50 usec
PL1 1.00 dB
SFO1 400.1329608 MHz

----- GRADIENT CHANNEL -----
GTSMPL SINE 500
OPF1 10.00 %
PL0 1000.00 usec
ND0 1
TD 328
SFO1 400.133 MHz
FIDRES 10.941208 Hz
SW 12.777 ppm
PRMODE QF
SI 1024
SP 400.1300000 MHz
MCW SINE
SSB 0
LB 0.00 Hz
GB 0
PC 1.40
SI 1024
MC1 QF
SC 400.1300000 MHz
MCW SINE
SSB 0
LB 0.00 Hz
GB 0

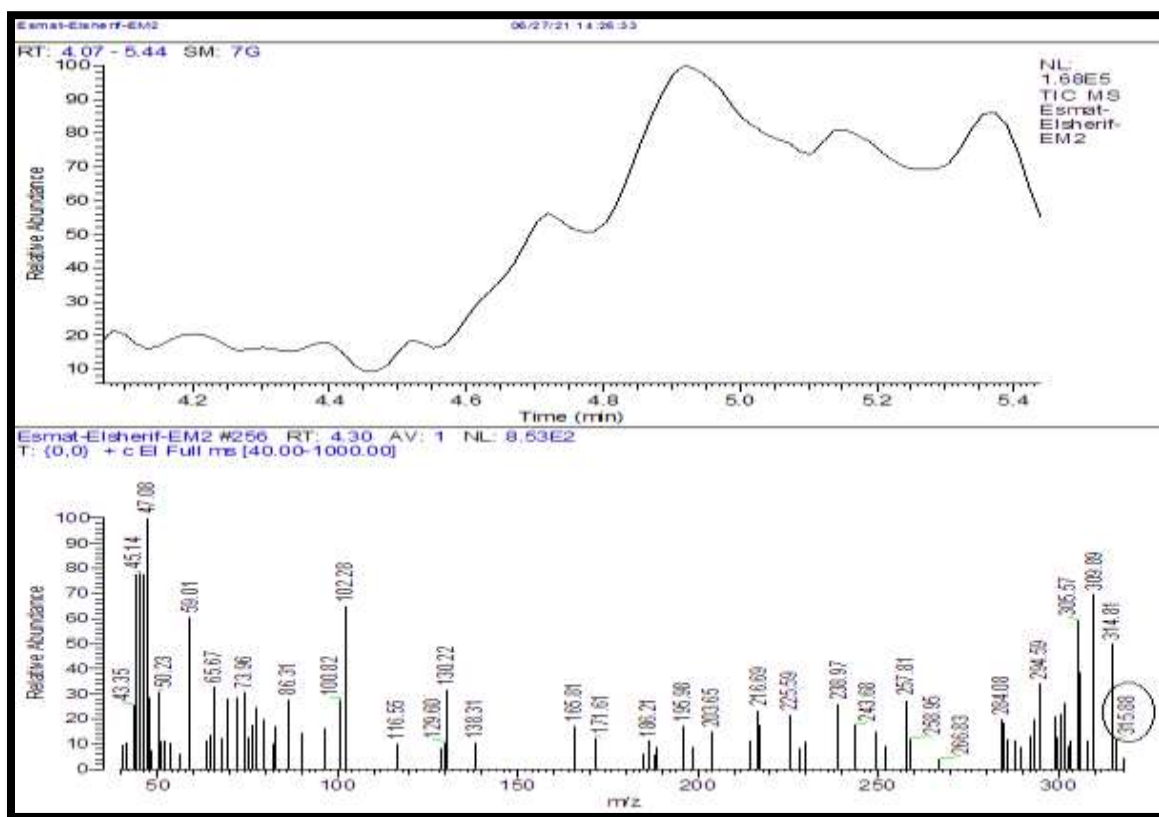
SI Fig. 4. ^{13}C NMR spectrum (DMSO- d_6) for compound 6a.



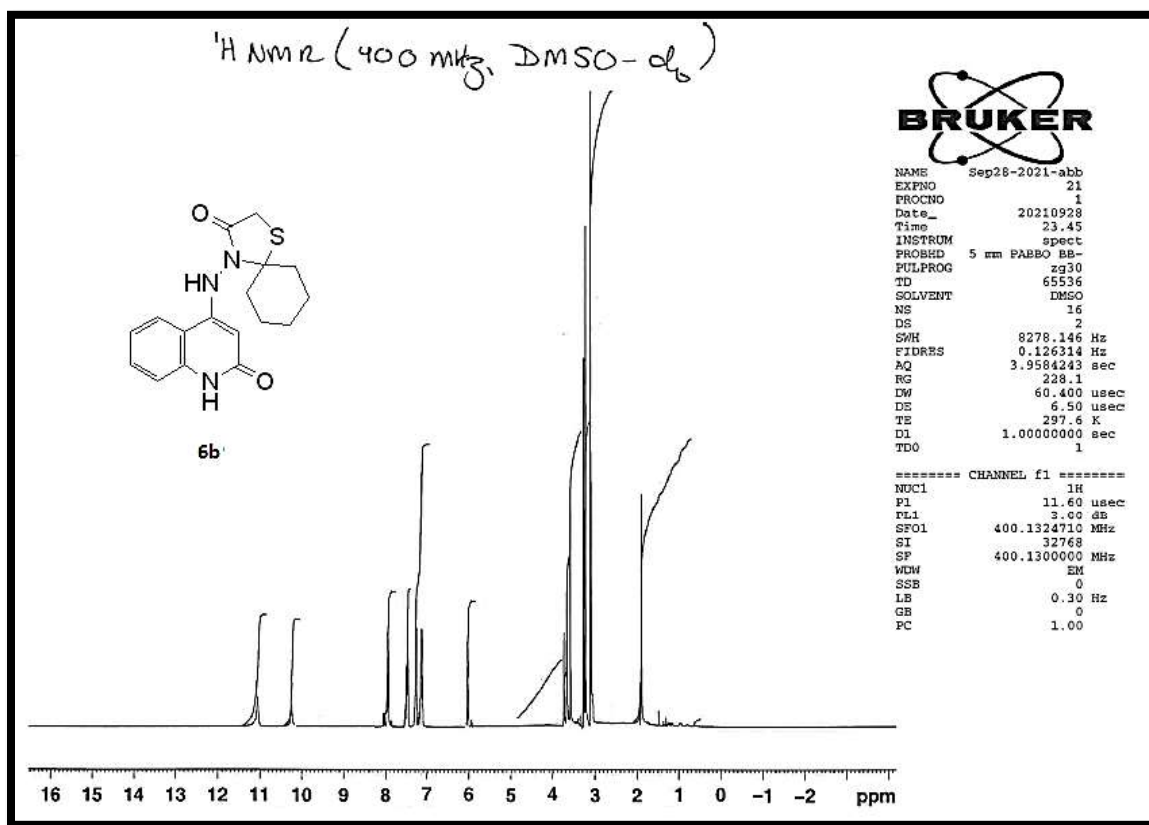
SI Fig. 5. ^{15}N NMR spectrum (DMSO- d_6) for compound 6a.



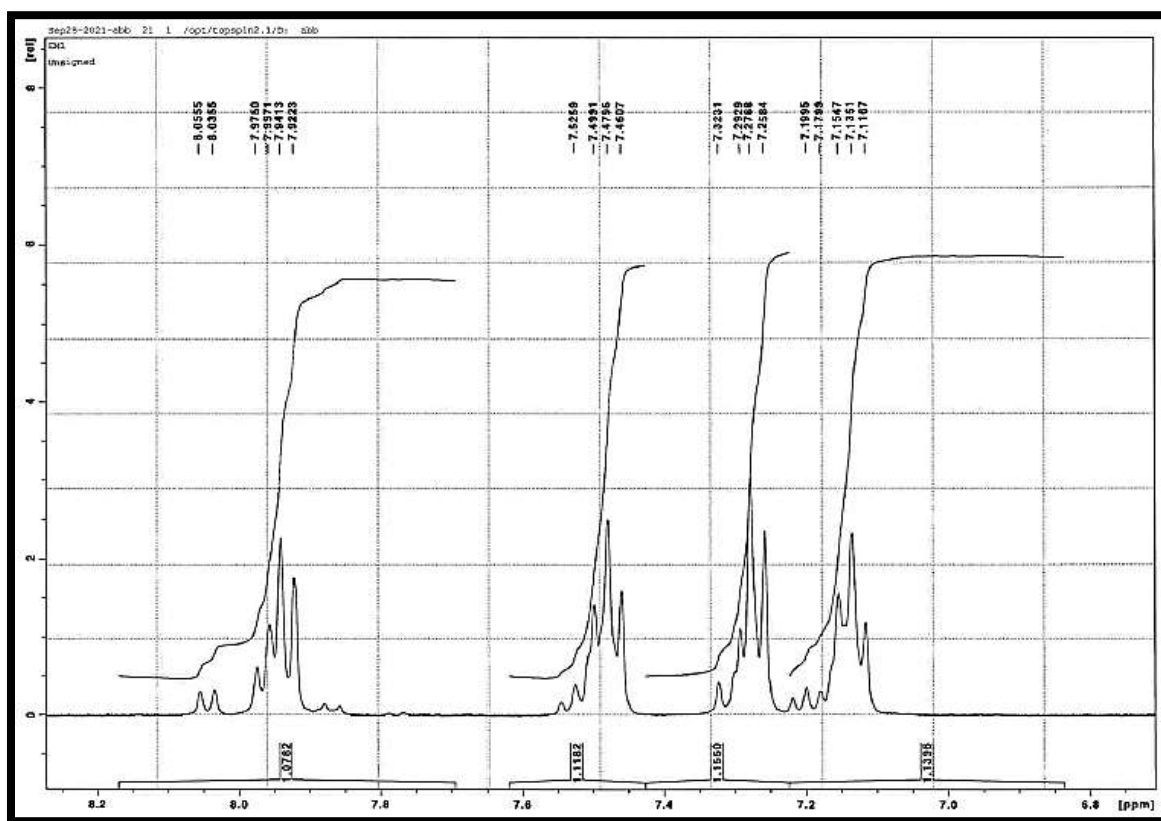
SI Fig. 6. Mass spectrometry for compound **6a**.



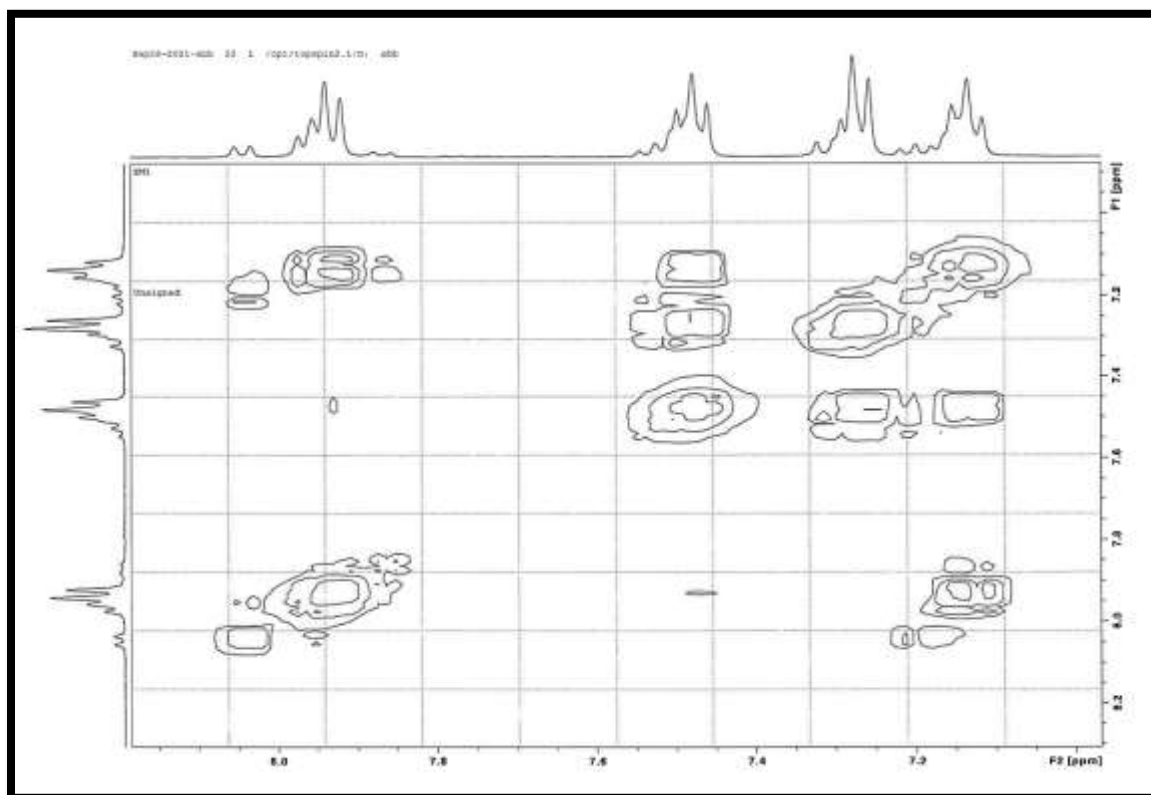
SI Fig. 7. ^1H NMR spectrum (DMSO- d_6) for compound **6b**.



