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In vitro and *in silico* approach to determine neuroprotective properties of iridoid glycosides from aerial parts of *Scrophularia amplexicaulis* by investigating their cholinesterase inhibition and anti-oxidant activities

Azadeh Hamedi [1,2], Gokhan Zengin [3], Abdurrahman Aktumsek [3], Zeliha Selamoglu [4], Ardalan Pasdaran [1,*]

[1]Medicinal Plants Processing Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[2]Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran
[3]Department of Biology, Science Faculty, Selcuk University, Campus, Konya, Turkey
[4]Department of Medical Biology, Faculty of Medicine, Nigde Ömer Halisdemir University, Campus, Nigde, Turkey
*corresponding author e-mail address: ***pasdaran@sums.ac.ir*** | Scopus ID 36195686700

ABSTRACT

Three iridoid glycosides included 6-*O*-methyl, 1-glucopyranosyl catalpol (Compound ***1***), 6-*O*-α-L (3"-*O*- *trans*, 4"-*O*- *trans* cinnamoyl)-rhamnopyranosyl catalpol (Compound ***2***) and scropolioside D (Compound ***3***) were isolated from aerial parts of *S. amplexicaulis* using chromatographic methods. The structures were determined by different spectroscopic data. The inhibitory effects (IC_{50} values) of the compounds on cholinesterase (AChE and BChE) was determined by *in-vitro* assays. Docking studied were performed to investigate receptor-ligands interactions. The antioxidant capacity was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, 2, 2'-azino-bis[3-ethylbenzothiazoline]-6-sulphonic acid (ABTS) radical cation, cupric ion reducing activity (CUPRAC), ferric reducing antioxidant power (FRAP), phosphomolybdenum and metal chelating assays. AChE and BChE were moderately inhibited by all of the investigated iridoid glycosides (compared to galantamine). Compound 2 and compound 3 showed comparable anti-oxidant effects with the Trolox as the control in phosphomolybdenum assay. Also, compound 1, showed acceptable activities in ABTS radical scavenging and Phosphomolybdenum assays compared to the control.

Keywords: *Anti-oxidant; Acetylcholinesterase; Butyrylcholinesterase; Iridoid glycosides; Alzheimer's disease; neuroprotective.*

1. INTRODUCTION

Different plants, mushrooms and natural products have been used in traditional medicine for treatment of various diseases [1-3]. Iridoid and iridoid glycosides have been reported as important metabolites of the Scrophulariaceae family [4]. Also, natural compounds have shown neuroprotective properties by a different mechanism including inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) as well as decreasing oxidative stresses [5-6]. Many neural diseases result from a change in concentration of neurotransmitters at the synaptic cleft that may lead to functional impairment of cholinergic systems. The cholinergic system is involved in processes regulating memory and learning. It has been shown that the usage of acetylcholinesterase (AChE) inhibitors, such as rivastigmine, donepezil, galantamine, and tacrine, have positive effects and improve cholinergic system impairment [7]. These therapeutics agents increase the availability of the neurotransmitter, acetylcholine, at the synaptic cleft [8]. Therefore, identification and development of AChE inhibitors are essential to the progress and advancement of novel therapeutics for management and treatment of Alzheimer's disease (AD). Moreover, these inhibitors may have putative therapeutic effects on other neurotransmitter-related neurological disorders, such as myasthenia gravis, Parkinson's disease, dementia, and ataxia [9]. Recent studies on other useful pathways in AD management and treatment may shed some light on the potential of butyrylcholinesterase (BChE) inhibitors [10]. Excessive BChE cortical level was shown to be directly proportional to increasing signs and symptoms of AD. In addition to the roles of AChE and BChE in neurodegenerative diseases, there has been reported evidence of the effect of oxidative stress on memory impairment [11]. Oxidation of the main cell compartments, such as lipids, proteins, and nucleic acids has led to some structural, dynamic, and functional changes in cells and in secretase activity. This may trigger a stream in β-amyloid formation [12]. The role of oxidative stress and damage in various neurodegenerative diseases has led to a focus on the inhibition of these processes as a target with small molecules [13-14]. Some iridoids isolated from different plants have been reported for their potent antioxidant activities [15-16]. Several studies have confirmed the useful effects of extracts, which include iridoids, on neurodegenerative problems [17-18]. Neuroprotective activity of diverse iridoids, including 8-*O*-*E*-*p*-methoxycinnamoyl harpagide, 8-*O*-*Z*-*p*-methoxycinnamoyl harpagide, 6'-*O*-*E*-*p*-methoxycinnamoyl harpagide, 6'-*O*-*Z*-*p*-methoxycinnamoyl harpagide, harpagide, *E*-harpagoside and *Z*-harpagoside have been reported previously [16]. This study was design to investigate neuroprotective potentials of 6-*O*-methyl,1-glucopyranosyl catalpol (***1***), 6-*O*-α-L (3"-*O*- *trans*, 4"-*O*- *trans* cinnamoyl)-rhamnopyranosyl catalpol (***2***) and scropolioside D (***3***) (three iridoid glycosides from aerial parts of *Scrophularia amplexicaulis*) by analyzing their cholinesterase inhibition activity as well as their anti-oxidant properties.

2. MATERIALS AND METHODS

2.1. Isolation of iridoid glycosides.

Iridoid glycosides (6-*O*-methyl, 1-glucopyranosyl catalpol, 6-*O*-α-L (3"-*O*- *trans*, 4"-*O*- *trans* cinnamoyl)-rhamnopyranosyl catalpol, and scropolioside D were isolated by the method described previously by Pasdaran et al. [18]. Briefly, the aerial parts of the plant were collected from East Azerbaijan province and its voucher specimen (2821) has been filed at the Herbarium of the Researches center for agriculture and natural resources, East Azerbaijan. The compounds were isolated by subjecting methanol extract to solid phase extraction (SPE) on a Sep-Pak (10 g) C18 cartridge followed by preparative HPLC (Dr. Mainsch GmbH ODS column 20 µM, 250 mm × 20 mm) using a gradient of MeOH: water mixture as mobile phase [17]. The structures of these compounds were determined by spectroscopic methods including ^{1}H-NMR, ^{13}C-NMR, 2 D NMR technique such as HMBC. Their spectra were assigned and compared with the previous reports on Iridoid glycosides on this plant [20-21].

2.2. Enzyme inhibitory activity.

2.2.1. Cholinesterase inhibition (In vitro approach).

Cholinesterase (ChE) inhibitory activity was measured using Ellman's method, as previously reported [21]. Sample solution (50 µL, 1-3 mM) was mixed with DTNB (5, 5-dithio-bis [2-nitrobenzoic] acid; 125 µL) and AChE (acetylcholinesterase; electric eel acetylcholinesterase, Type-VI-S, EC 3.1.1.7, Sigma) or BChE (butyrylcholinesterase; horse serum butyrylcholinesterase, EC 3.1.1.8, Sigma) solution (25 µL) in Tris-HCl buffer (pH 8.0) in a 96-well microplate and incubated for 15-min at 25 °C. Then, the reaction was initiated with the addition of acetylthiocholine iodide (ATCI) or butyrylthiocholine chloride (BTCl; 25 µL). Similarly, a blank solution was prepared by adding sample solution to all reaction reagents without enzyme (AChE or BChE) solution. The absorbance of samples and blank solutions were read at 405 nm after 10-min incubation at 25 °C. Galantamine was used as a standard. The absorbance of the blank solution was subtracted from that of the sample and the cholinesterase inhibitory was expressed IC_{50} values.

2.2.2. Receptor–ligand molecular docking (In silico approach).

The PDB file of different acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were collected from Protein Database. The isolated iridoid glycosides were energy minimized with Avogadro free software. Preparation of the receptors (included water molecules elimination, active box identification, etc.) was done by Discovery Studio 2016 Client. Interaction analysis between isolated compounds and active box of acetylcholinesterase and butyrylcholinesterase receptors was carried out using PyMOL (version 1.4.1), which generated with a size of 24 × 24 × 24 at 1Å grid spacing. Auto Dock Vina was used for the isolated compounds selectivity and binding manner with AChE and BChE receptors. The MM2 energy minimization was performed for all compounds prior to docking. During the docking process, additional modifications (such as hydrogen atoms and Kollman charges) were added. Grid space of AChE and BChE active site were generated with a size of 24 × 24 × 24 at 1Å grid spacing in AutoDock Vina software. The positional root-mean-square deviation values (rmsd < 3.0) and free energy of binding of the compounds were collected.

2.3. *In Vitro* antioxidant assays.

2.3.1. Phosphomolybdenum method.

Phosphomolybdenum method was used to evaluate the total antioxidant activity of the isolated iridoids [22]. A reagent solution includes 4 mM ammonium molybdate, 28 mM sodium phosphate, and 0.6 M sulfuric acid was used in combination with 0.3 mL of sample solutions, after a 90 minutes incubation at 95 °C absorbance of these samples was read at 695 nm. Half maximal effective concentration (EC_{50}) was used for samples total antioxidant potential expression. All measurements were done as triplicate. Trolox was used as a positive standard. The antioxidant capacities were expressed as the number of equivalents of trolox.
Sample solution (0.3 mL, 1-5 mg/mL) was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The sample absorbance was read at 695 nm after a 90-min incubation at 95 °C. Trolox was used as a standard. The total antioxidant capacity was expressed as EC_{50} values (the effective concentration at which the absorbance was 0.5).

2.3.2. Radical scavenging activity.

2.3.2.1. DPPH radical scavenging activity. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was estimated according to methodology by Zengin et al [21]. 4 mL of 0.004% of DPPH solution (methanolic solution) was mixed with 1 mL of different concentrations of sample solutions. After a 30 minutes incubation at 25 ° C absorbance of samples was read at 517 nm. The half maximal inhibitory concentration (IC_{50}) was used for compression between samples and trolox as standard.

Sample solution (1 mL, 1-5 mg/mL) was added to a 4 mL of 0.004% methanol solution of DPPH. The sample absorbance was read at 517 nm after a 30-min incubation at room temperature in the dark. Trolox was used as a standard. DPPH radical scavenging activity was expressed as IC_{50} values (%50 of free radical/ enzyme inhibition).

2.3.2.2. ABTS radical cation scavenging activity. The scavenging activity against ABTS radical cation (2, 2'-azino-bis[3-ethylbenzothiazoline]-6-sulphonic acid) was measured according to the method of Lee et al. [23]. For producing of 2, 2'-azino-bis [3-ethylbenzothiazoline]-6-sulphonic acid radical cation a mixture of potassium persulphate (2.45) mM and ABTS solution (7 mM) was prepared. After 4–8 h in the dark at room temperature $ABTS^{+}$ was produced. 2 mL of diluted $ABTS^{+}$ methanol solution (with 0.700±0.02 absorbance at 734 nm) was mixed with 1 mL of different concentrations of sample solutions. After 30 minutes of incubation at 25 ° C, absorbance of samples was read at 734 nm in comparison to trolox as standard.

Briefly, $ABTS^{+}$ was produced directly by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and then allowing the mixture to stand for 12-16 h in the dark at room temperature. Prior to performing the assay, the $ABTS^{+}$ solution was diluted with methanol to an absorbance of 0.700±0.02 at 734 nm. The sample solution (1 mL, 1-5 mg/mL) was added to the ABTS solution (2 mL) and mixed. The sample absorbance was read at 734 nm after a 30-min incubation at room temperature. Trolox was used as a standard. ABTS radical scavenging activity was expressed as IC_{50} values.

2.3.3. Reducing power.

2.3.3.1. CUPRAC assay. The cupric ion reducing activity (CUPRAC) was determined according to the method of Zengin et al., (2014) [22]. The sample solution (0.5 mL, 1-5 mg/mL) was added to the premixed reaction mixture containing $CuCl_2$ (1 mL, 10 mM), neocuproine (1 mL, 7.5 mM), and NH_4Ac buffer (1 mL, 1 M, pH 7.0). Similarly, a blank solution was prepared by adding a sample solution (0.5 mL) to the premixed reaction mixture (3 mL) without $CuCl_2$. Then, the absorbance of the sample and of the blank solutions was read at 450 nm after 30-min incubation at room temperature. Trolox was used as a standard. The results of CUPRAC were expressed as EC_{50} values.

2.3.3.2. FRAP assay. The ferric reducing antioxidant power (FRAP) assay was conducted with slight modifications according to Zengin et al., (2015) [24]. Sample solution (0.1 mL, 1-5 mg/mL) was added to premixed FRAP reagent (2 mL) containing acetate buffer (0.3 M, pH 3.6), 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ; 10 mM) in 40 mM HCl and ferric chloride (20 mM) in a ratio of 10:1:1 (v/v/v). Subsequently, the sample absorbance was read at 593 nm after 30-min incubation at room temperature. Trolox was used as a standard. The results of FRAP were expressed as EC_{50} values.

2.3.3.3. Metal chelating activity on ferrous ions. The metal chelating activity on ferrous ions was determined according to Zengin et al., (2015) [22]. Briefly, sample solution (2 mL, 1-5 mg/mL) was added to $FeCl_2$ solution (0.05 mL; 2 mM). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). Similarly, a blank solution was prepared by adding sample solution (2 mL) to $FeCl_2$ solution (0.05 mL; 2 mM) and water (0.2 mL) without ferrozine. Thereafter, the sample and blank absorbance were read at 562 nm after a 10-min incubation at room temperature. The absorbance of the blank solution was subtracted from that of the sample. EDTA (disodium edetate) was used as a standard. Metal chelating activity was expressed as IC_{50} values.

3. RESULTS

The spectra of the isolated iridoid glycosides (supplementary file No: 1) were assigned and compared with the previous reports on Iridoid glycosides of this plant [9, 23]. The isolated molecules were identified as 6-O-methyl, 1-glucopyranosyl catalpol, 6-O-α-L (3"-O- trans, 4"-O- trans cinnamoyl)-rhamnopyranosyl catalpol and scropolioside D. The 1D and 2D NMR data and chemical structures of the compounds are shown in Table 1-2 and Figure 1.

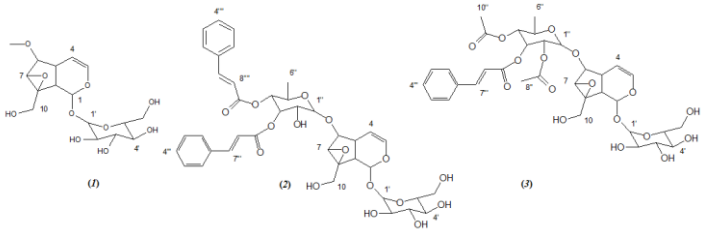

Figure 1. Chemical structures of 6-*O*-methyl, 1-glucopyranosyl catalpol (***1***), 6-*O*-α-L (3"-*O- trans*, 4"-*O- trans* cinnamoyl)-rhamnopyranosyl catalpol (***2***) and scropolioside D (***3***), the iridoid glycosides from *S. amplexicaulis.*

The cholinesterase inhibitory abilities of the isolated compounds were evaluated by IC_{50} values (Table 3 and Figure 2). From these results, scropolioside D was the most active on both AChE (1.02 mM) and BChE (1.06 mM), followed by 6-*O*-α-L (3"-*O- trans*, 4"-*O- trans* cinnamoyl)-rhamnopyranosyl catalpol (1.03 and 1.10 mM) and 6-*O*-methyl, 1-glucopyranosyl catalpol (1.17 and 1.12 mM). However, galanthamine inhibited these enzymes at considerable lower concentrations. Limited work has been conducted on the cholinesterase inhibitory activity of iridoid compounds. Yoon et al. showed that 6'-*O*-cinnamoylharpagide, harpagoside, and 8-*O*-(*cis*-cinnamoyl) harpagide can be used as BChE and AChE inhibitors, although among these compounds only 6'-*O*-cinnamoylharpagide showed a remarkable effect [25-26]. Other isolated iridoid glycoside from leaves of *Pseuderanthemum carruthersii* (Seem.) Guill.var. *atropurpureum* (Bull.) Fosb. 5 β, 6 β –dihydroxyantirrhide showed the AChE inhibitory effect [27]. An *in vitro* cholinesterase enzyme assay showed some activity on BChE and AChE among the known iridoid compounds, loganin and morroniside [28]. Another iridoid, 10-hydroxymajoroside obtained from *Plantago cornuti* Gouan L. also showed a moderate inhibitory effect on AChE [29]. Again, catalpol, which is an iridoid glucoside, was reported as a useful neuroprotective agent against Alzheimer's disease [30]. New studies on this family may provide new horizons for managing neurodegenerative diseases.

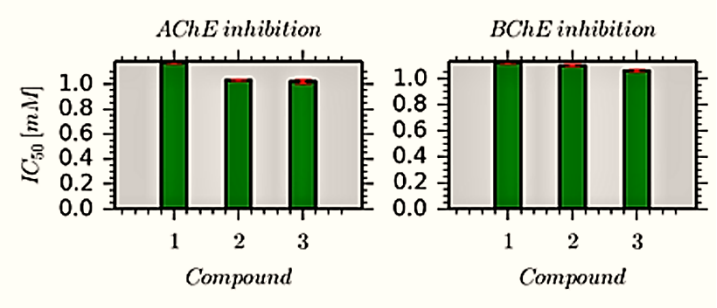

Figure 2. AChE and BChE inhibitory abilities of 6-*O*-methyl, 1-glucopyranosyl catalpol (***1***), 6-*O*-α-L (3"-*O- trans*, 4"-*O- trans* cinnamoyl)-rhamnopyranosyl catalpol (***2***) and scropolioside D (***3***). Values are expressed are means ±SD.

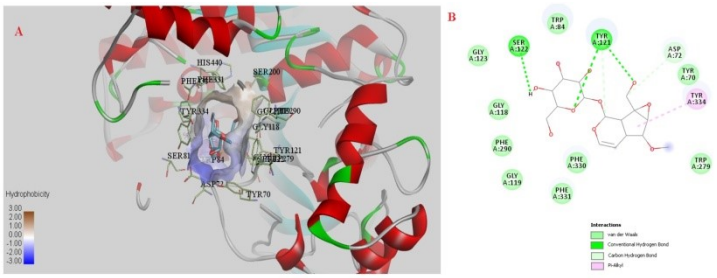

Figure 3. (A) The 6-*O*-methyl, 1-glucopyranosyl catalpol (compound ***1***) in center of the active box of 1oce, the active box amino acids residues, and hydrophobicity map of active box. (B) The 2D diagram of the amino acids residues interactions with 6-*O*-methyl, 1-glucopyranosyl catalpol. Hydrophobic and hydrogen binding condition observed in 1oce binding pocket.

Different docking results of various AChE inhibitors were revealed that hydrophobic interactions between inhibitor ligands skeleton were observed with Tyr-70, Gly-123, Trp-84, Trp-279, Phe-330, Phe-331, Tyr-334 [36, 37]. The docking findings of the previous studies support the experimental result. Although the hydrophobic amino acid residues which located at the peripheral position of AChE active box involved in most interactions

between ligand and this class of enzyme, but in addition to these interactions, hydrogen bonding between the different parts of compound 1 and Tyr-121, Ser-122, Asp-72 amino acids may be acts as an additional important connections which occurred in AChE active box (Figure 3, Table 4).

Table 1. ^{1}H-NMR, ^{13}C-NMR data of 6-*O*-methyl, 1-glucopyranosyl catalpol (***1***), 6-*O*-α-L (3"-*O*- *trans*, 4"-*O*- *trans* cinnamoyl)-rhamnopyranosyl catalpol (***2***) and scropolioside D (***3***) *.

	δ H			δ C		
	Compound 1	Compound 2	Compound 3	Compound 1	Compound 2	Compound 3
1	5-5.2	4.80 d (7.8)	4.89 d (9.4)	93.94	95.14	93.72
2	-	_	_	-	_	_
3	6.38 dd (6, 1.6)	6.43 dd (1.4, 6)	6.42 dd (1.4, 6)	140.65	141.74	141.09
4	5-5.2	5.13 dd (6, 1.4)	5.04 dd (6, 4.5)	102.62	102.41	101.76
5	2.34 m	2.57m	2.51m	35.93	36.01	35.73
6	-	4.08 d (8)	4.09 d (8)	87.03	84.20	83.42
7	3.78 brs	3.73 brs	3.79 brs	56.87	57.88	57.99
8	-	_	_	65.35	65.34	65.14
9	2.59 dd (1.6, 7.6)	2.65 dd (9.5,8)	2.60 dd (9.4,8)	41.70	42.70	41.85
10	3.85, 4.19 dd (13.2, 13.2)	4.21 dd (11,1.6)	4.20,385 d (13)	61.27	58.56	59.98
11	3.49 s	-	-	58.05	-	-
1'	4.81 d (7.6)	4.80 d (7.9)	4.83 d (7.8)	98.27	99.42	98.29
2'	3.1-3.4 m	3.36-3.49m	3.36-3.49m	73.28	73.26	73.41
3'	3.1-3.4 m	3.36-3.49m	3.36-3.49m	76.97	77.50	77.23
4'	3.1-3.4 m	3.36-3.49m	3.36-3.49m	70.13	70.94	70.85
5'	3.1-3.4 m	3.36-3.49m	3.36-3.49m	76.09	76.38	76.25
6'	3.68, 3.94 dd (12, 12)	3.77-3.87m	3.77_4.2 m	6.02	61.43	61.57
1''	_	5.14 d (1.5)	5.13d(1.5)	_	95.68	96.25
2''	_	5.41 dd (1.8,3.4)	5.31brs	_	69.41	69.35
3''	_	5.52 dd (6.8,10)	5.39 d (9.6)	_	70.11	70.37
4''	_	5.34 t (9.8)	5.16 dd (9)	_	68.78	68.89
5''	_	_	_	_	66.54	66.63
6''	_	1.28 d (6.2)	1.21 d (6)	_	16.40	16.30
7''	_	_	_	_	170.05	170.21
8''	_	_	2.10 s	_	19.58	19.33
9''	_	_	_	_	_	170.43
10''	_	_	1.99 s	_	_	19.33
1'''	_	_	_	_	130.81	130.41
2'''	_	7.48m	7.48m	_	128.08	128.00
3'''	_	7.34m	7.34m	_	128.45	128.67
4'''	_	7.34m	7.34m	_	134.01	134.07
5'''	_	7.34m	7.34m	_	128.45	128.67
6'''	_	7.48m	7.48m	_	128.08	128.00
7'''	_	7.76 d (16)	7.69 d (16)	_	144.87	145.97
8'''	_	6.56 d (16)	6.48 d (16)	_	116.39	116.48
9'''	_	_	_	_	166.41	165.82
1''''	_	_	_	_	130.51	_
2''''	_	7.48m	_	_	128.08	_
3''''	_	7.34m	_	_	128.45	_
4''''	_	7.34m	_	_	134.01	_
5''''	_	7.34m	_	_	128.45	_
6''''	_	7.48m	_	_	128.08	_
7''''	_	7.66 d (16)	_	_	144.87	_
8''''	_	6.43 d (16)	_	_	116.39	_
9''''	-	_	_	-	165.87	_

* Chemical shift of protons and carbons were assessed based on HMBC data.