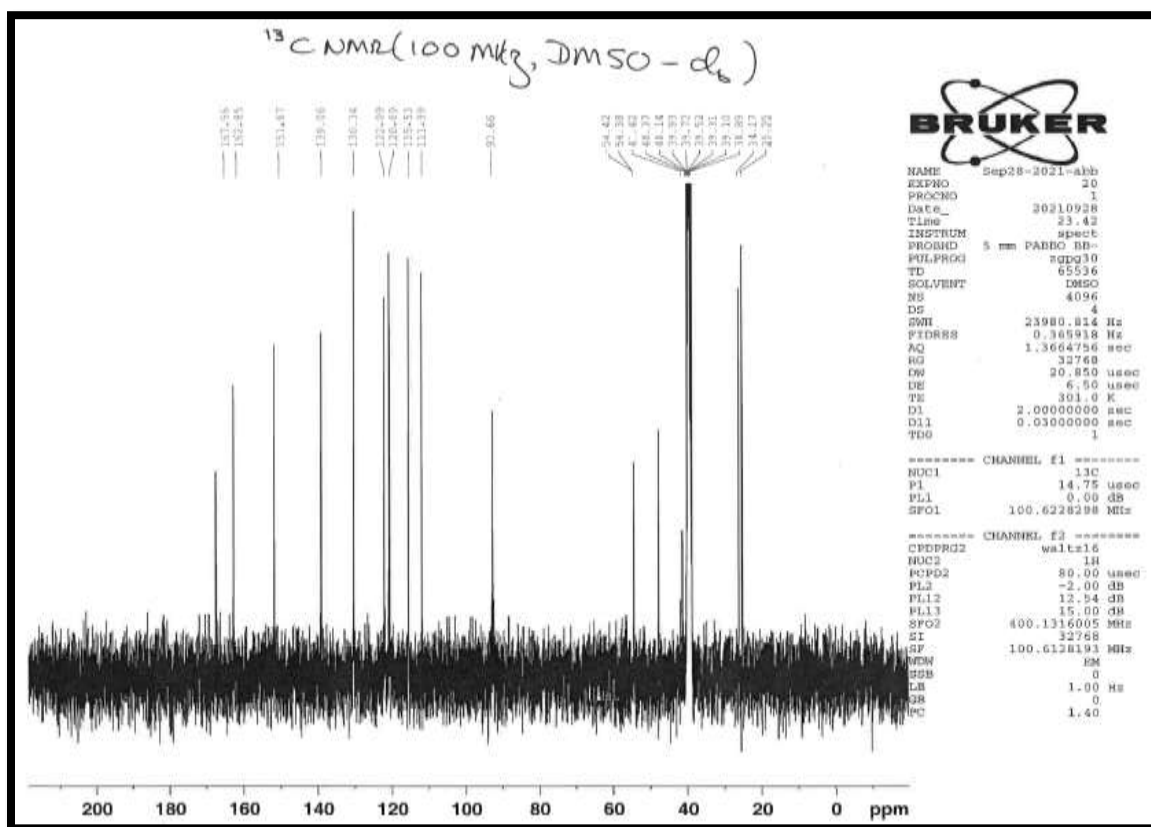
SI Fig. 8. Part of ^1H NMR spectrum (DMSO- d_6) for compound **6b**.



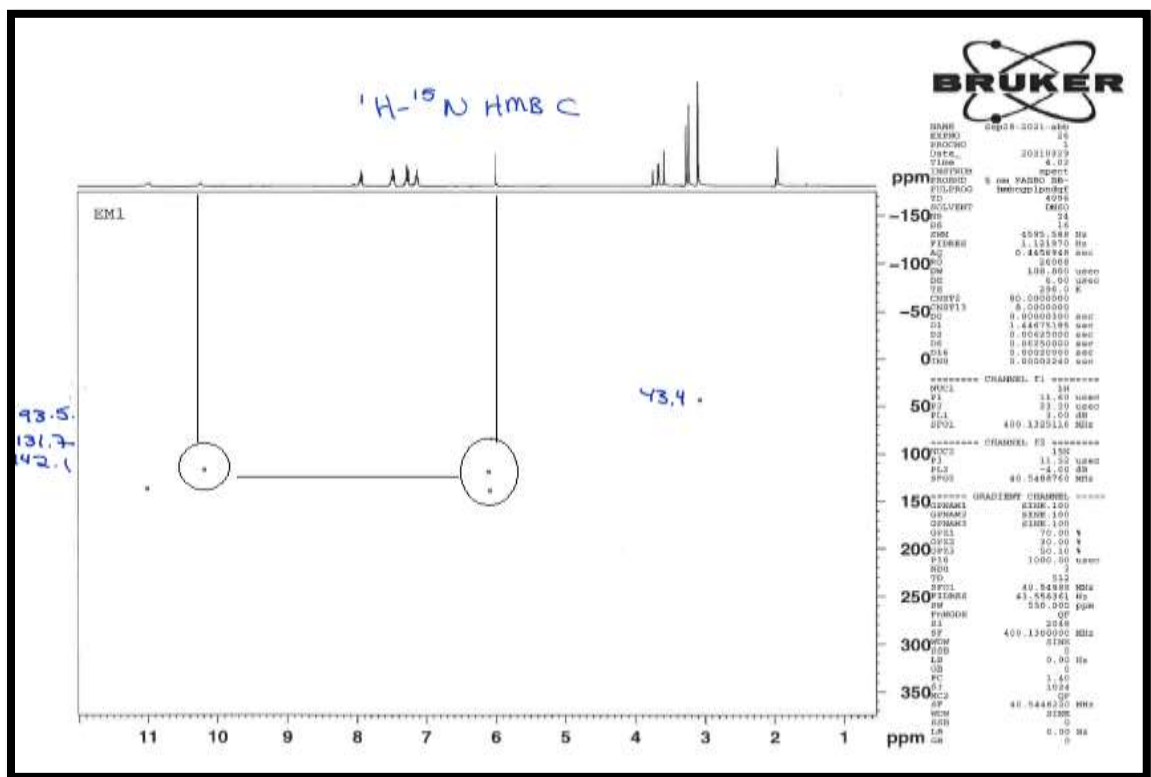
SI Fig. 9. Part of ^1H - ^1H Cosy spectrum spectrum (DMSO- d_6) for compound **6b**.



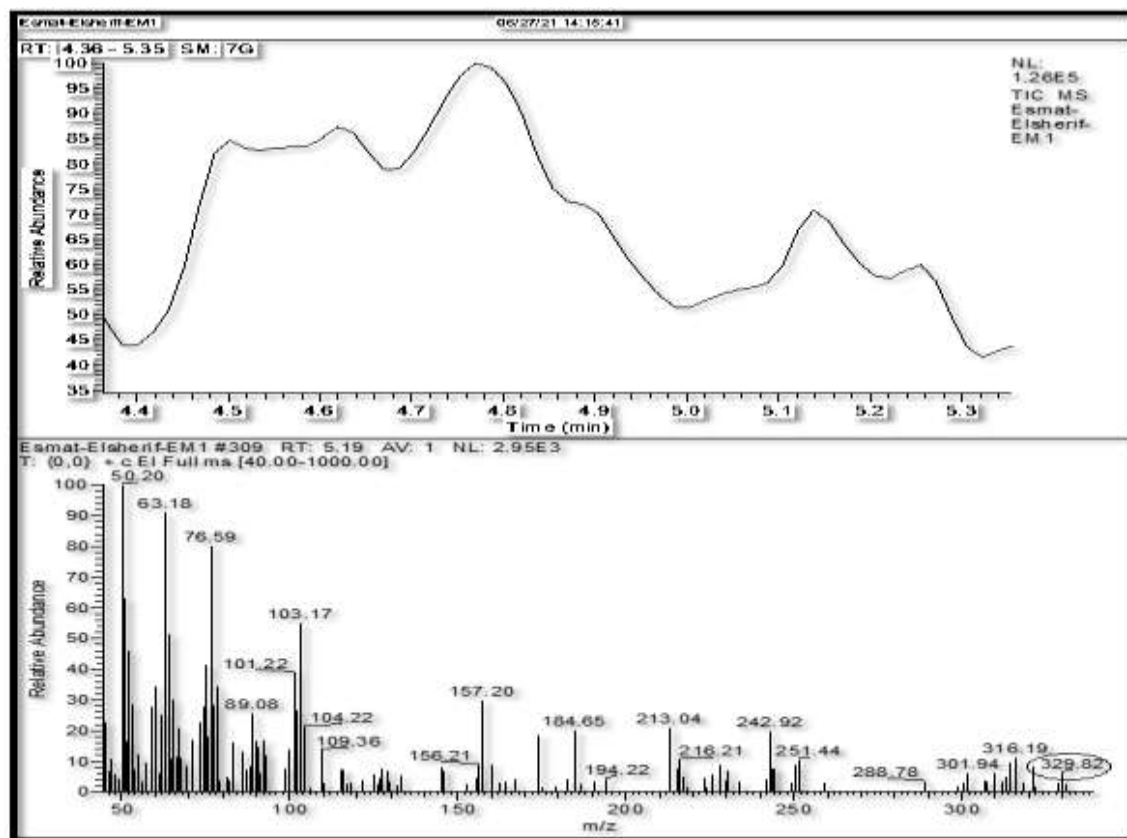
SI Fig. 10. ^{13}C NMR spectrum (DMSO- d_6) for compound **6b**.



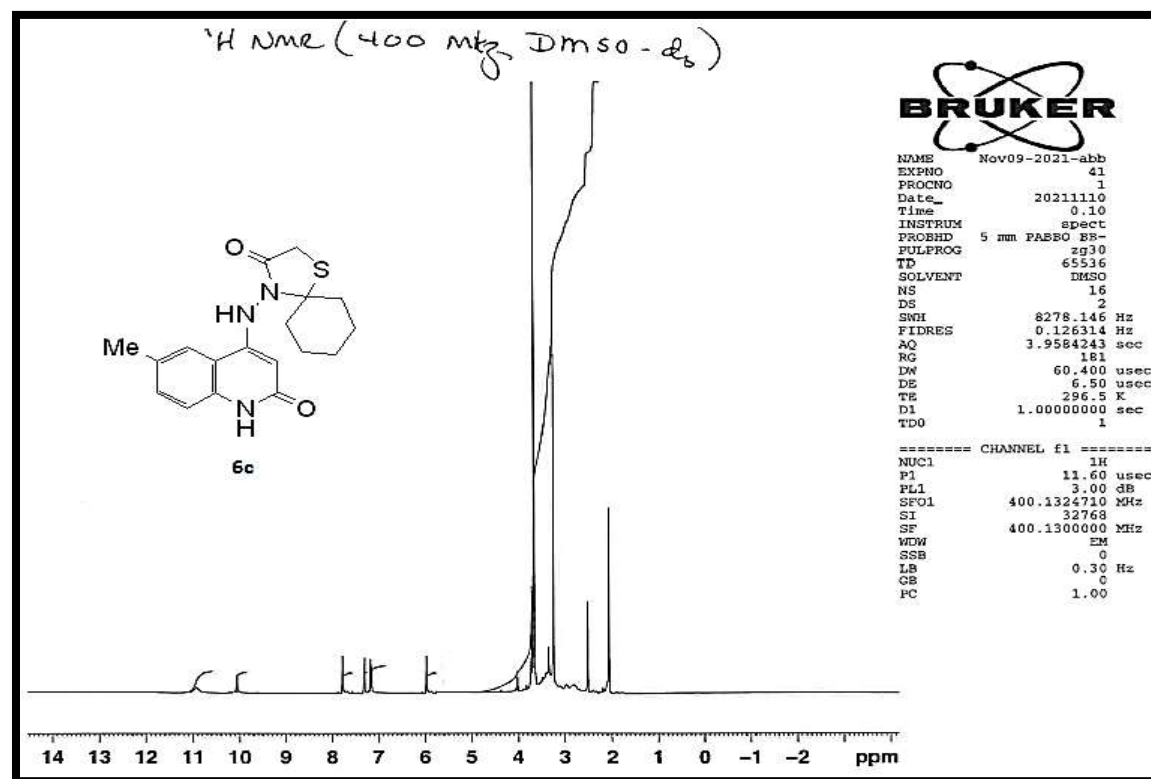
SI Fig. 11. ^{15}N NMR spectrum (DMSO- d_6) for compound **6b**.



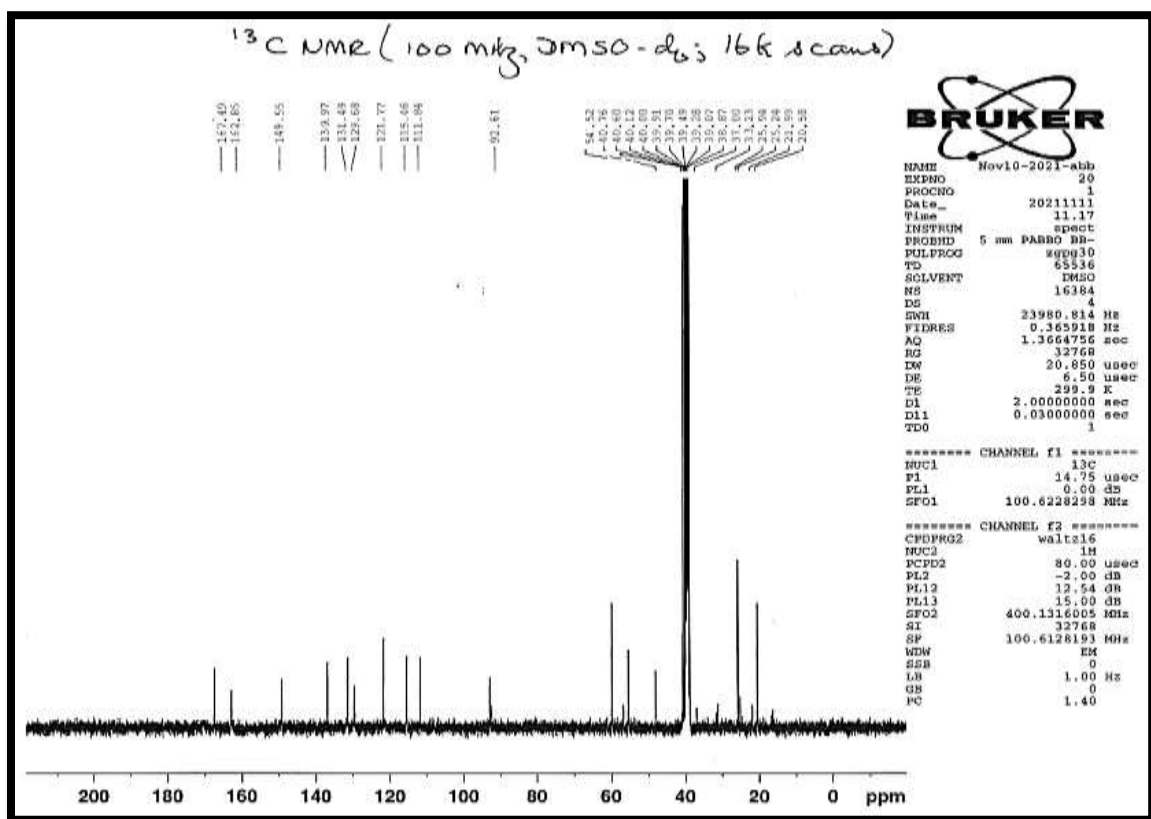
SI Fig. 12. Mass spectrometry for compound **6b**.