The antioxidant abilities of the iridoids were evaluated by different test systems including free radical scavenging (DPPH and ABTS), reducing power (CUPRAC and FRAP), phosphomolybdenum and metal chelating assays. The results of antioxidant capacity assays were evaluated using IC_{50} (DPPH, ABTS and metal chelating) and EC_{50} (phosphomolybdenum, CUPRAC and FRAP) values. The lower IC_{50} and EC_{50} values indicate stronger antioxidant activity. The results are given in Table 5 and Figure 4. 6-*O*-α-L (3"-*O*- *trans*, 4"-*O*- *trans* cinnamoyl)-rhamnopyranosyl catalpol (EC_{50}: 0.79±0.02 mg/mL) and Scropolioside D (EC_{50}: 1.37±0.03 mg/mL) showed comparable anti-oxidant effects with the Trolox as the control (EC_{50}: 0.55±0.09 mg/mL) in phosphomolybdenum assay. But, 6-*O*-methyl, 1-glucopyranosyl catalpol showed acceptable activities in ABTS radical scavenging (IC_{50}: 2.57±0.01 mg/mL) and Phosphomolybdenum assays (IC_{50}: 3.89±0.06 mg/mL) compared to the control (IC_{50}: 0.44±0.05 mg/mL and EC_{50}: 0.55±0.09 mg/mL, respectively).

Table 2. HMBC data of 6-*O*-methyl, 1-glucopyranosyl catalpol (***1***), 6-*O*-α-L (3"-*O*- *trans*, 4"-*O*- *trans* cinnamoyl)-rhamnopyranosyl catalpol (***2***) and scropolioside D (***3***).

	^{1}H-NMR	^{13}C-NMR	$^{2}J_{CH}$	$^{3}J_{CH}$	^{1}H-NMR	^{13}C-NMR	$^{2}J_{CH}$	$^{3}J_{CH}$
			Compound *3*				**Compound *2***	
1	4.89 d (9.4)	93.72	C-9	C-8, C-1'	4.80 d (7.8)	95.14		C-1'
2	_	_	_	_	_	_		
3	6.42 dd (1.4, 6)	141.09	C-4	C-1	6.43 dd (1.4, 6)	141.74	C-4	C-1, C-5
4	5.04 dd (6, 4.5)	101.76	C-3, C-5	C-9	5.13 dd (6, 1.4)	102.41	C-3, C-5	C-9
5	2.51 m	35.73	C-9, C-5, C-4	C-1	2.57 m	36.01	C-9	
6	4.09 d (8)	83.42	C-5, C-7	C-1'', C4, C-8	4.08 d (8)	84.20	C-5, C-7	C-1'', C-4
7	3.79 brs	57.99	C-8, C-6	C-9, C-5	3.73 brs	57.88	C-6	C-5
8	_	65.14	_	_	_	65.34		
9	2.60 dd (9.4,8)	41.85	C-5, C-1, C-8	C-7, C-6	2.65 dd (9.5,8)	42.70	C-8, C-1	C-7, C-6
10	4.20,385 d (13)	59.98	C-8	C-9, C-7	4.21dd (11,1.6)	58.56		
1'	4.83 d (7.8)	98.29	C-2'	C-1, C3'	4.80 d (7.9)	99.42		C-1
2'	3.36-3.49m	73.41	_	_	3.36-3.49m	73.26	_	_
3'	3.36-3.49m	77.23	_	_	3.36-3.49m	77.50	_	_
4'	3.36-3.49m	70.85	_	_	3.36-3.49m	70.94	_	_
5'	3.36-3.49m	76.25	_	_	3.36-3.49m	76.38	_	_
6'	3.77 4.2 m	61.57	_	_	3.77-3.87m	61.43	_	_
1''	5.13 d (1.5)	96.25	_	C-6, C-5''	5.14 d (1.5)	95.68	C-2''	C-6, C-5''
2''	5.31 brs	69.35	C-3'', C1'' C-9''	C-4''	5.41 dd (1.8,3.4)	69.41	C-7'', C-3''	C-4''
3''	5.39 d (9.6)	70.37	C-2'', C9'''	C-5''	5.52 dd (6.8,10)	70.11	C-2'', C9'''	_
4''	5.16 dd (9)	68.89	C-5'', C-3''	C-6'', C-7''	5.34 t (9.8)	68.78	C-5'', C-3''	C-6'', C-9''''
5''	_	66.63	_	_	_	66.54	_	_
6''	1.21 d (6)	16.30	C-5''	C-4 ''	1.28 d (6.2)	16.40	C-5''	C-4 ''
7''	_	170.21	_	_	_	170.05	_	_
8''	2.10 s	19.33	C-7''	_	2.14 s	19.58	C-7''	_
9''	_	170.43	_	_	_	_	_	_
10''	1.99 s	19.33	C-9''	_	_	_	_	_
1'''	_	130.41	_	_	_	130.81	_	_
2'''	7.48m	128.00	C-3'''	_	7.48m	128.08	C-3'''	_
3'''	7.34m	128.67	C-2''', C-4'''	_	7.34m	128.45	C-2''', C-4'''	_
4'''	7.34m	134.07	C-5'''	C-2'''	7.34m	134.01	C-5'''	C-2'''
5'''	7.34m	128.67	C-6''', C-4'''	_	7.34m	128.45	C-6''', C-4'''	_
6'''	7.48m	128.00	C-5'''	C-7'''	7.48m	128.08	C-5'''	C-7'''
7'''	7.69 d (16)	145.97	C-1''', C-8'''	C-9'''	7.76 d (16)	144.87	C-1''', C-8'''	C-9'''
8'''	6.48 d (16)	116.48	C-9''', C-7'''	C-3'', C-1'''	6.56 d (16)	116.39	C-9''', C-7'''	C-3'', C-1'''
9'''	_	165.82	_	_	_	166.41	_	_
1''''	_	_	_	_	_	130.51	_	_
2''''	_	_	_	_	7.48m	128.08	C-3''''	_
3''''	_	_	_	_	7.34m	128.45	C-2'''', C-4''''	_
4''''	_	_	_	_	7.34m	134.01	C-5''''	C-2''''
5''''	_	_	_	_	7.34m	128.45	C-6'''', C-4''''	_
6''''	_	_	_	_	7.48m	128.08	C-5''''	C-7''''
7''''	_	_	_	_	7.66 d (16)	144.87	C-1'''', C-8''''	C-9''''
8''''	_	_	_	_	6.43 d (16)	116.39	C-9'''', C-7''''	C-1''''
9''''	_	_	_	_	_	165.87	_	_

The iridoids exhibited very low DPPH scavenging activity (IC_{50} value: >5 mg/mL for compound 1) and two of them were not active on DPPH. Lack of antioxidant activity in DPPH radical scavenging activity was previously observed in many studies on iridoids [16, 31-32]. The DPPH radical scavenging activity mechanistically depends on the proton-donating ability of the chemical compounds to the DPPH radical. One of the effective parts in any chemical structure is the nucleophilic core like the hydroxyl group that these parts consist of the impressive role in DPPH radical scavenging activity [33]. Based on this, the presence of hydroxyl groups by substitution on the aromatic ring may neutralize free radicals. The absence of these nucleophilic cores could reduce the antioxidant potential of compounds in the DPPH assay [34]. Thus, isolated iridoids do not have any significant DPPH radical scavenging potential. The absence of phenol functional groups in their structural body may explain this situation. The importance of phenol functional groups in DPPH radical scavenging activity has been also shown in 8-*O*-Caffeoyl massenoside isolated from *Caryopteris odorata* [35].

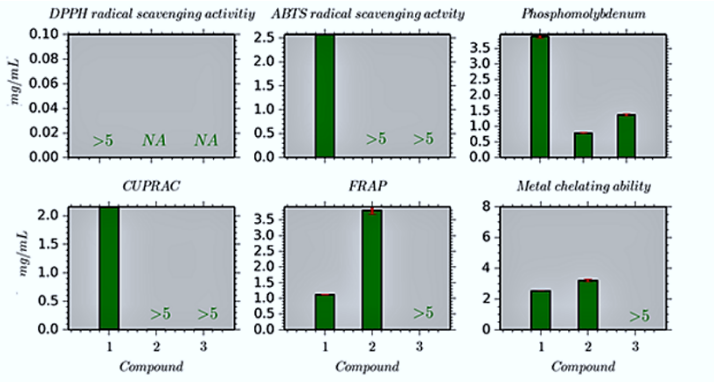

Figure 4. Antioxidant abilities of 6-*O*-methyl, 1-glucopyranosyl catalpol (***1***), 6-*O*-α-L (3"-*O*- *trans*, 4"-*O*- *trans* cinnamoyl)-rhamnopyranosyl catalpol (***2***) and scropolioside D (***3***). The figure indicates EC_{50} values for CUPRAC, FRAP and phosphomolybdenum assays and IC_{50} values for DPPH, ABTS and metal chelating assays. Values expressed are means ±SD. NA: not active.

Consideration of metal chelating ability, FRAP, CUPRAC, and ABTS radical scavenging activity of the isolated compounds

showed that 6-*O*-methyl, 1-glucopyranosyl catalpol has the highest activity between these iridoid glycosides (Table 5 and Figure 4). However, 6-*O*-α-L (3"-*O*- *trans*, 4"-*O*- *trans* cinnamoyl)-rhamnopyranosyl catalpol exerted the best activity in phosphomolybdenum assay, followed by scropolioside D and 6-*O*-methyl, 1-glucopyranosyl catalpol.

Table 3. AChE and BChE inhibitory effects of the 6-*O*-methyl (***1***), 1-glucopyranosyl catalpol, 6-*O*-α-L (3"-*O*- *trans*, 4"-*O*- *trans* cinnamoyl)-rhamnopyranosyl catalpol (***2***) and scropolioside D (***3***) (IC_{50}: mM).

Samples	AChE inhibition	BChE inhibition
Compound ***1***	1.17 ± 0.01*	1.12 ± 0.01
Compound ***2***	1.03 ± 0.01	1.10 ± 0.01
Compound ***3***	1.02 ± 0.02	1.06 ± 0.01
Galanthamine	0.01 ± 0.001	0.02 ± 0.001

*Values expressed are means ± SD.

Table 4. Various acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) receptor free binding energies calculated values (EFEB) and root-mean-square deviation of atomic positions (rmsd l.b. & u.b.) of ligands, 6-O-methyl, 1-glucopyranosyl catalpol (***1***), 6-O-α-L (3"-O- trans, 4"-O- trans cinnamoyl)-rhamnopyranosyl catalpol (***2***) and scropolioside D (***3***).

Receptors	PDB ligand	Comp. 1			Comp. 2			Comp. 3					
BChE	EFEB (kcal/mol)	rmsd l.b.	rmsd u.b.	EFEB (kcal/mol)	rmsd l.b.	rmsd u.b.	EFEB (kcal/mol)	rmsd l.b.	rmsd u.b.	EFEB (kcal/mol)	rmsd l.b.	rmsd u.b.
4bds	-7.5	1.194	2.885	-8.6	1.423	1.563	-	-	-	-	-	-
4tpk	-12.6	1.887	2.318	-9.5	1.965	-	-7.5	1.150	2.570	-8.1	0.000	0.000
5dyw	-15.0	0.000	0.000	-9.1	1.953	2.424	-7.3	1.133	2.422	-10.0	0.000	0.000
5klr	-7.0	0.000	0.000	-4.9	1.728	2.189	-7.1	1.854	1.871	-12.0	0.000	0.000
6eqp	-11.4	1.978	2.438	-9.5	1.907	-	-6.1	0.000	0.000	-	-	-
6f7q	-15.9	0.000	0.000	-9.6	1.906	-	-	-	-	-	-	-
6qae	-8.9	0.000	0.000	-9.5	1.995	-	-	-	-	-	-	-
AChE	EFEB (kcal/mol)	rmsd l.b.	rmsd u.b.	EFEB (kcal/mol)	rmsd l.b.	rmsd u.b.	EFEB (kcal/mol)	rmsd l.b.	rmsd u.b.	EFEB (kcal/mol)	rmsd l.b.	rmsd u.b.
1dx6	-10.6	0.000	0.000	-9.3	2.479	-	-	-	-	-	-	-
1oce	-8.0	1.276	1.327	-9.3	1.219	1.281	-	-	-	-	-	-
1qti	-11.0	0.000	0.000	-8.2	2.273	-	-	-	-	-	-	-
4bdt	-13.6	0.000	0.000	-5.2	0.000	0.000	-	-	-	-	-	-
4ey6	-10.9	0.000	0.000	-8.0	0.000	0.000	-	-	-	-	-	-
4ey7	-15.9	0.000	0.000	-7.5	1.992	3.000	-3.8	0.000	0.000	-	-	-
4m0e	-6.8	1.900	2.704	-6.8	1.987	-	-4.1	0.000	0.000	-	-	-
6cqy	-9.4	0.000	0.000	-7.7	1.233	1.430	-7.0	2.312	2.870	-8.4	0.000	0.000

Table 5. Antioxidant abilities of the isolated compounds (IC_{50} or EC_{50}: mg/mL)*.

Assays	Compound *1*	Compound *2*	Compound *3*	Trolox	EDTA
DPPH radical scavenging activity **	>5	NA	NA	0.20 ± 0.02	NT
ABTS radical scavenging activity **	2.57 ± 0.01	>5	>5	0.44 ± 0.05	NT
Phosphomolybdenum ***	3.89 ± 0.06	0.79 ± 0.02	1.37 ± 0.03	0.55 ± 0.09	NT
CUPRAC ***	2.15 ± 0.05	>5	>5	0.19 ± 0.01	NT
FRAP ***	1.12 ± 0.01	3.80 ± 0.12	>5	0.09 ± 0.01	NT
Metal chelating ability **	2.53 ± 0.01	3.20 ± 0.08	>5	NT	0.02 ± 0.01

* Values expressed are means ±SD. NA, not active. NT, not tested.
** All results were expressed as the IC_{50} value (mg/mL).
*** All results were expressed as the EC_{50} value (mg/mL).

Some studies have shown that iridoids exhibit antioxidant potentials. For example, 8-*O*-*trans*-cinnamoyl shanzhiside methylester and 8-*O*-Caffeoyl massenoside (isolated from *Caryopteris odorata)* have shown antioxidant potential in phosphomolybdenum and FRAP assays, respectively. In another study, three iridoid glycosides, 6'-*O*-*trans*-caffeoylnegundoside, 2'-*O*-*p*-hydroxybenzoyl- 6'-*O*-*trans*- caffeoylgardoside, and 2'-*O*-*p*-hydroxybenzoyl- 6'-*O*-*trans*- caffeoyl-8-*epi*-loganic acid (isolated from *Vitex altissima*) have shown antioxidant effects on superoxide free radical [35]. Similar activity has also been observed from asperuloside and catalpol in lipid peroxidation assay and FRAP test, respectively [31, 36]. Antioxidant activity estimation of the iridoids, utilizing a variety of methods, is needed to explain structure-activity relationships (SAR) of these natural compounds and elucidate the effects of substitutions on this relationship. Limited investigation has been conducted on SAR of iridoid antioxidant potential. Based on Gousiadou et al., minor iridoids from *Scutellaria albida* ssp. *albida* showed antioxidant activity on lipid peroxidation and DPPH assays [37]. This may be related to the number of hydroxyl and methoxy groups on the aromatic portion of molecule. Deacylation led to a decrease in potential, yet the acyl moiety position made no difference in this potential. The number of hydroxyl groups on the aromatic moiety did not increase activity regardless of the presence of free hydroxyl groups on the main iridoid cores. Non-rigidity could exhibit a moderate interaction potential with DPPH [37-40]. Other results suggest that the double bond in the iridoid skeleton did not increase the antioxidant activity of compounds [25].

To the best of our knowledge, the present study is the first report on the biological activity of 6-O-methyl, 1-glucopyranosyl catalpol, 6-O-α-L (3"-O- trans, 4"-O- trans cinnamoyl)-rhamnopyranosyl catalpol, and scropolioside D. Considering the moderate anti-oxidant and cholinesterase inhibition properties of these molecules and the possibility of the synergistic effects of these activities, the components could be valuable objects for more *in vivo* studies for designing new hit compounds in the pharmaceutical arena.

4. CONCLUSIONS

In this study, three iridoid glycosides were isolated from aerial parts of *S. amplexicaulis* and their potential neuroprotective properties were investigated. Overall, 6-*O*-methyl, 1-glucopyranosyl catalpol, 6-*O*-α-L (3"-*O*- *trans*, 4"-*O*- *trans* cinnamoyl)-rhamnopyranosyl catalpol and scropolioside D may serve neuroprotective effects by acceptable anti-oxidant properties and moderate cholinesterase inhibition effects.