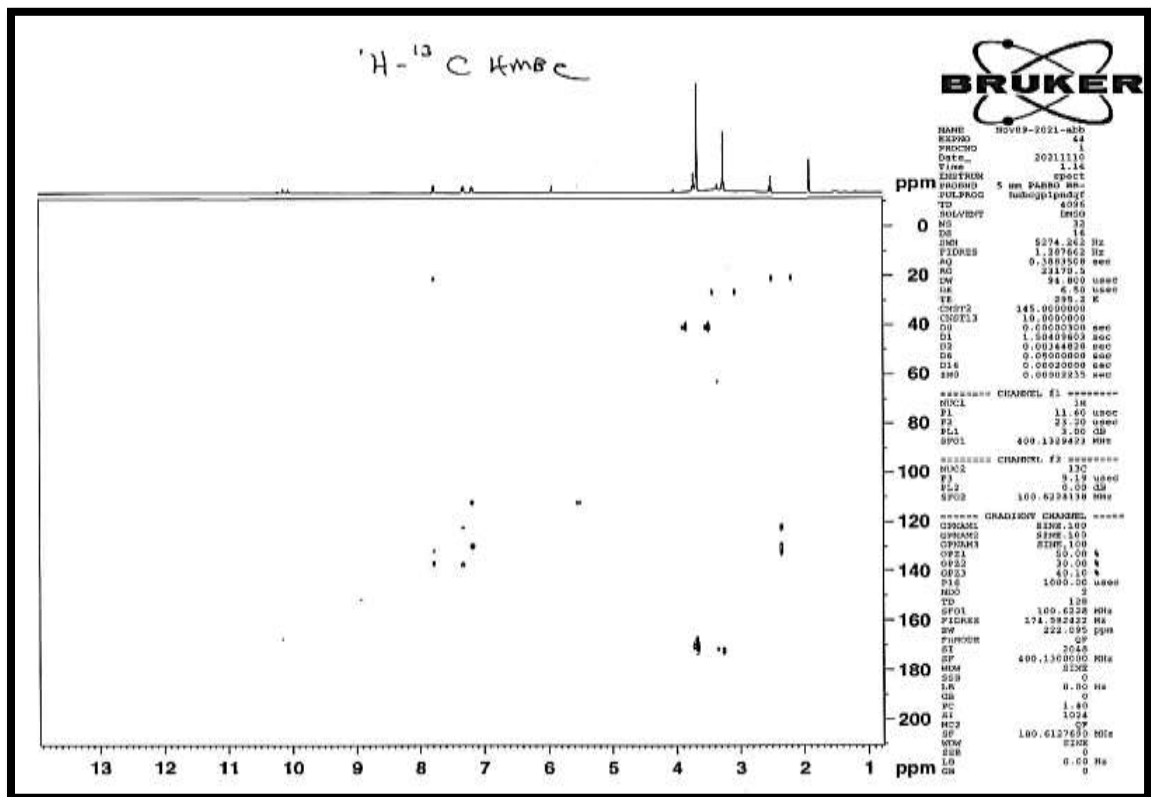
SI Fig. 13. ^1H NMR spectrum (DMSO- d_6) for compound **6c**.



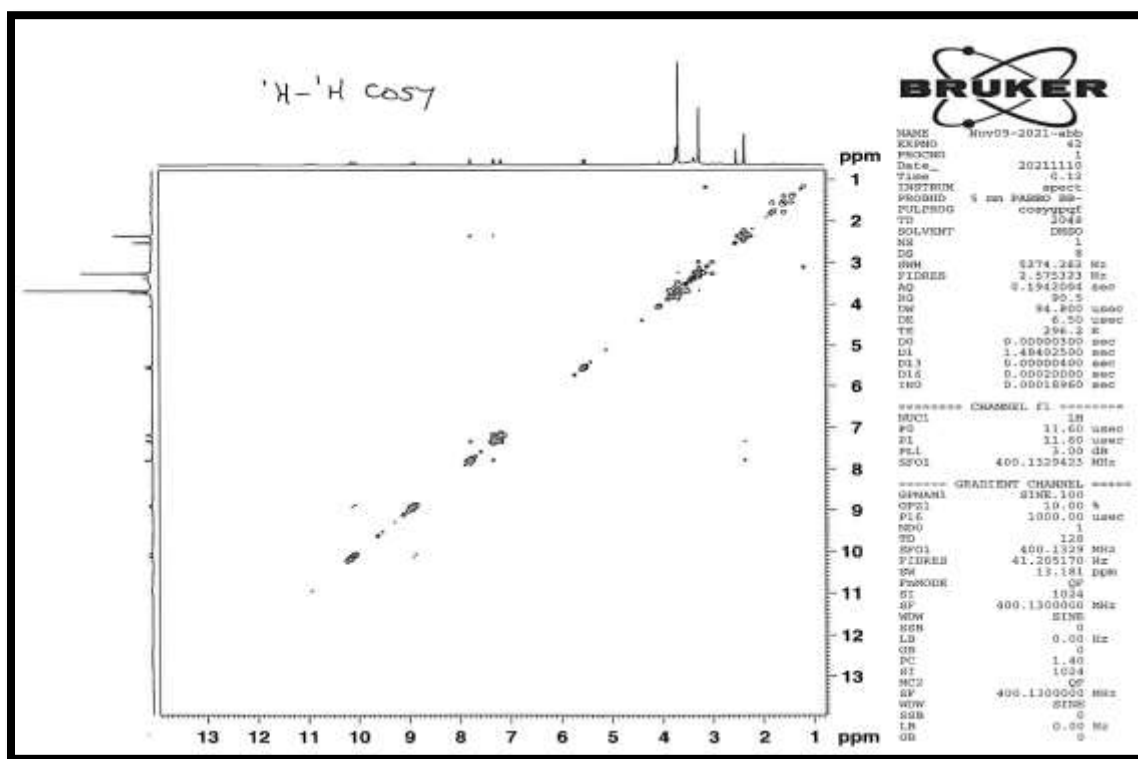
SI Fig. 14. ^{13}C NMR spectrum (DMSO- d_6) for compound 6c.



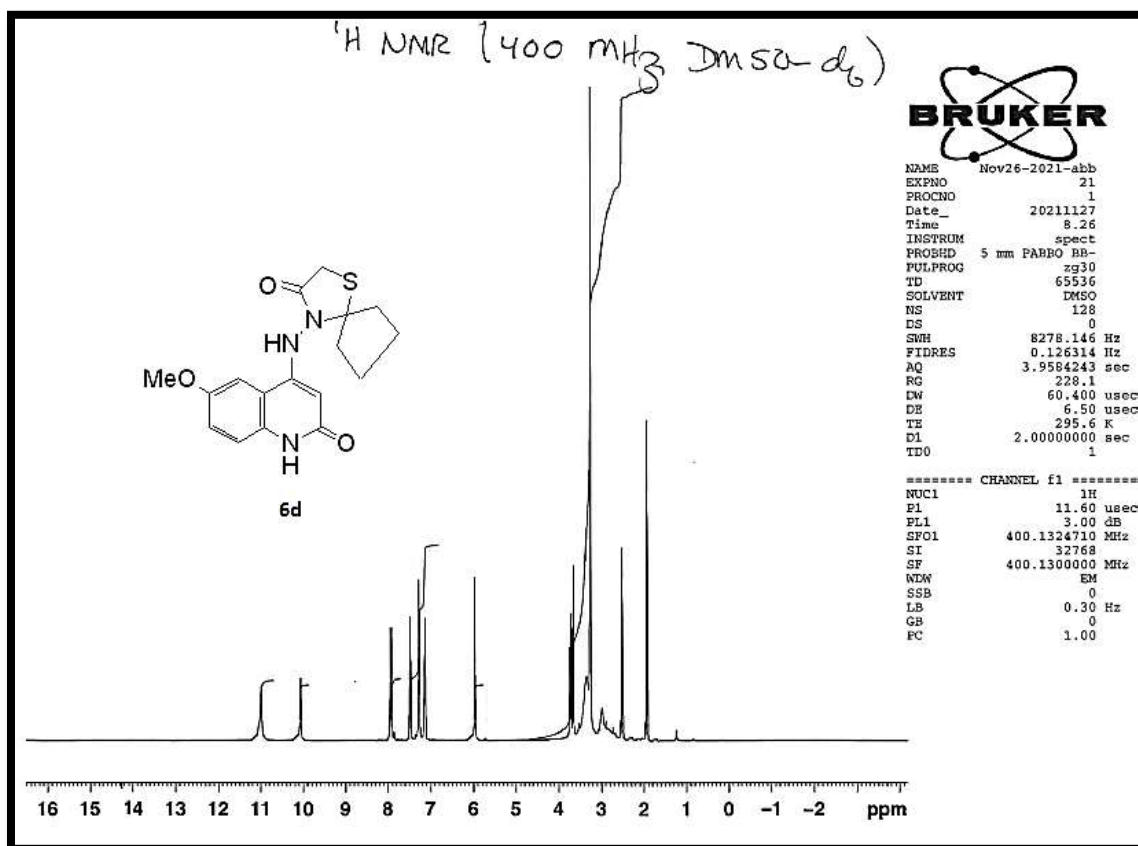
SI Fig. 15. ^1H - ^{13}C HMBC spectrum (DMSO- d_6) for compound 6c.



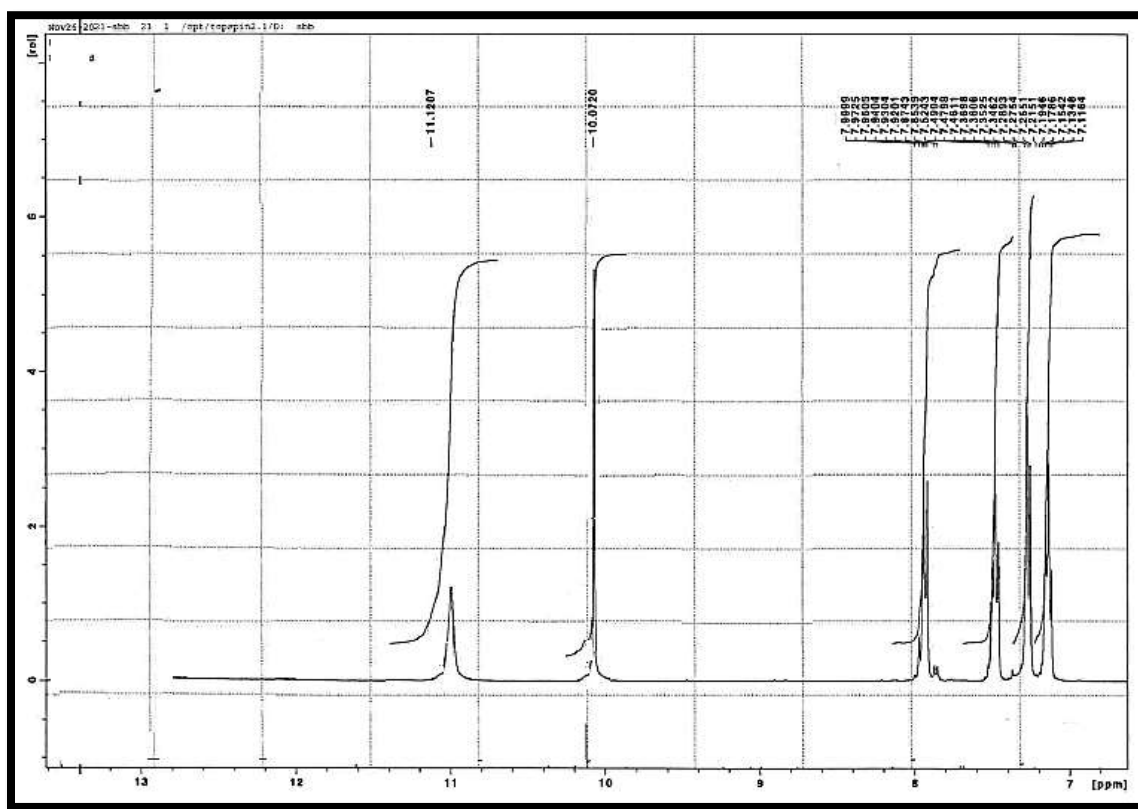
SI Fig. 16. ^1H - ^1H Cosy spectrum (DMSO- d_6) for compound **6c**.