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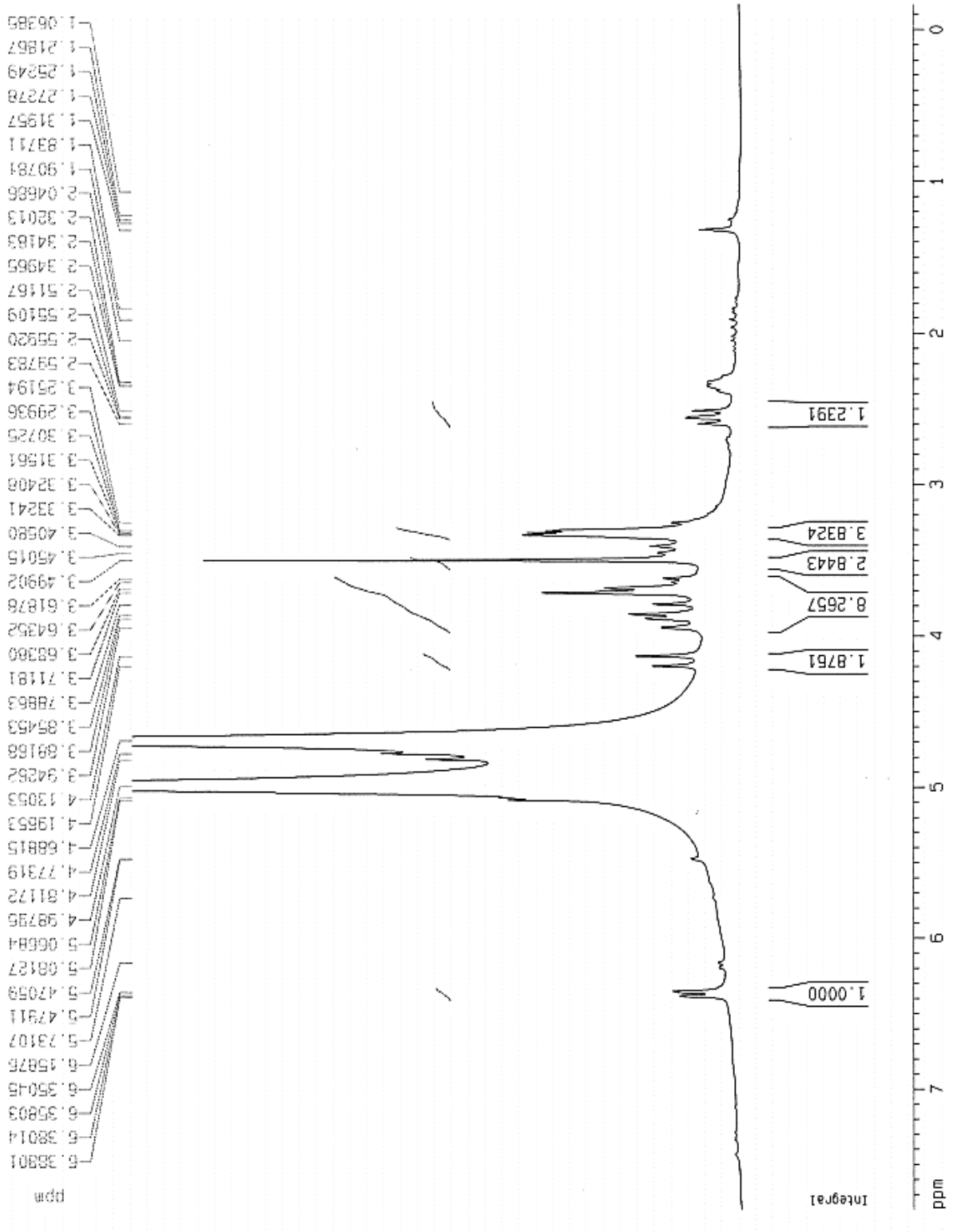
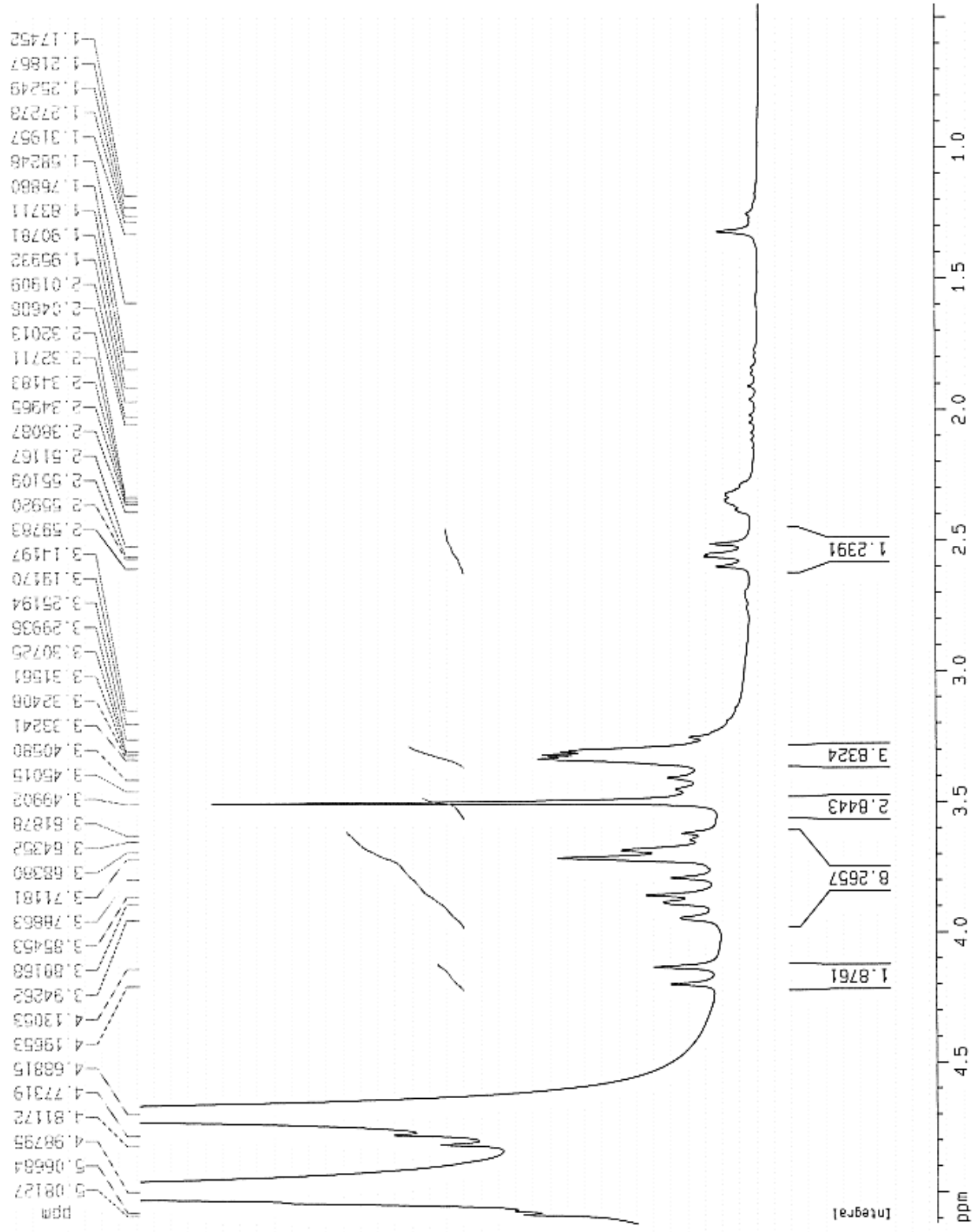
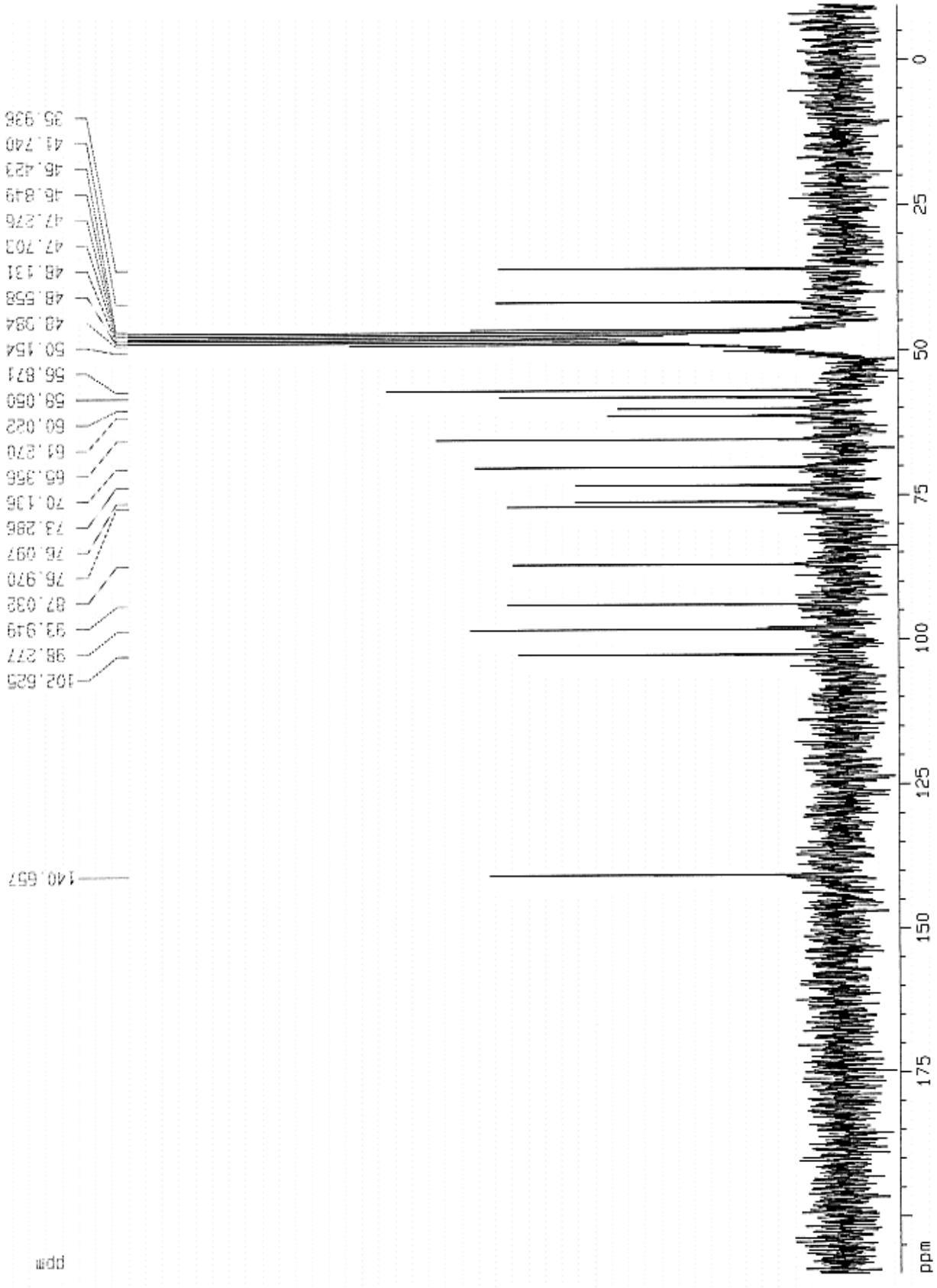
Supplementary materials

1D and 2D NMR spectrum of 6-*O*-methyl, 1-glucopyranosyl catalpol (*1*), 6-*O*-α-L (3"-*O*- *trans*, 4"-*O*- *trans* cinnamoyl)-rhamnopyranosyl catalpol (*2*) and scropolioside D (*3*):

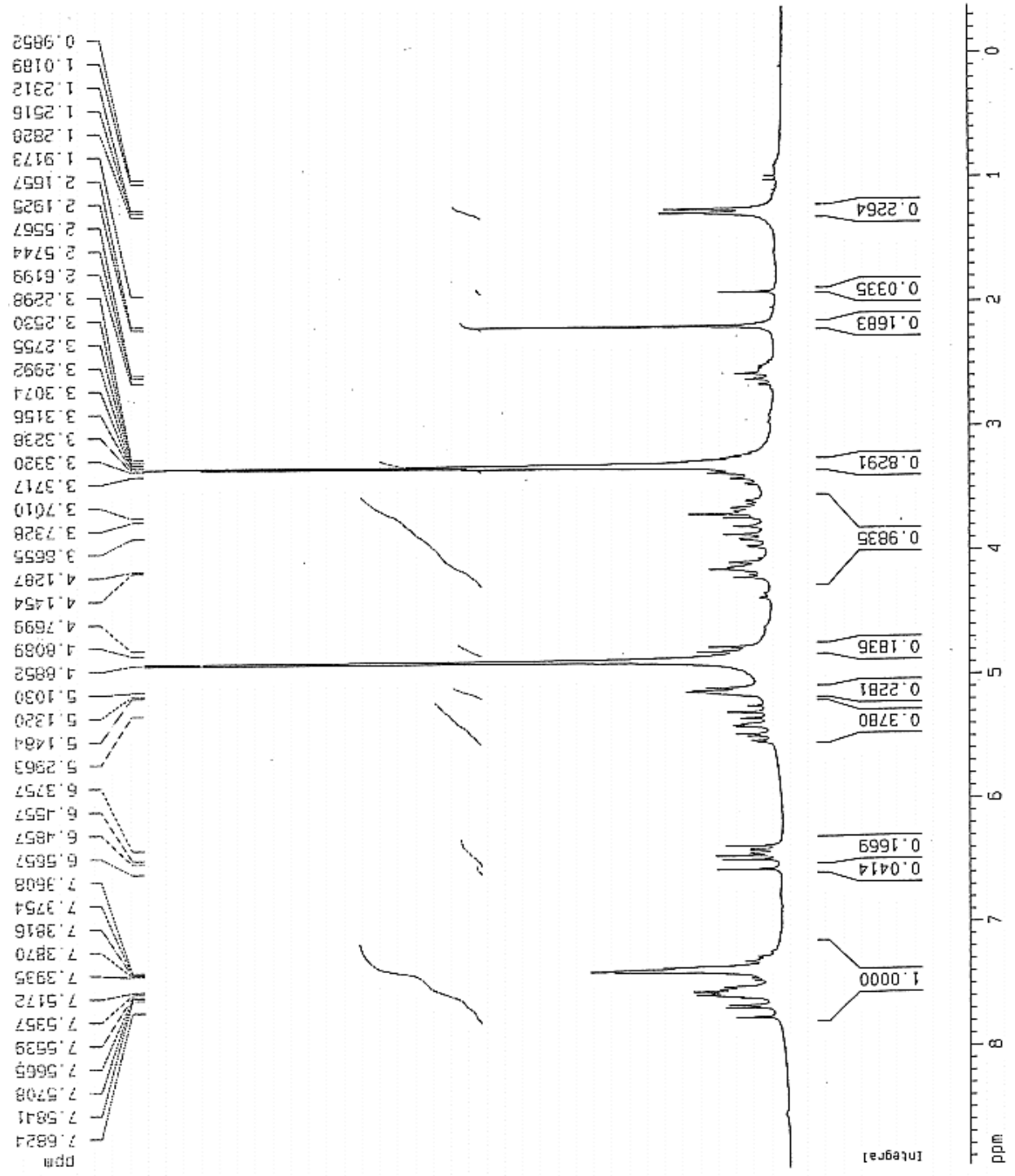
Compound ***1***. ^{1}H-NMR, ^{13}C-NMR spectrum of 6-*O*-methyl, 1-glucopyranosyl catalpol (***1***).