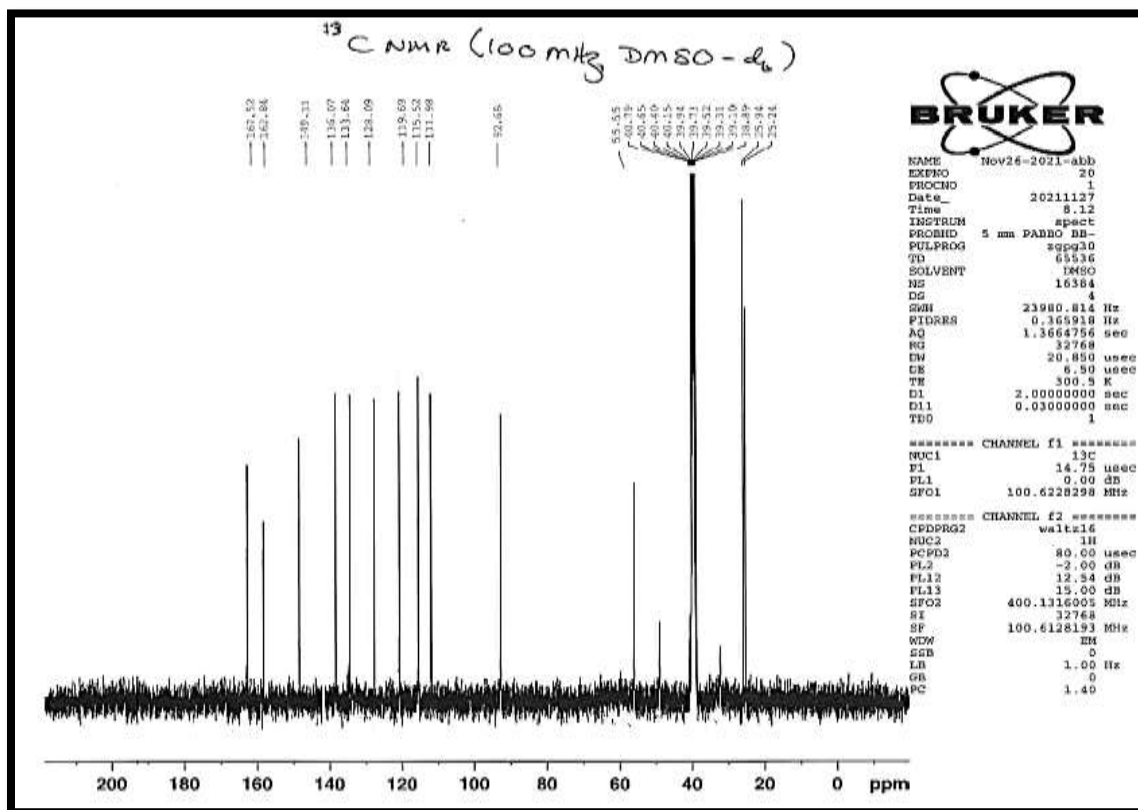
SI Fig. 17. ^1H NMR spectrum (DMSO- d_6) for compound **6d**.



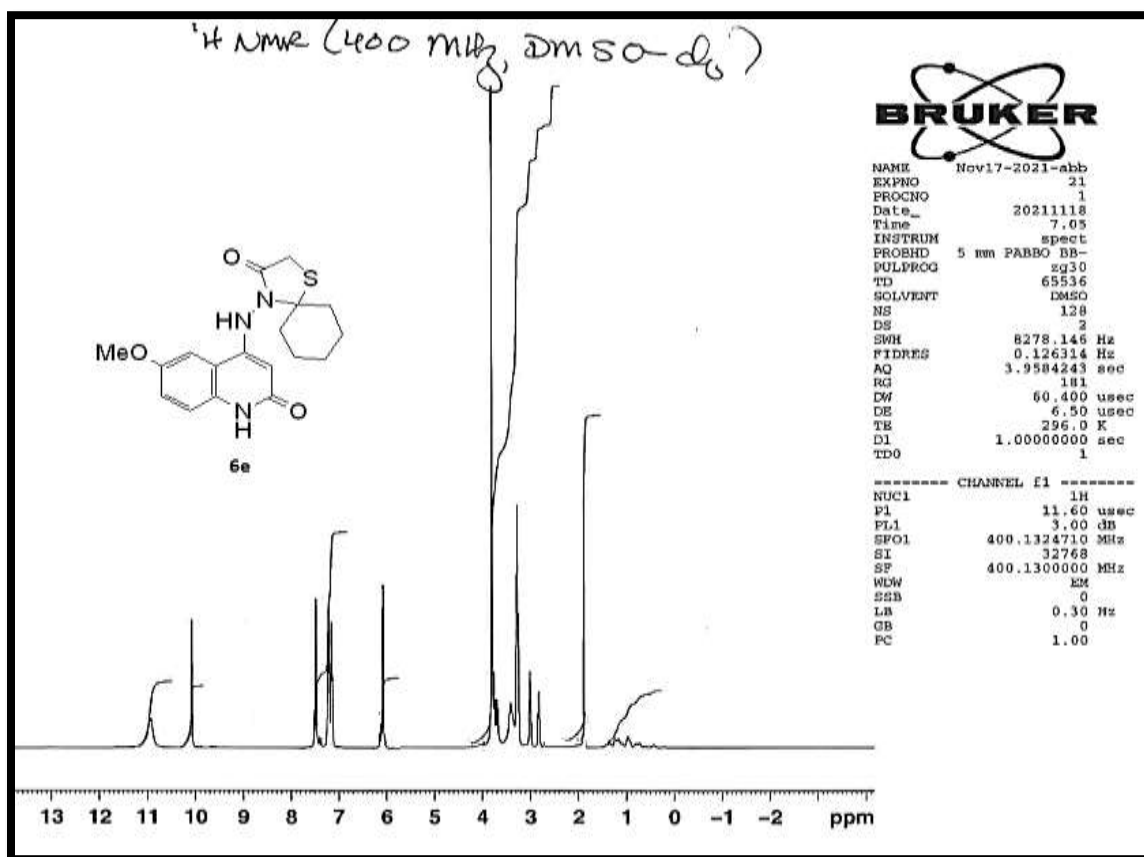
SI Fig. 18. ^1H - ^{13}C HMBC spectrum (DMSO- d_6) for compound **6d**.



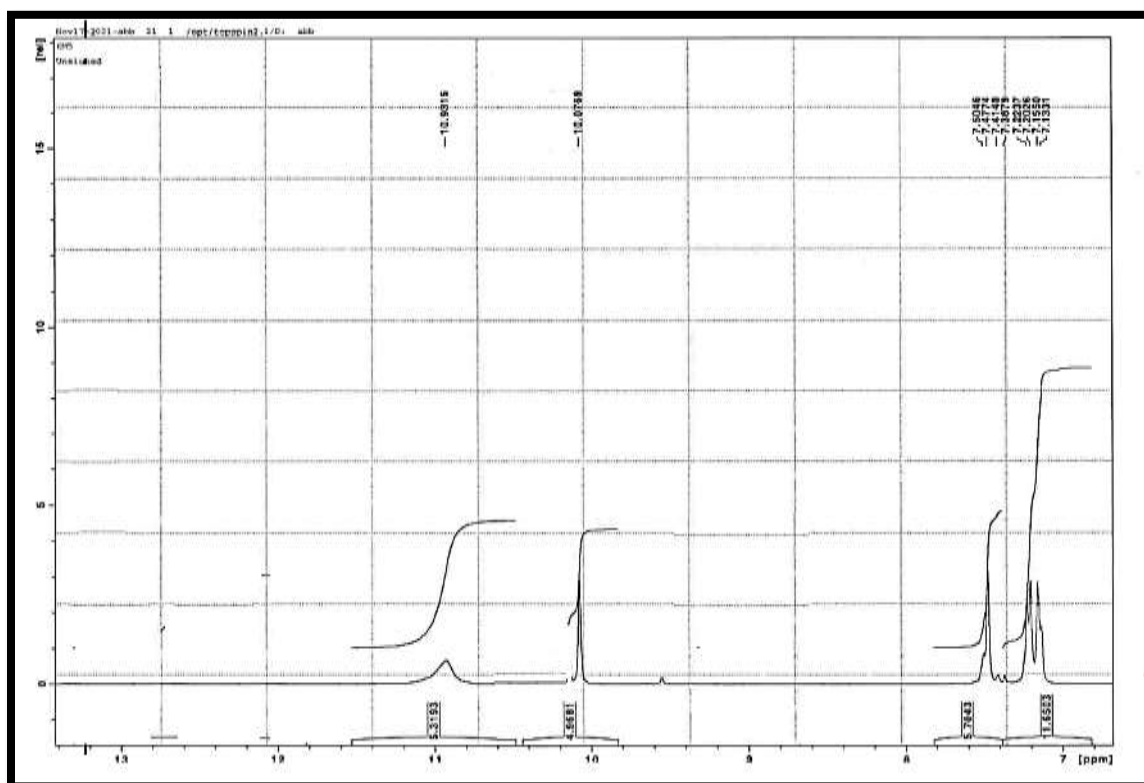
SI Fig. 19. ^{13}C NMR spectrum (DMSO- d_6) for compound **6d**.



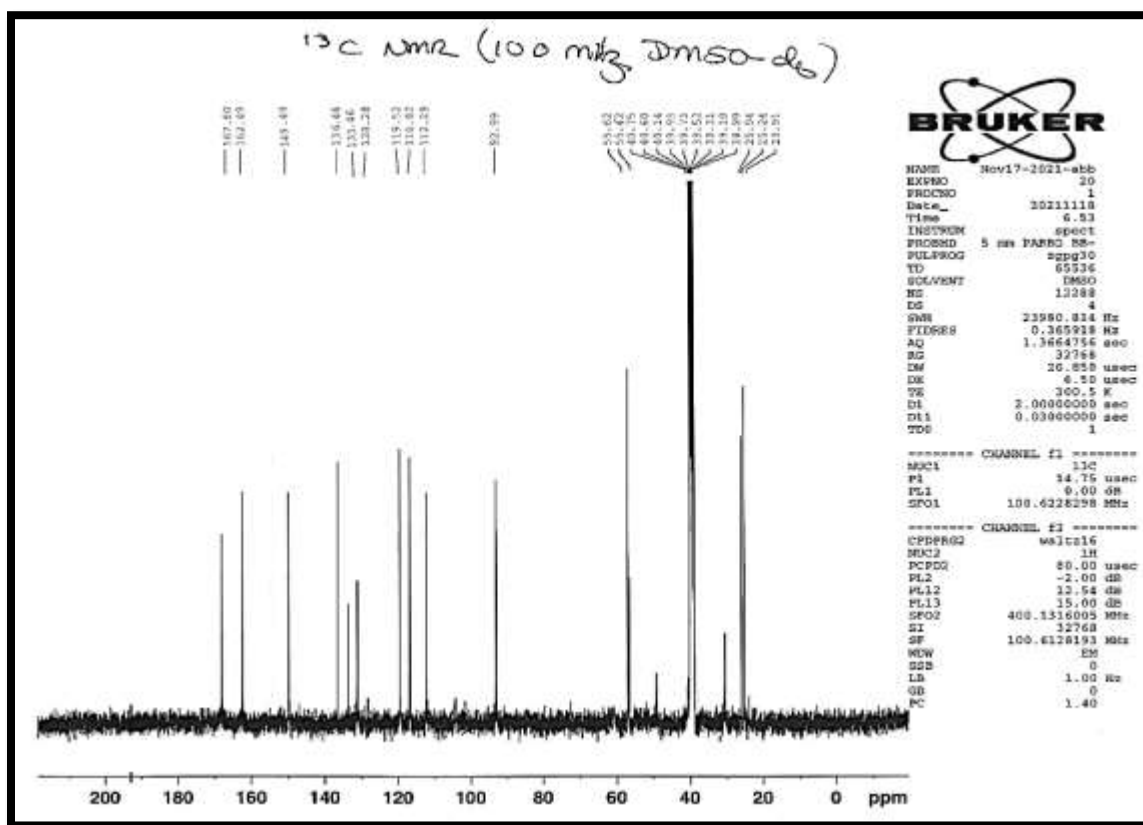
SI Fig. 20. ^1H NMR spectrum (DMSO- d_6) for compound 6e.



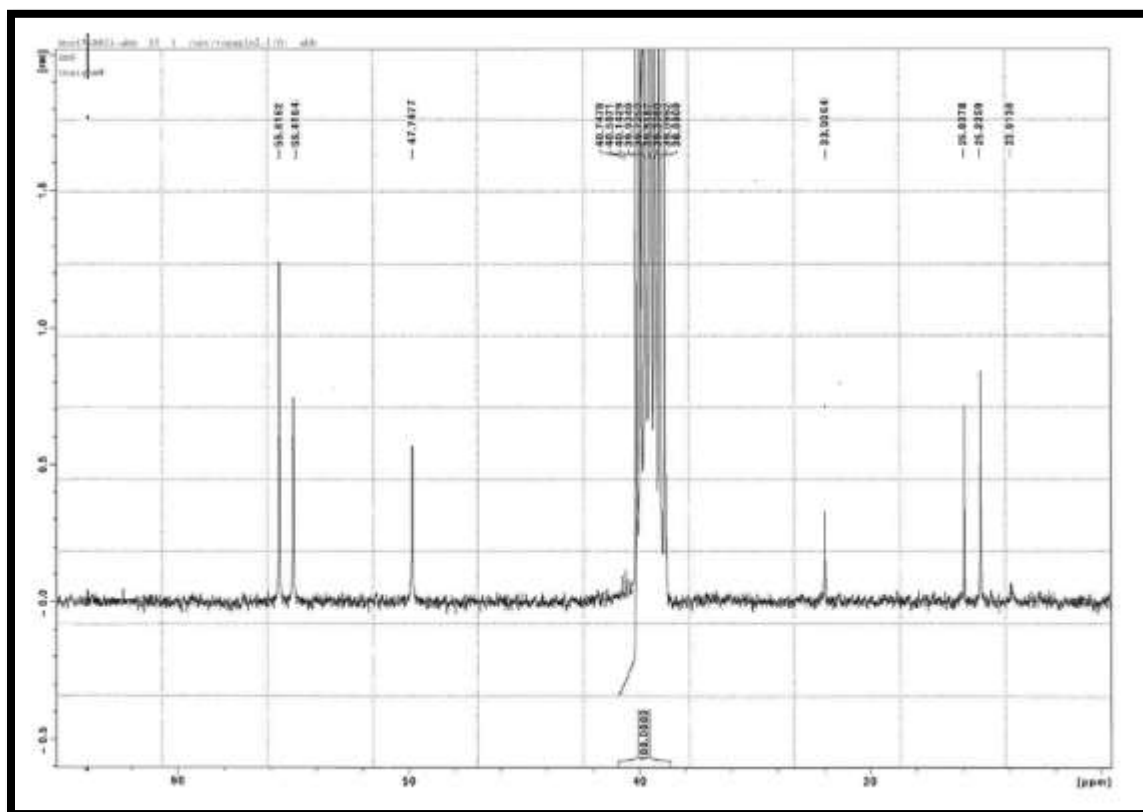
SI Fig. 21. Part of ^1H NMR spectrum (DMSO- d_6) for compound 6e.



SI Fig. 22. ^{13}C NMR spectrum (DMSO- d_6) for compound **6e**.



SI Fig. 23. Part of ^{13}C NMR spectrum ($\text{DMSO}-d_6$) for compound **6e**.



¹H NMR (400 MHz, DMSO-d₆)

BRUKER

```

NAME      Dec01-2021-abb
EXPNO     21
PROCNO    1
Date_     20211202
Time      8.25
INSTRUM   spect
PROBHD    5 mm PABBO BB-
PULPROG   zg30
TD         65536
SOLVENT   DMSO
NS         128
DS         0
SWH        8278.146 Hz
FIDRES     0.126314 Hz
AQ         3.9584243 sec
RG         228.1
DW         60.400 usec
DE         6.50 usec
TE         295.2 K
D1         2.00000000 sec
TD0        1

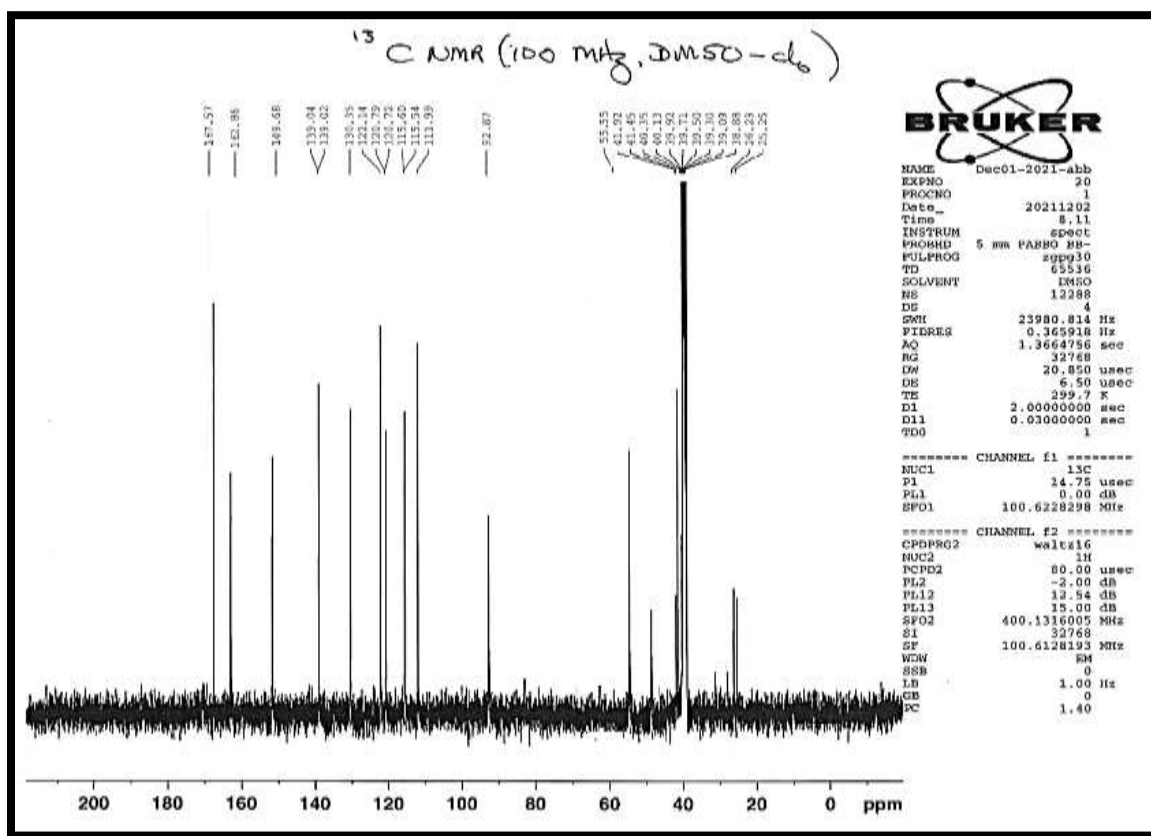
===== CHANNEL f1 =====
NUC1       1H
P1         11.60 usec
PL1        3.00 dB
SFO1       400.1324710 MHz
SI         32768
SF         400.1300000 MHz
WDW        EM
SSB        0
LB         0.30 Hz
GB         0
PC         1.00

```

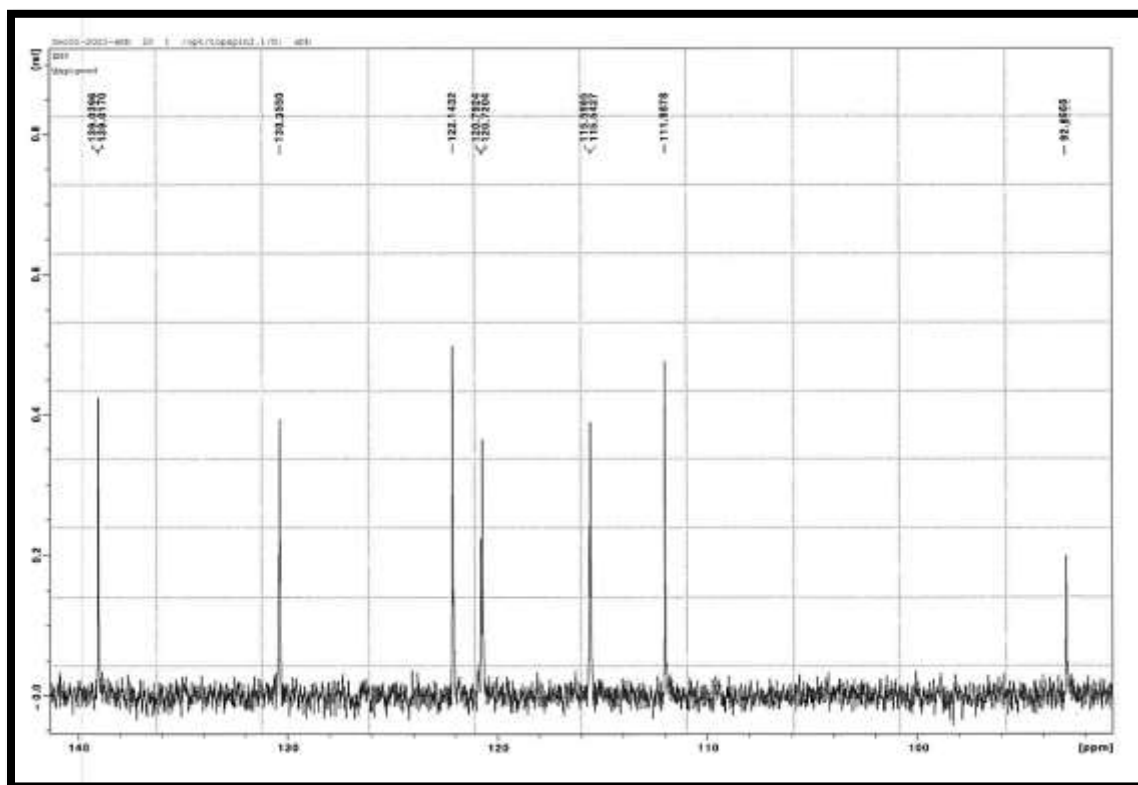
7a

COc1ccc2c(c1)c(=O)[nH]c3c2nc(=O)[nH]3C4(C(C(C(C(C4)C)C)C)C)C

SI Fig. 26. ^{13}C NMR spectrum (DMSO- d_6) for compound 7a.



SI Fig. 27. Part of ^{13}C NMR spectrum (DMSO- d_6) for compound 7a.



¹H NMR (400 MHz, DMSO-d₆)

7b

COC1=CC=C2C(=C1)N(C(=O)N2C3CCN(C3Cc4ccccc4)C5CCSC5=O)C(=O)N

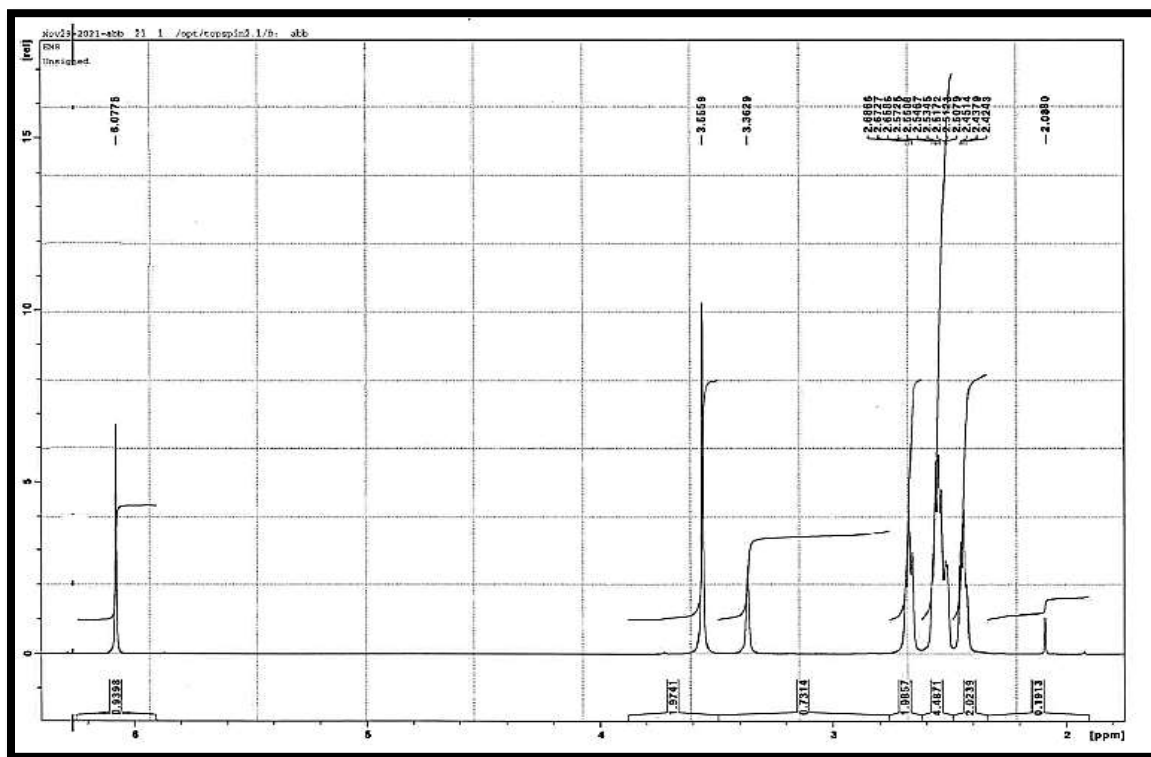
Chemical structure of 7b is shown. The spectrum displays peaks in the aromatic region (7.0-8.0 ppm), a methoxy singlet (~3.8 ppm), and a complex aliphatic region (2.5-3.5 ppm). Integration values are provided below the baseline.