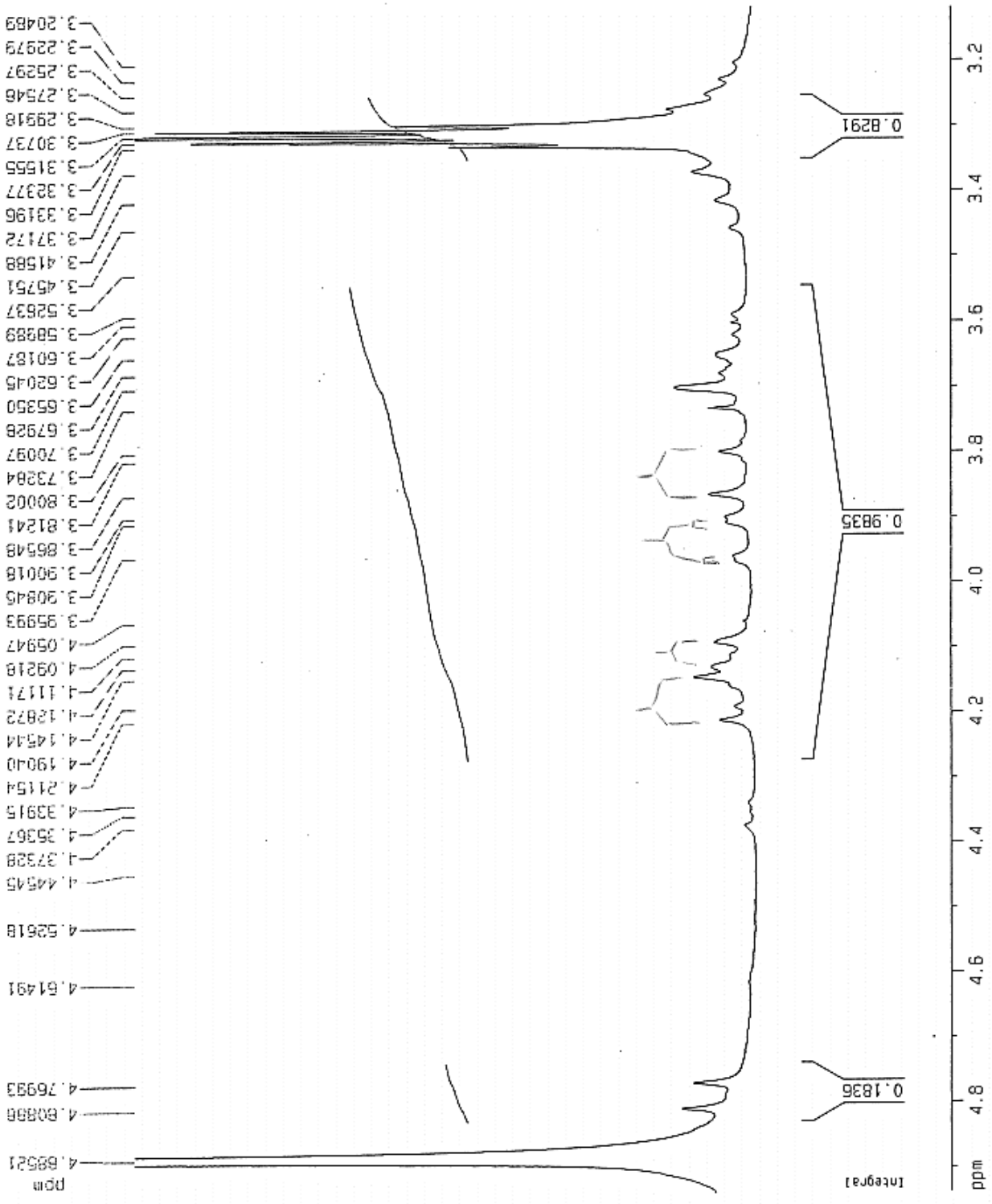


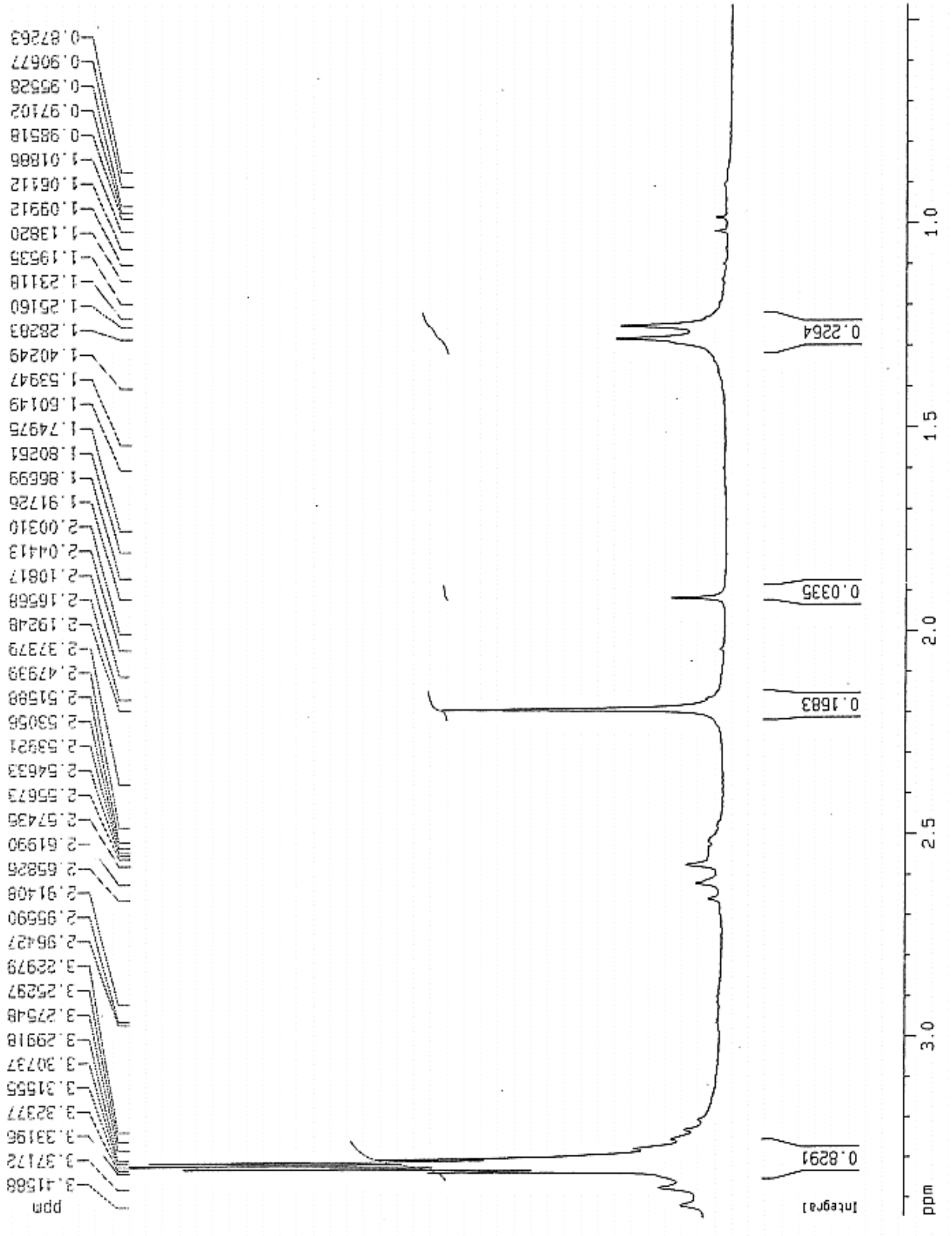

1.2391
3.8324
2.8443
8.2657
1.8761
Integral
ppm
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5

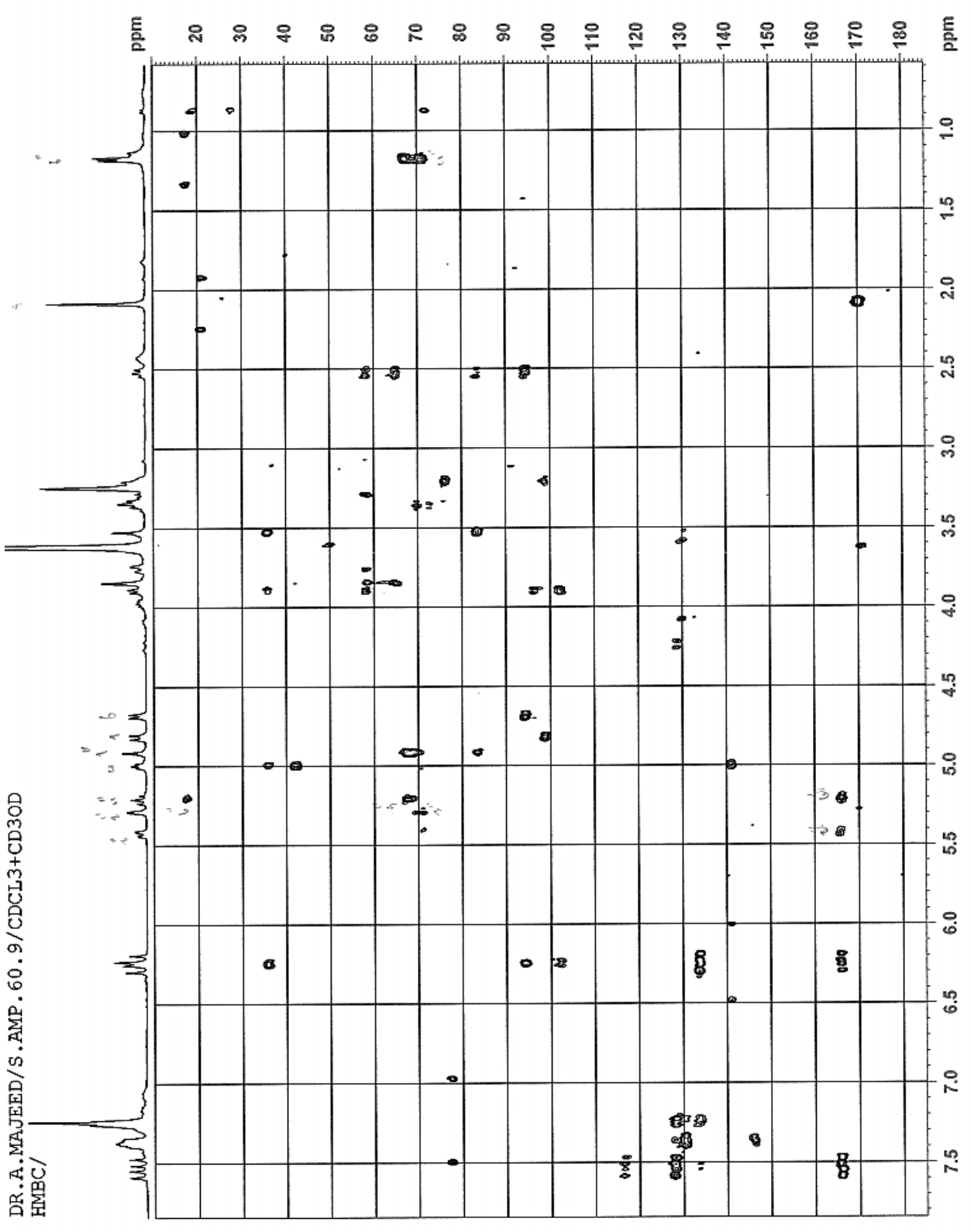

35.936
41.740
46.423
46.849
47.276
47.703
48.131
48.558
48.984
50.154
56.871
58.060
60.022
61.270
65.356
70.136
73.286
76.097
76.970
87.032
93.949
98.277
102.625
140.657
0
25
50
75
100
125
150
175
ppm