Chemical Shift (ppm)	Integration
8.00	0.10
7.80	0.10
7.60	0.10
7.40	0.10
7.20	0.10
7.00	0.10
6.80	0.10
6.60	0.10
6.40	0.10
6.20	0.10
6.00	0.10
5.80	0.10
5.60	0.10
5.40	0.10
5.20	0.10
5.00	0.10
4.80	0.10
4.60	0.10
4.40	0.10
4.20	0.10
4.00	0.10
3.80	0.10
3.60	0.10
3.40	0.10
3.20	0.10
3.00	0.10
2.80	0.10
2.60	0.10
2.40	0.10
2.20	0.10
2.00	0.10
1.80	0.10
1.60	0.10
1.40	0.10
1.20	0.10
1.00	0.10
0.80	0.10
0.60	0.10
0.40	0.10
0.20	0.10
0.00	0.10

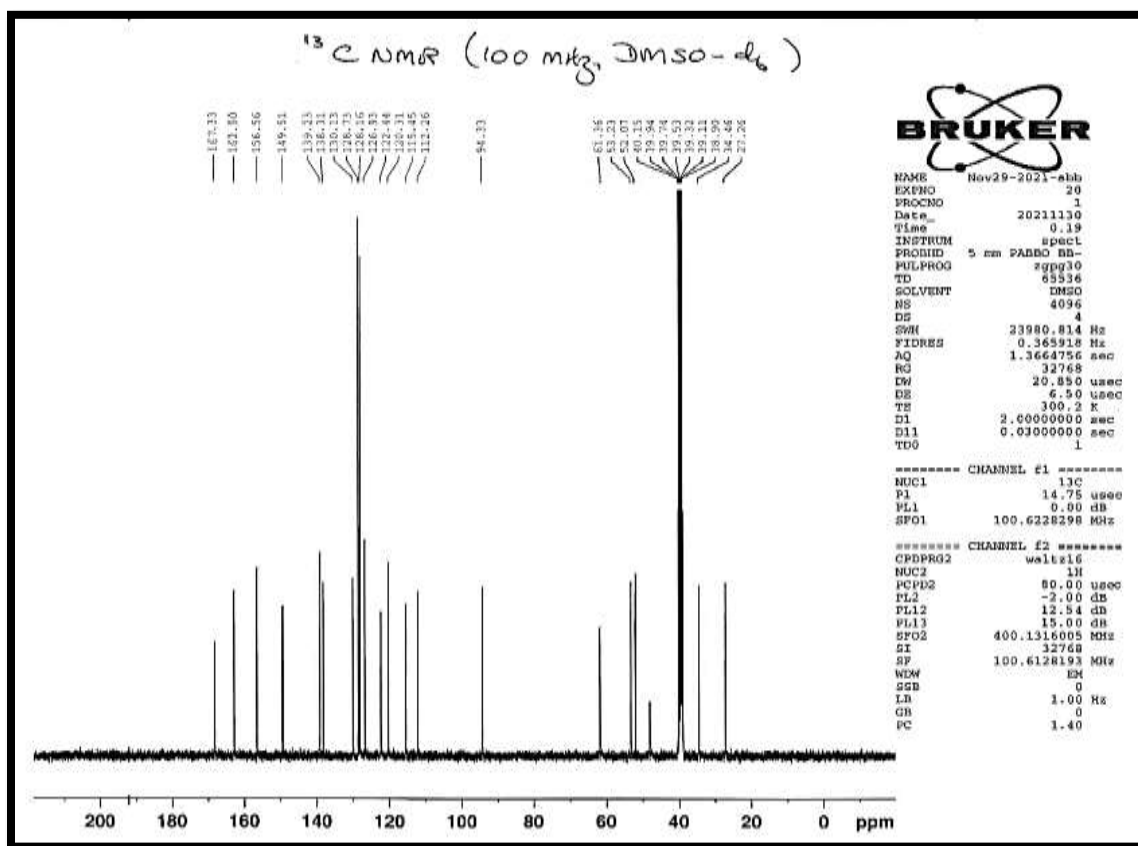
ppm

1H NMR spectrum of compound 21 in CDCl₃. The spectrum shows peaks at 11.0380, 10.0303, 7.8554, and 7.8551 ppm. Integration values are 0.1970, 0.3856, 1.0097, 0.6554, 3.3349, 2.0000, and 1.0310. The x-axis is labeled [ppm] and ranges from 11 to 7.5. The y-axis is labeled [a.u.] and ranges from 0 to 15.

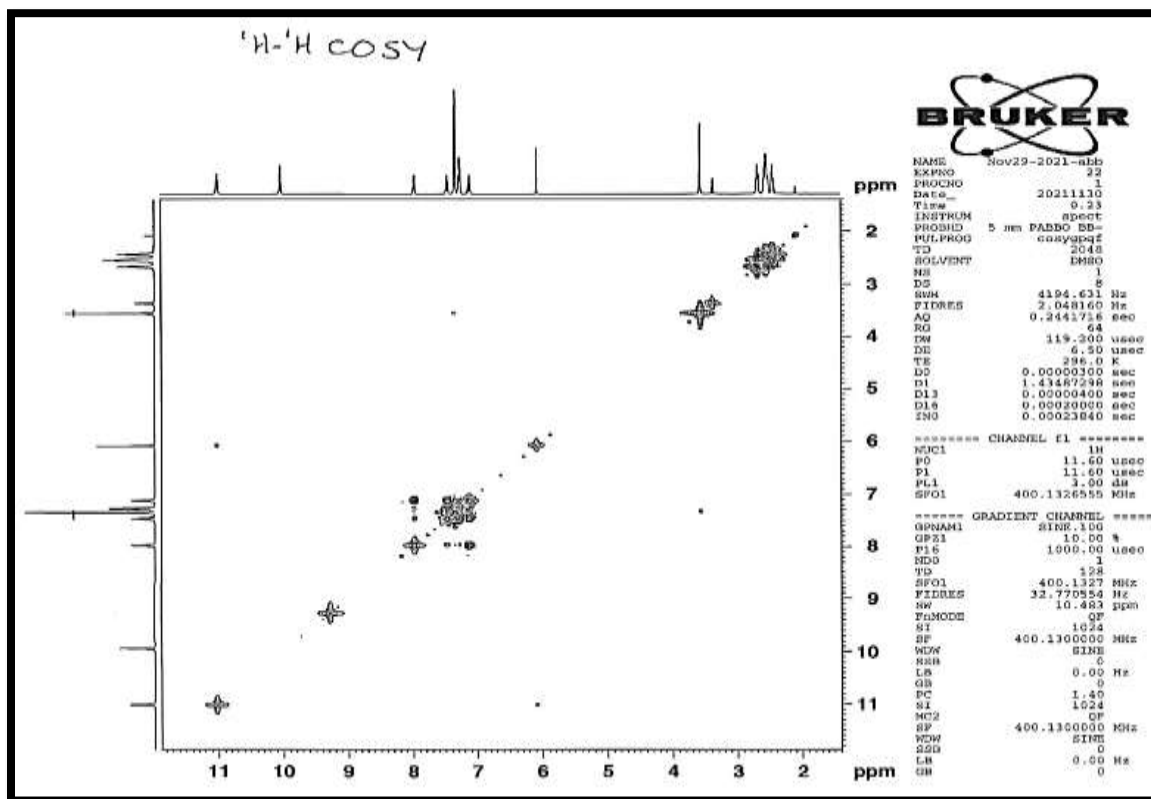
SI Fig. 30. Part of ^1H NMR spectrum (DMSO- d_6) for compound **7b**.



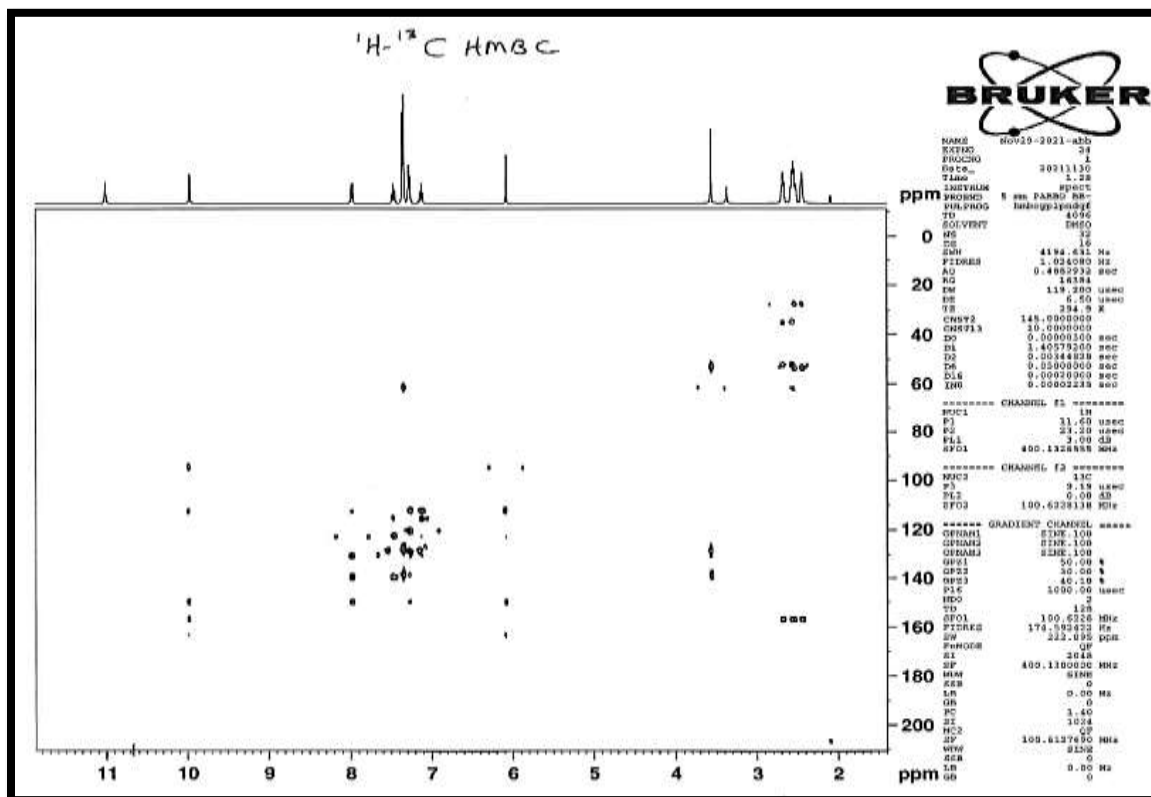
SI Fig. 32. ^{13}C NMR spectrum (DMSO- d_6) for compound **7b**.



SI Fig. 33. ^1H - ^1H Cosy spectrum (DMSO- d_6) for compound **7b**.



SI Fig. 34. ^1H - ^{13}C HMBC spectrum (DMSO- d_6) for compound **7b**.



[illegible]

¹H-¹⁵N HMBSC

BRUKER

NAME: 100729-2021-400
EXPNO: 20
PROCNO: 20211110
DATE_: 4.10
TIME: 10:00
INSTRUM: spect
PROBHD: 5 mm PABBO BBO
PULPROG: zgpg30
TD: 4096
SOLVENT: DMSO
NS: 16
DS: 16
SWH: 4192.431 MHz
FIDRES: 1.024888 Hz
AQ: 0.488532 sec
RG: 2608
DQ: 119.420 usad
DE: 6.00 usec
TE: 300.2 K
CMT2: 80.000000
CMT3: 0.000000
U0: 1.40579200 usec
D1: 0.00454000 sec
D2: 0.06200000 sec
D16: 0.00420000 sec
D17: 0.00002240 sec
===== CHANNEL f1 =====
NUC1: 15N
P1: 11.00 usec
PL1: 23.20 dB
RF1: 400.1326889 MHz
===== CHANNEL f2 =====
NUC2: 1H
P2: 11.52 usec
PL2: 4.00 dB
RF2: 500.1360520 MHz
===== GRADIENT CHANNEL =====
GPM1: 20.00 Hz
GPM2: 20.00 Hz
GPM3: 20.00 Hz
GPM4: 20.00 Hz
GPM5: 20.00 Hz
GPM6: 20.00 Hz
GPM7: 20.00 Hz
GPM8: 20.00 Hz
GPM9: 20.00 Hz
GPM10: 20.00 Hz
GPM11: 20.00 Hz
GPM12: 20.00 Hz
GPM13: 20.00 Hz
GPM14: 20.00 Hz
GPM15: 20.00 Hz
GPM16: 20.00 Hz
GPM17: 20.00 Hz
GPM18: 20.00 Hz
GPM19: 20.00 Hz
GPM20: 20.00 Hz
GPM21: 20.00 Hz
GPM22: 20.00 Hz
GPM23: 20.00 Hz
GPM24: 20.00 Hz
GPM25: 20.00 Hz
GPM26: 20.00 Hz
GPM27: 20.00 Hz
GPM28: 20.00 Hz
GPM29: 20.00 Hz
GPM30: 20.00 Hz
GPM31: 20.00 Hz
GPM32: 20.00 Hz
GPM33: 20.00 Hz
GPM34: 20.00 Hz
GPM35: 20.00 Hz
GPM36: 20.00 Hz
GPM37: 20.00 Hz
GPM38: 20.00 Hz
GPM39: 20.00 Hz
GPM40: 20.00 Hz
GPM41: 20.00 Hz
GPM42: 20.00 Hz
GPM43: 20.00 Hz
GPM44: 20.00 Hz
GPM45: 20.00 Hz
GPM46: 20.00 Hz
GPM47: 20.00 Hz
GPM48: 20.00 Hz
GPM49: 20.00 Hz
GPM50: 20.00 Hz
GPM51: 20.00 Hz
GPM52: 20.00 Hz
GPM53: 20.00 Hz
GPM54: 20.00 Hz
GPM55: 20.00 Hz
GPM56: 20.00 Hz
GPM57: 20.00 Hz
GPM58: 20.00 Hz
GPM59: 20.00 Hz
GPM60: 20.00 Hz
GPM61: 20.00 Hz
GPM62: 20.00 Hz
GPM63: 20.00 Hz
GPM64: 20.00 Hz
GPM65: 20.00 Hz
GPM66: 20.00 Hz
GPM67: 20.00 Hz
GPM68: 20.00 Hz
GPM69: 20.00 Hz
GPM70: 20.00 Hz
GPM71: 20.00 Hz
GPM72: 20.00 Hz
GPM73: 20.00 Hz
GPM74: 20.00 Hz
GPM75: 20.00 Hz
GPM76: 20.00 Hz
GPM77: 20.00 Hz
GPM78: 20.00 Hz
GPM79: 20.00 Hz
GPM80: 20.00 Hz
GPM81: 20.00 Hz
GPM82: 20.00 Hz
GPM83: 20.00 Hz
GPM84: 20.00 Hz
GPM85: 20.00 Hz
GPM86: 20.00 Hz
GPM87: 20.00 Hz
GPM88: 20.00 Hz
GPM89: 20.00 Hz
GPM90: 20.00 Hz
GPM91: 20.00 Hz
GPM92: 20.00 Hz
GPM93: 20.00 Hz
GPM94: 20.00 Hz
GPM95: 20.00 Hz
GPM96: 20.00 Hz
GPM97: 20.00 Hz
GPM98: 20.00 Hz
GPM99: 20.00 Hz
GPM100: 20.00 Hz
GPM101: 20.00 Hz
GPM102: 20.00 Hz
GPM103: 20.00 Hz
GPM104: 20.00 Hz
GPM105: 20.00 Hz
GPM106: 20.00 Hz
GPM107: 20.00 Hz
GPM108: 20.00 Hz
GPM109: 20.00 Hz
GPM110: 20.00 Hz
GPM111: 20.00 Hz
GPM112: 20.00 Hz
GPM113: 20.00 Hz
GPM114: 20.00 Hz
GPM115: 20.00 Hz
GPM116: 20.00 Hz
GPM117: 20.00 Hz
GPM118: 20.00 Hz
GPM119: 20.00 Hz
GPM120: 20.00 Hz
GPM121: 20.00 Hz
GPM122: 20.00 Hz
GPM123: 20.00 Hz
GPM124: 20.00 Hz
GPM125: 20.00 Hz
GPM126: 20.00 Hz
GPM127: 20.00 Hz
GPM128: 20.00 Hz
GPM129: 20.00 Hz
GPM130: 20.00 Hz
GPM131: 20.00 Hz
GPM132: 20.00 Hz
GPM133: 20.00 Hz
GPM134: 20.00 Hz
GPM135: 20.00 Hz
GPM136: 20.00 Hz
GPM137: 20.00 Hz
GPM138: 20.00 Hz
GPM139: 20.00 Hz
GPM140: 20.00 Hz
GPM141: 20.00 Hz
GPM142: 20.00 Hz
GPM143: 20.00 Hz
GPM144: 20.00 Hz
GPM145: 20.00 Hz
GPM146: 20.00 Hz
GPM147: 20.00 Hz
GPM148: 20.00 Hz
GPM149: 20.00 Hz
GPM150: 20.00 Hz
GPM151: 20.00 Hz
GPM152: 20.00 Hz
GPM153: 20.00 Hz
GPM154: 20.00 Hz
GPM155: 20.00 Hz
GPM156: 20.00 Hz
GPM157: 20.00 Hz
GPM158: 20.00 Hz
GPM159: 20.00 Hz
GPM160: 20.00 Hz
GPM161: 20.00 Hz
GPM162: 20.00 Hz
GPM163: 20.00 Hz
GPM164: 20.00 Hz
GPM165: 20.00 Hz
GPM166: 20.00 Hz
GPM167: 20.00 Hz
GPM168: 20.00 Hz
GPM169: 20.00 Hz
GPM170: 20.00 Hz
GPM171: 20.00 Hz
GPM172: 20.00 Hz
GPM173: 20.00 Hz
GPM174: 20.00 Hz
GPM175: 20.00 Hz
GPM176: 20.00 Hz
GPM177: 20.00 Hz
GPM178: 20.00 Hz
GPM179: 20.00 Hz
GPM180: 20.00 Hz
GPM181: 20.00 Hz
GPM182: 20.00 Hz
GPM183: 20.00 Hz
GPM184: 20.00 Hz
GPM185: 20.00 Hz
GPM186: 20.00 Hz
GPM187: 20.00 Hz
GPM188: 20.00 Hz
GPM189: 20.00 Hz
GPM190: 20.00 Hz
GPM191: 20.00 Hz
GPM192: 20.00 Hz
GPM193: 20.00 Hz
GPM194: 20.00 Hz
GPM195: 20.00 Hz
GPM196: 20.00 Hz
GPM197: 20.00 Hz
GPM198: 20.00 Hz
GPM199: 20.00 Hz
GPM200: 20.00 Hz
GPM201: 20.00 Hz
GPM202: 20.00 Hz
GPM203: 20.00 Hz
GPM204: 20.00 Hz
GPM205: 20.00 Hz
GPM206: 20.00 Hz
GPM207: 20.00 Hz
GPM208: 20.00 Hz
GPM209: 20.00 Hz
GPM210: 20.00 Hz
GPM211: 20.00 Hz
GPM212: 20.00 Hz
GPM213: 20.00 Hz
GPM214: 20.00 Hz
GPM215: 20.00 Hz
GPM216: 20.00 Hz
GPM217: 20.00 Hz
GPM218: 20.00 Hz
GPM219: 20.00 Hz
GPM220: 20.00 Hz
GPM221: 20.00 Hz
GPM222: 20.00 Hz
GPM223: 20.00 Hz
GPM224: 20.00 Hz
GPM225: 20.00 Hz
GPM226: 20.00 Hz
GPM227: 20.00 Hz
GPM228: 20.00 Hz
GPM229: 20.00 Hz
GPM230: 20.00 Hz
GPM231: 20.00 Hz
GPM232: 20.00 Hz
GPM233: 20.00 Hz
GPM234: 20.00 Hz
GPM235: 20.00 Hz
GPM236: 20.00 Hz
GPM237: 20.00 Hz
GPM238: 20.00 Hz
GPM239: 20.00 Hz
GPM240

¹H-¹⁵N HSQC

132.6
141.9

BRUKER

NAME Mov2-2011-asm
EXPNO 25
PROCNO 1
DATE_ 20211110
TIME 3.42
INSTRUM spect
PROBHD 5 mm PABO BB-
PULPROG zgpg30
TD 1024
SOLVENT DMSO
NS 8
DS 1
SWH 4194.631 Hz
FIDRES 4.094319 Hz
AQ 0.1221108 sec
RG 320
DWE 119.200 usec
TE 300.2 K
JMOD 0.0000000 sec
D1 1.471998000 sec
D4 0.6027779 sec
D5 0.0300000 sec
D11 0.0000400 sec
D12 0.0000000 sec
D13 0.0000000 sec
D14 0.0000000 sec
D15 0.0000000 sec
D16 0.0000000 sec
D17 0.0000000 sec
D18 0.0000000 sec
D19 0.0000000 sec
D20 0.0000000 sec
D21 0.0000000 sec
D22 0.0000000 sec
D23 0.0000000 sec
D24 0.0000000 sec
D25 0.0000000 sec
D26 0.0000000 sec
D27 0.0000000 sec
D28 0.0000000 sec
D29 0.0000000 sec
D30 0.0000000 sec
D31 0.0000000 sec
D32 0.0000000 sec
D33 0.0000000 sec
D34 0.0000000 sec
D35 0.0000000 sec
D36 0.0000000 sec
D37 0.0000000 sec
D38 0.0000000 sec
D39 0.0000000 sec
D40 0.0000000 sec
D41 0.0000000 sec
D42 0.0000000 sec
D43 0.0000000 sec
D44 0.0000000 sec
D45 0.0000000 sec
D46 0.0000000 sec
D47 0.0000000 sec
D48 0.0000000 sec
D49 0.0000000 sec
D50 0.0000000 sec
D51 0.0000000 sec
D52 0.0000000 sec
D53 0.0000000 sec
D54 0.0000000 sec
D55 0.0000000 sec
D56 0.0000000 sec
D57 0.0000000 sec
D58 0.0000000 sec
D59 0.0000000 sec
D60 0.0000000 sec
D61 0.0000000 sec
D62 0.0000000 sec
D63 0.0000000 sec
D64 0.0000000 sec
D65 0.0000000 sec
D66 0.0000000 sec
D67 0.0000000 sec
D68 0.0000000 sec
D69 0.0000000 sec
D70 0.0000000 sec
D71 0.0000000 sec
D72 0.0000000 sec
D73 0.0000000 sec
D74 0.0000000 sec
D75 0.0000000 sec
D76 0.0000000 sec
D77 0.0000000 sec
D78 0.0000000 sec
D79 0.0000000 sec
D80 0.0000000 sec
D81 0.0000000 sec
D82 0.0000000 sec
D83 0.0000000 sec
D84 0.0000000 sec
D85 0.0000000 sec
D86 0.0000000 sec
D87 0.0000000 sec
D88 0.0000000 sec
D89 0.0000000 sec
D90 0.0000000 sec
D91 0.0000000 sec
D92 0.0000000 sec
D93 0.0000000 sec
D94 0.0000000 sec
D95 0.0000000 sec
D96 0.0000000 sec
D97 0.0000000 sec
D98 0.0000000 sec
D99 0.0000000 sec
D100 0.0000000 sec
D101 0.0000000 sec
D102 0.0000000 sec
D103 0.0000000 sec
D104 0.0000000 sec
D105 0.0000000 sec
D106 0.0000000 sec
D107 0.0000000 sec
D108 0.0000000 sec
D109 0.0000000 sec
D110 0.0000000 sec
D111 0.0000000 sec
D112 0.0000000 sec
D113 0.0000000 sec
D114 0.0000000 sec
D115 0.0000000 sec
D116 0.0000000 sec
D117 0.0000000 sec
D118 0.0000000 sec
D119 0.0000000 sec
D120 0.0000000 sec
D121 0.0000000 sec
D122 0.0000000 sec
D123 0.0000000 sec
D124 0.0000000 sec
D125 0.0000000 sec
D126 0.0000000 sec
D127 0.0000000 sec
D128 0.0000000 sec
D129 0.0000000 sec
D130 0.0000000 sec
D131 0.0000000 sec
D132 0.0000000 sec
D133 0.0000000 sec
D134 0.0000000 sec
D135 0.0000000 sec
D136 0.0000000 sec
D137 0.0000000 sec
D138 0.0000000 sec
D139 0.0000000 sec
D140 0.0000000 sec
D141 0.0000000 sec
D142 0.0000000 sec
D143 0.0000000 sec
D144 0.0000000 sec
D145 0.0000000 sec
D146 0.0000000 sec
D147 0.0000000 sec
D148 0.0000000 sec
D149 0.0000000 sec
D150 0.0000000 sec
D151 0.0000000 sec
D152 0.0000000 sec
D153 0.0000000 sec
D154 0.0000000 sec
D155 0.0000000 sec
D156 0.0000000 sec
D157 0.0000000 sec
D158 0.0000000 sec
D159 0.0000000 sec
D160 0.0000000 sec
D161 0.0000000 sec
D162 0.0000000 sec
D163 0.0000000 sec
D164 0.0000000 sec
D165 0.0000000 sec
D166 0.0000000 sec
D167 0.0000000 sec
D168 0.0000000 sec
D169 0.0000000 sec
D170 0.0000000 sec
D171 0.0000000 sec
D172 0.0000000 sec
D173 0.0000000 sec
D174 0.0000000 sec
D175 0.0000000 sec
D176 0.0000000 sec
D177 0.0000000 sec
D178 0.0000000 sec
D179 0.0000000 sec
D180 0.0000000 sec
D181 0.0000000 sec
D182 0.0000000 sec
D183 0.0000000 sec
D184 0.0000000 sec
D185 0.0000000 sec
D186 0.0000000 sec
D187 0.0000000 sec
D188 0.0000000 sec
D189 0.0000000 sec
D190 0.0000000 sec
D191 0.0000000 sec
D192 0.0000000 sec
D193 0.0000000 sec
D194 0.0000000 sec
D195 0.0000000 sec
D196 0.0000000 sec
D197 0.0000000 sec
D198 0.0000000 sec
D199 0.0000000 sec
D200 0.0000000 sec
D201 0.0000000 sec
D202 0.0000000 sec
D203 0.0000000 sec
D204 0.0000000 sec
D205 0.0000000 sec
D206 0.0000000 sec
D207 0.0000000 sec
D208 0.0000000 sec
D209 0.0000000 sec
D210 0.0000000 sec
D211 0.0000000 sec
D212 0.0000000 sec
D213 0.0000000 sec
D214 0.0000000 sec
D215 0.0000000 sec
D216 0.0000000 sec
D217 0.0000000 sec
D218 0.0000000 sec
D219 0.0000000 sec
D220 0.0000000 sec
D221 0.0000000 sec
D222 0.0000000 sec
D223 0.0000000 sec
D224 0.0000000 sec
D225 0.0000000 sec
D226 0.0000000 sec
D227 0.0000000 sec
D228 0.0000000 sec
D229 0.0000000 sec
D230 0.0000000 sec
D231 0.000000

Appendix A

4. Experimental

General Details

Analytical grade chemicals and solvents were used. The reactions monitored by thin layer chromatography on Merck alumina-backed TLC plates aluminum sheets. Melting points were determined on Stuart electro-thermal melting point apparatus and were uncorrected. NMR spectra were recorded on Bruker AV-400 spectrometer in DMSO- d_6 as solvent and TMS as internal standard, at Florida Institute of Technology, Chemistry Department, 150 W University Blvd, Melbourne, FL 32901, USA. Melting point carried out by using Stuart Electro-Thermal apparatus and uncorrected. Chemical shifts are expressed in δ (ppm) versus internal (TMS) = 0 ppm for ^1H NMR and ^{13}C NMR, and external liquid ammonia = 0 ppm for ^{15}N . Correlations were established using ^1H - ^1H COSY, ^1H - ^{13}C , ^1H - ^{15}N HSQC and HMBC experiments. Chemical shifts (δ) are reported in parts per million (ppm) relative to Tetramethylsilane (TMS). Splitting patterns are denoted as follows: singlet (s), broad (b), doublet (d), triplet (t), multiplet (m), quartet (q), broad of singlet (bs) and doublet of doublets (dd) and stated in Hertz (Hz). Elemental analyses were carried out on a Perkin Elmer device at the Microanalytical Institute of Organic Chemistry, Karlsruhe Institute of Technology, Karlsruhe, Germany. The mass spectra were recorded on a Finnigan Fab 70 eV at Al-Azhar University, Egypt.