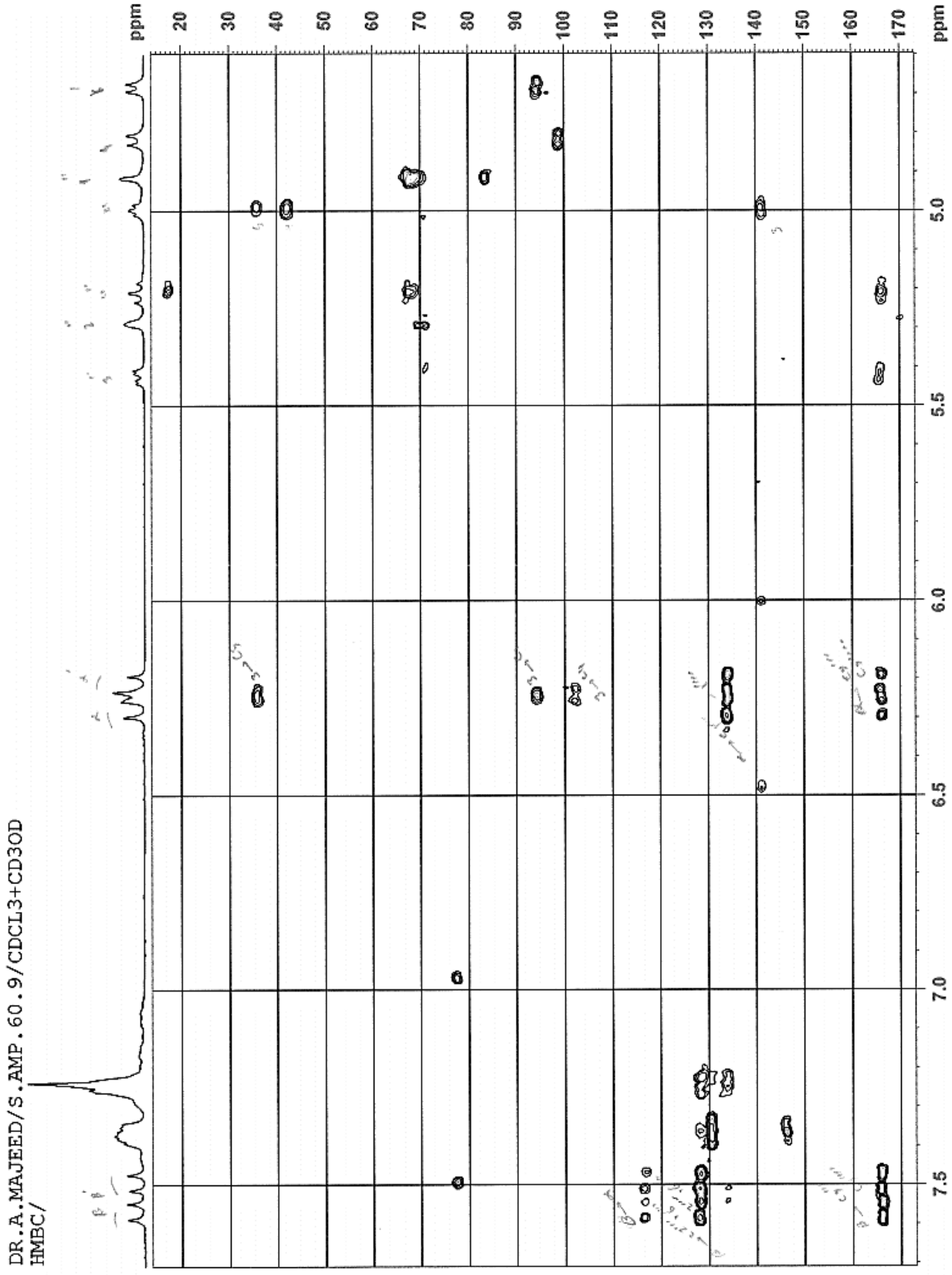
Compound ***2***. ^{1}H-NMR, HMBC spectrum of 6-*O*-α-L (3"-*O*- *trans*, 4"-*O*- *trans* cinnamoyl)-rhamnopyranosyl catalpol (***2***).