4.2. Biological evaluation

4.2.1. Cytotoxic activity using MTT Assay and evaluation of IC₅₀

4.2.1.1. MTT assay

MTT assay was performed to investigate the effect of the synthesized compounds on mammary epithelial cells (MCF-10A). The cells were propagated in medium consisting of Ham's F-12 medium/ Dulbecco's modified Eagle's medium (DMEM) (1:1) supplemented with 10% foetal calf serum, 2 mM glutamine, insulin (10 µg/mL), hydrocortisone (500 ng/mL) and epidermal growth factor (20 ng/mL). Trypsin ethylenediamine tetra acetic acid (EDTA) was used to passage the cells after every 2-3 days. 96-well flat-bottomed cell culture plates were used to seed the cells at a density of 10^4 cells mL⁻¹. The medium was aspirated from all the wells of culture plates after 24 h followed by the addition of synthesized compounds (in 200 µL medium to yield a final concentration of 0.1% (v/v) dimethyl sulfoxide) into individual wells of the plates. Four wells were designated to a single compound. The plates were allowed to incubate at 37°C for 96 h. Afterwards, the medium was aspirated and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (0.4 mg/mL) in medium was added to each well and subsequently incubated for 3 h. The medium was aspirated and 150 µL dimethyl sulfoxide (DMSO) was added to each well. The plates were vortexed followed by the measurement of absorbance at 540 nm on a microplate reader. The results were presented as inhibition (%) of proliferation in contrast to controls comprising 0.1% DMSO.

4.2.1.2. Assay for antiproliferative effect

To explore the antiproliferative potential of compounds propidium iodide fluorescence assay was performed using different cell lines such as Panc-1 (pancreas cancer cell line), MCF-7 (breast cancer cell line), HT-29 (colon cancer cell line) and A-549 (epithelial cancer cell line), respectively. To calculate the total nuclear DNA, a fluorescent dye (propidium iodide, PI) is used which can attach to the DNA, thus offering a quick and precise technique. PI cannot pass through the cell membrane and its signal intensity can be considered as directly proportional to quantity of cellular DNA. Cells whose cell membranes are damaged or have changed permeability are counted as dead ones. The assay was performed by seeding the cells of different cell lines at a density of 3000-7500 cells/well (in 200 µl medium) in culture plates followed by incubation for 24h at 37 °C in humidified 5% CO₂/95% air atmospheric conditions. The medium was removed; the compounds were added to the plates at 10 µM concentrations (in 0.1% DMSO) in triplicates, followed by incubation for 48h. DMSO (0.1%) was used as control. After incubation, medium was removed followed by the addition of PI (25 µl, 50 µg/mL in water/medium) to each well of the plates. At -80 °C, the plates were allowed to freeze for 24 h, followed by thawing at 25 °C. A fluorometer (Polar-Star BMG Tech) was used to record the readings at excitation and emission wavelengths of 530 and 620 nm for each well. The percentage cytotoxicity of compounds was calculated using the following formula:

$$\% \text{ Cytotoxicity} = \frac{A_c - A_{TC}}{A_c} \times 100$$

Where A_{TC} = Absorbance of treated cells and A_c = Absorbance of control. Erlotinib was used as positive control in the assay.

4.2.2. EGFR inhibitory assay

Baculoviral expression vectors including pBlueBacHis2B and pFASTBacHTc were used separately to clone 1.6 kb cDNA coding for EGFR cytoplasmic domain (EGFR-CD, amino acids 645–1186). 5' upstream to the EGFR sequence comprised a sequence that encoded (His)₆. Sf-9 cells were infected for 72h for protein expression. The pellets of Sf-9 cells were solubilized in a buffer containing sodium vanadate (100 μ M), aprotinin (10 μ g/mL), triton (1%), HEPES buffer (50mM), ammonium molybdate (10 μ M), benzamidine HCl (16 μ g/mL), NaCl (10 mM), leupeptin (10 μ g/mL) and pepstatin (10 μ g/mL) at 0°C for 20 min at pH 7.4, followed by centrifugation for 20 min. To eliminate the nonspecifically bound material, a Ni-NTA super flow packed column was used to pass through and wash the crude extract supernatant first with 10 μ M and then with 100 mM imidazole. Histidine-linked proteins were first eluted with 250 and then with 500 μ M imidazole subsequent to dialysis against NaCl (50 μ M), HEPES (20 μ M), glycerol (10%) and 1 μ g/mL each of aprotinin, leupeptin and pepstatin for 120 mins. The purification was performed either at 4 °C or on ice. To record autophosphorylation level, EGFR kinase assay was carried out on the basis of DELFIA/Time-Resolved Fluorometry. The compounds were first dissolved in DMSO absolute, subsequent to dilution to appropriate concentration using HEPES (25 μ M) at pH 7.4. Each compound (10 μ L) was incubated with recombinant enzyme (10 μ L, 5 ng for EGFR, 1:80 dilution in 100 mM HEPES) for 10 min at 25°C, subsequent to the addition of 5X buffer (10 μ L, containing 2 mM MnCl₂, 100 μ M Na₃VO₄, 20 μ M HEPES and 1 μ M DTT) and ATP-MgCl₂ (20 μ L, containing 0.1 μ M ATP and 50 μ M MgCl₂) and incubation for 1h. The negative and positive controls were included in each plate by the incubation of enzyme either with or without ATP-MgCl₂. The liquid was removed after incubation and the plates were washed thrice using wash buffer. Europium-tagged antiphosphotyrosine antibody (75 μ L, 400

ng) was added to each well followed by incubation of 1h and then washing of the plates using buffer. The enhancement solution was added to each well and the signal was recorded at excitation and emission wavelengths of 340 at 615 nm. The autophosphorylation percentage inhibition by compounds was calculated using the following equation:

$$100\% - [(negative\ control)/(positive\ control) - (negative\ control)]$$

Using the curves of percentage inhibition of eight concentrations of each compound, IC₅₀ was calculated. Majority of signals detected by antiphosphotyrosine antibody were from EGFR because the enzyme preparation contained low impurities.

4.2.3. BRAF kinase assay

V^{600E} mutant BRAF kinase assay was performed to investigate the activity of tested compounds against BRAF. Mouse full-length GST-tagged BRAF^{V600E} (7.5 ng, Invitrogen, PV3849) was pre-incubated with drug (1 µL) and assay dilution buffer (4 µL) for 60 mins at 25°C. In assay dilution buffer, a solution (5 µL) containing MgCl₂ (30 µM), ATP (200 µM), recombinant human full length (200 ng) and *N*-terminal His-tagged MEK1 (Invitrogen) was added to start the assay, subsequent to incubation for 25 mins at 25°C. The assay was stopped using 5X protein denaturing buffer (LDS) solution (5 µL). To further denature the protein, heat (70° C) was applied for 5 mins. 4-12% precast NuPage gel plates (Invitrogen) were used to carry out electrophoresis (at 200 V). 10 µL of each reaction was loaded into the precast plates and electrophoresis was allowed to proceed. After completion of electrophoresis, the front part of the precast gel plate (holding hot ATP) was cut and afterwards cast-off. The dried gel was developed using a phosphor screen. A reaction without active enzyme was used as negative control while that containing no inhibitor served as positive control. To study the effect of compounds on cell-

based pERK1/2 activity in cancer cells, commercially available ELISA kits (Invitrogen) were used according to manufacturer's instructions.

4.2.3. Caspase-3 activation assay

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. Add 100 µl of the *Standard Diluent Buffer* to the zero standard wells. Well(s) reserved for chromogen blank should be left empty. Add 100 µl of standards and controls or diluted samples to the appropriate microtiter wells. The sample dilution chosen should be optimized for each experimental system. Tap gently on side of plate to mix. Cover wells with *plate cover* and incubate for 2 hours at room temperature. Thoroughly aspirate or decant solution from wells and discard the liquid, Wash wells 4 times. Pipette 100 µl of *Caspase-3 (Active) Detection Antibody* solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix. Cover plate with *plate cover* and incubate for 1 hour at room temperature. Thoroughly aspirate or decant solution from wells and discard the liquid, Wash wells 4 times. Add 100 µl Anti-Rabbit IgG HRP Working Solution to each well except the chromogen blank(s). Prepare the working dilution as described in Preparing IgG HRP. Cover wells with the *plate cover* and incubate for 30 minutes at room temperature. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. Add 100 µl of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue. Incubate for 30 minutes at room temperature and in the dark. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored, and the substrate reaction stopped before the O.D. of the positive wells exceeds the limits of the

instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested. Add 100 µl of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µl each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 2 hours after adding the *Stop Solution*. Use a curve fitting software to generate the standard curve. A four-parameter algorithm provides the best standard curve fit. Read the concentrations for unknown samples and controls from the standard curve. Multiply value(s) obtained for sample(s) by the appropriate dilution factor to correct for the dilution in step 3. Samples producing signals greater than that of the highest standard should be diluted in *Standard Diluent Buffer* and reanalyzed.

4.2.4. Caspase-8 activation assay

Cells were obtained from American Type Culture Collection, cells were grown in RPMI 1640 containing 10% fetal bovine serum at 37°C, stimulated with the compounds to be tested for caspase8, and lysed with Cell Extraction Buffer. This lysate was diluted in Standard Diluent Buffer over the range of the assay and measured for human active caspase-8 content. (*Cells are Plated in a density of $1.2 - 1.8 \times 10,000$ cells/well in a volume of 100µl complete growth medium + 100 ul of the tested compound per well in a 96-well plate for 24 hours before the enzyme assay for Tubulin.*). The absorbance of each microwell was read on a spectro-photometer at 450 nm. A standard curve is prepared from 7human Caspase-8 standard dilutions and human Caspase-8 concentration determined.

4.2.5. Bax activation assay

Bring all reagents, except the human Bax- α Standard, to room temperature for at least 30 minutes prior to opening. The human Bax- α Standard solution should not be left at room temperature for more than 10 minutes. All standards, controls and samples should be run in duplicate. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C. Pipet 100 μ L of Assay Buffer into the S0 (0 pg/mL standard) wells. Pipet 100 μ L of Standards #1 through #6 into the appropriate wells. Pipet 100 μ L of the Samples into the appropriate wells. Tap the plate gently to mix the contents. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm. Empty the contents of the wells and wash by adding 400 μ L of wash solution to every well. Repeat the wash 4 more times for a total of **5 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer. Pipet 100 μ L of yellow Antibody into each well, except the Blank. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm. Empty the contents of the wells and wash by adding 400 μ L of wash solution to every well. Repeat the wash 4 more times for a total of **5 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer. Add 100 μ L of blue Conjugate to each well, except the Blank. Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm. Empty the contents of the wells and wash by adding 400 μ L of wash solution to every well. Repeat the wash 4 more times for a total of **5 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer. Pipet 100 μ L of Substrate Solution into each well. Incubate for 30 minutes at room temperature on a plate shaker at ~500

rpm. Pipet 100 μ L Stop Solution to each well. Blank the plate reader against the Blank wells, read the optical density at 450 nm. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample. Using linear graph paper, plot the Average Net OD for each standard versus Bax concentration in each standard. Approximate a straight line through the points. The concentration of Bax in the unknowns can be determined by interpolation.

4.2.6. Bcl-2 inhibition assay

Mix all reagents thoroughly without foaming before use. Wash the microwells twice with approximately 300 μ L Wash Buffer per well with thorough aspiration of microwell contents between washes. Take caution not to scratch the surface of the microwells. After the last wash, empty the wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 mins. Do not allow wells to dry. Add 100 μ L of Sample Diluent in duplicate to all standard wells and to the blank wells. Prepare standard (1:2 dilution) in duplicate ranging from 32 ng/mL to 0.5 ng/mL. Add 100 μ L of Sample Diluent, in duplicate, to the blank wells. Add 80 μ L of Sample Diluent, in duplicate, to the sample wells. Add 20 μ L of each Sample, in duplicate, to the designated wells. Add 50 μ L of diluted biotin-conjugate to all wells, including the blank wells. Cover with a plate cover and incubate at room temperature, on a microplate shaker at 100 rpm if available, for 2 hours. Remove plate cover and empty the wells. Wash microwell strips 3 times as described in step 2. Add 100 μ L of diluted Streptavidin-HRP to all wells, including the blank wells. Cover with a plate cover and incubate at room temperature, on a microplate shaker at 100 rpm if available, for 1 hour. Remove plate cover and empty the wells. Wash microwell strips 3 times as described in step 2. Proceed to the next step.

Pipette 100 μ l of mixed TMB Substrate Solution to all wells, including the blanks. Incubate the microwell strips at room temperature (18° to 25°C) for about 15 minutes, if available on a rotator set at 100 rpm. Avoid direct exposure to intense light. The point, at which the substrate reaction is stopped, is often determined by the ELISA reader. Many ELISA readers record absorbance only up to 2.0 O.D. Therefore, the color development within individual microwells must be watched by the person running the assay and the substrate reaction stopped before positive wells are no longer properly detectable. Stop the enzyme reaction by quickly pipetting 100 μ L of Stop Solution into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wavelength.

4.3. Statistical analysis

Computerized Prism 5 program was used to statistically analyzed data using one-way ANOVA test followed by Tukey's as post ANOVA for multiple comparison at $P \leq .05$. Data were presented as mean \pm SEM.