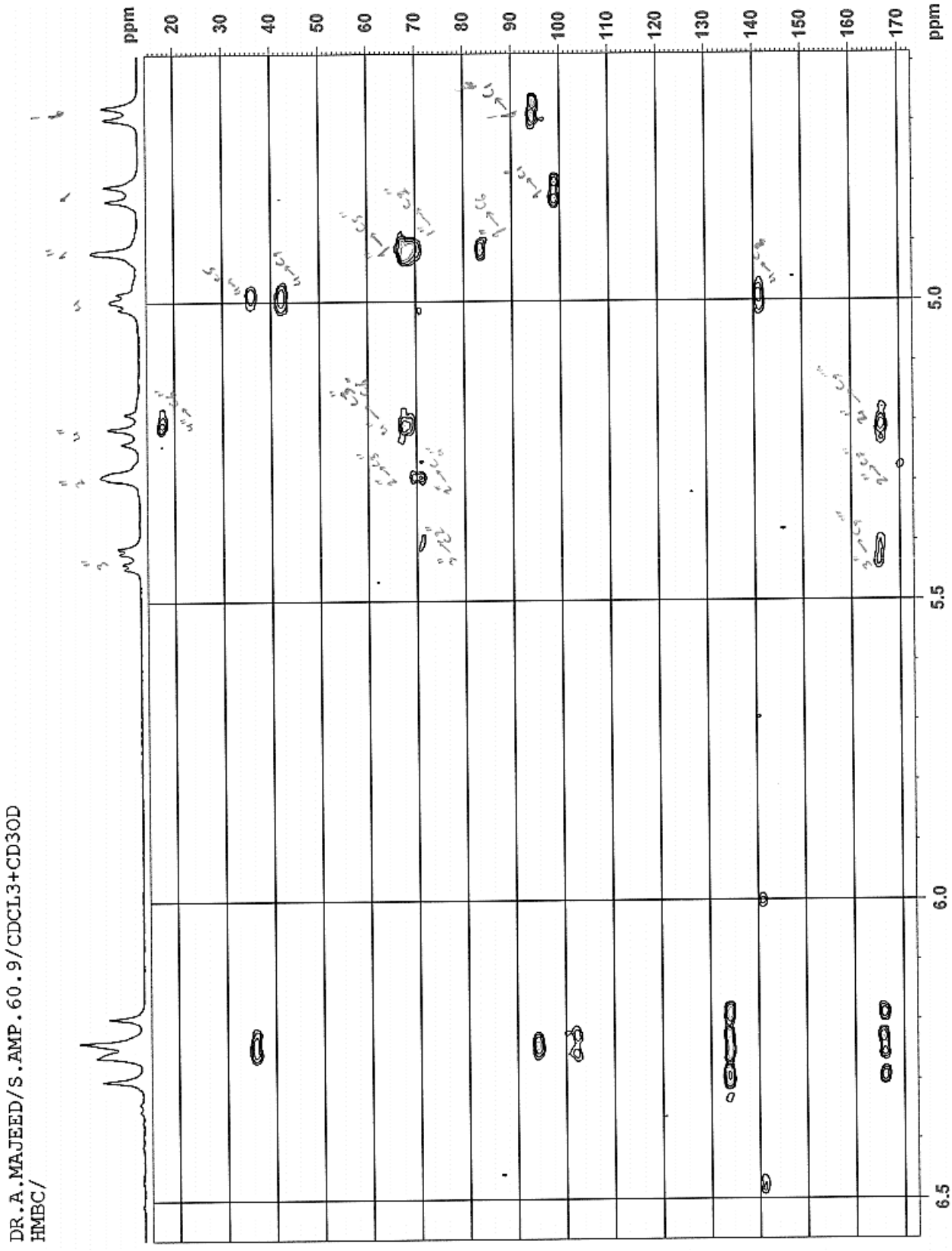


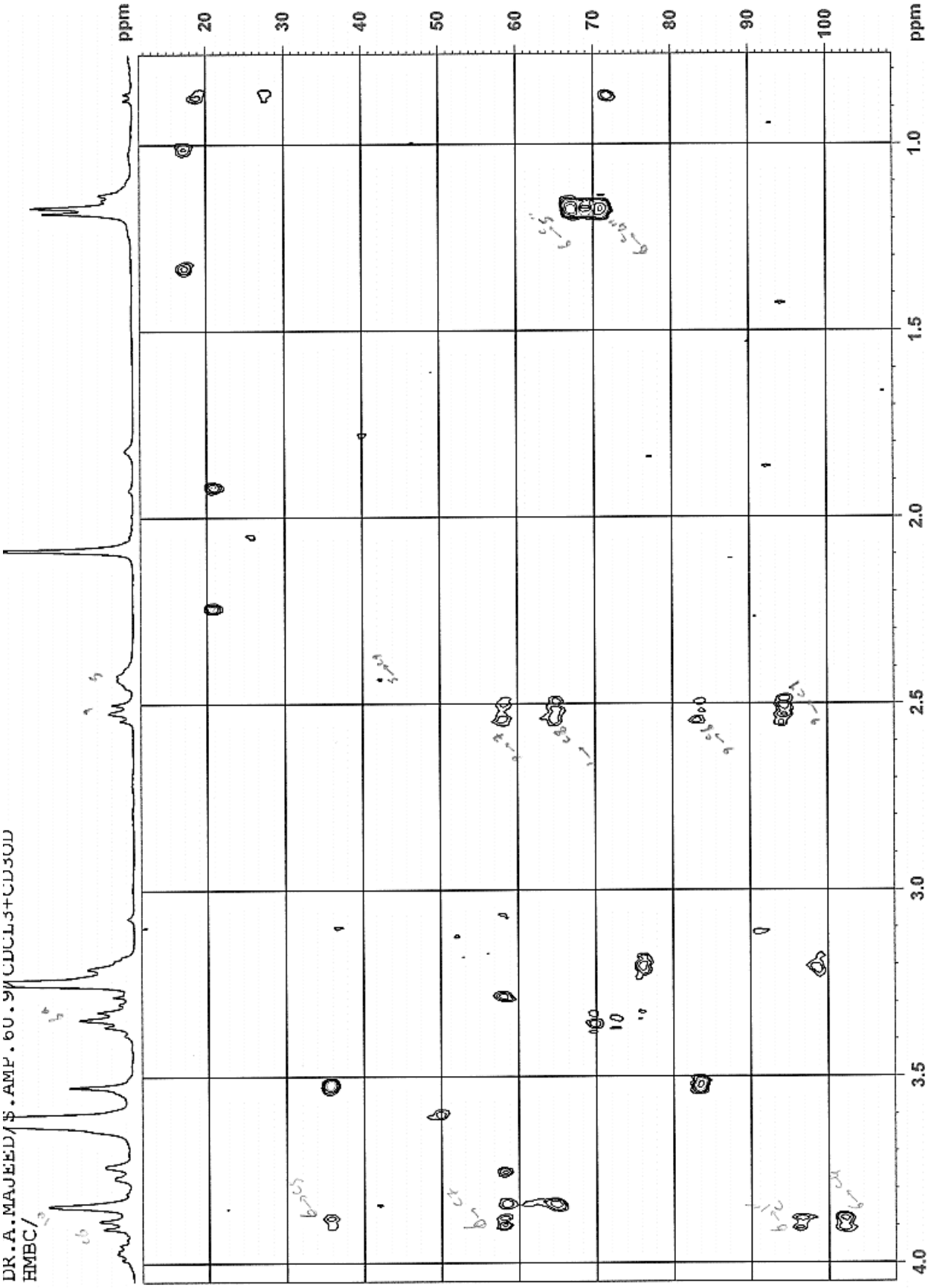

0.8291
0.9835
0.1836
integral
ppm
3.2
3.4
3.6
3.8
4.0
4.2
4.4
4.6
4.8


0.87263
0.90677
0.95528
0.97102
0.98518
1.01886
1.06112
1.09912
1.13820
1.19535
1.23118
1.25160
1.28203
1.40249
1.53947
1.60149
1.74975
1.80261
1.86699
1.91726
2.00310
2.04413
2.10817
2.16568
2.19248
2.37379
2.47939
2.51589
2.53056
2.53921
2.54633
2.55673
2.57435
2.61990
2.65825
2.91408
2.95590
2.96427
3.22979
3.25297
3.27548
3.29918
3.30737
3.31555
3.32377
3.33196
3.37172
3.41568
ppm
0.2264
0.0335
0.1683
0.8291
Integral
1.0
1.5
2.0
2.5
3.0
ppm

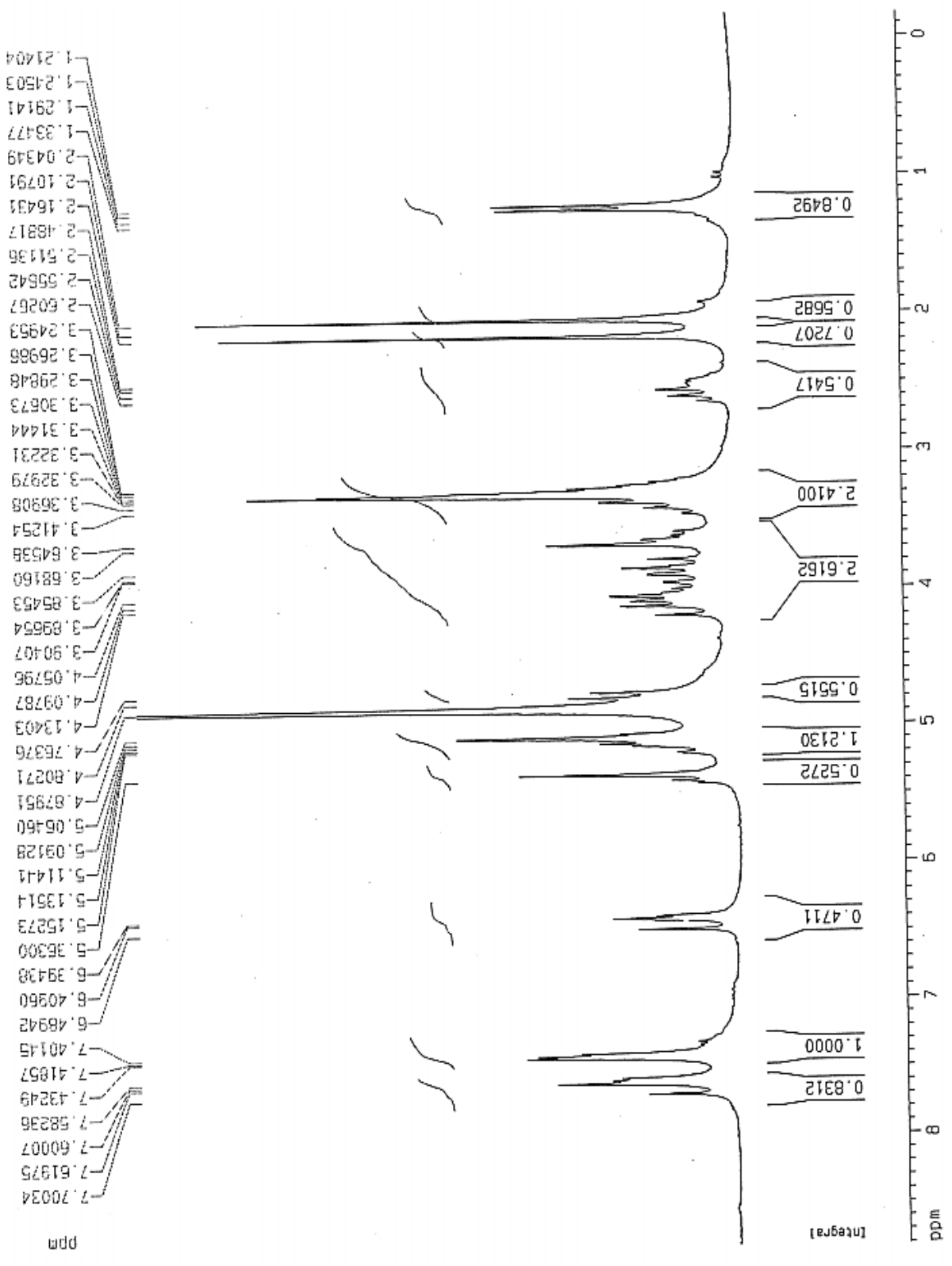

DR.A.MAJEED/S.AMP.60.9/CDCL3+CD3OD
HMBC/
ppm
ppm

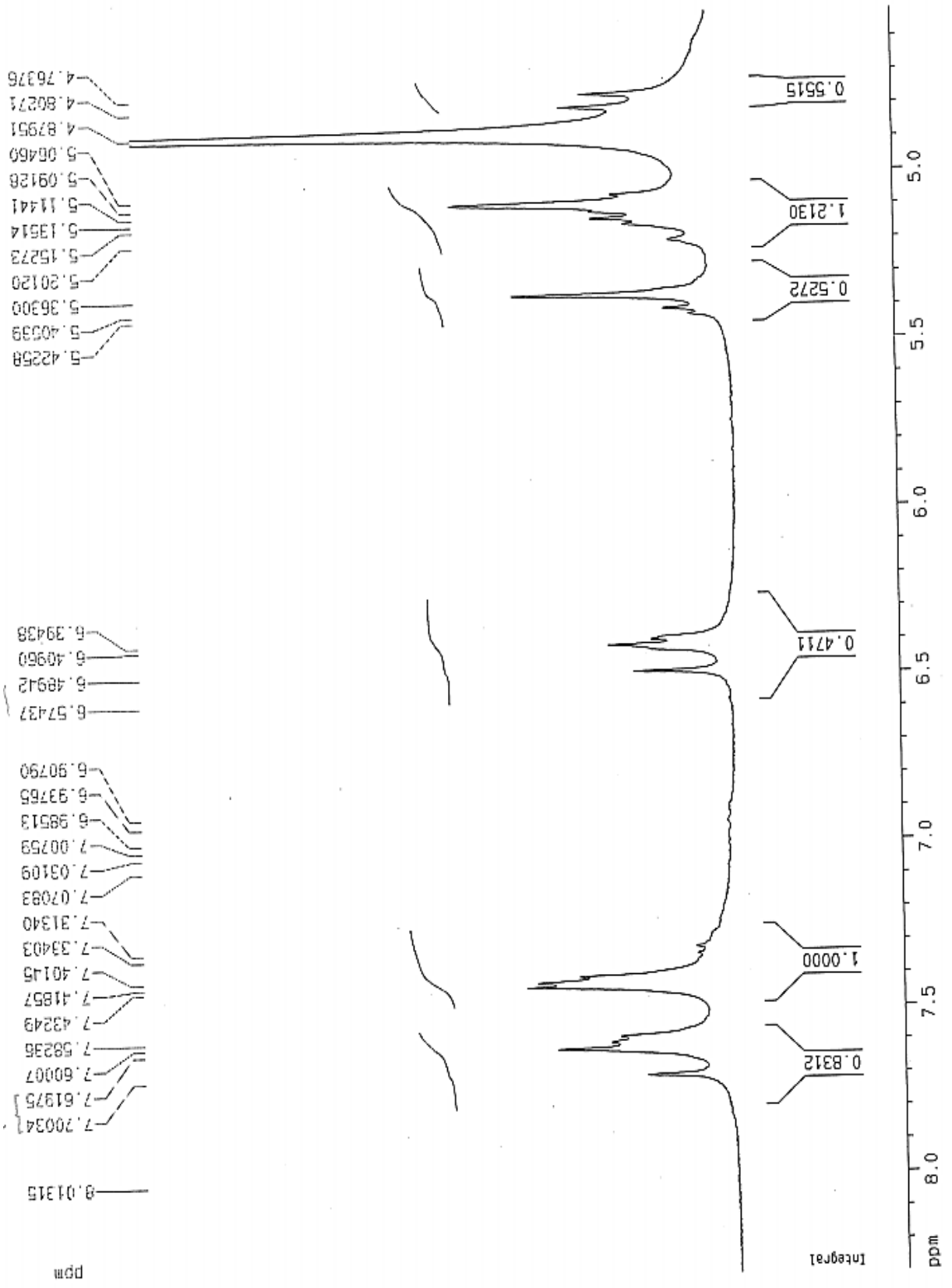

DR.A.MAJEED/S.AMP.60.9/CDCL3+CD3OD
HMBC/
ppm
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
ppm
5.0
5.5
6.0
6.5
7.0
7.5

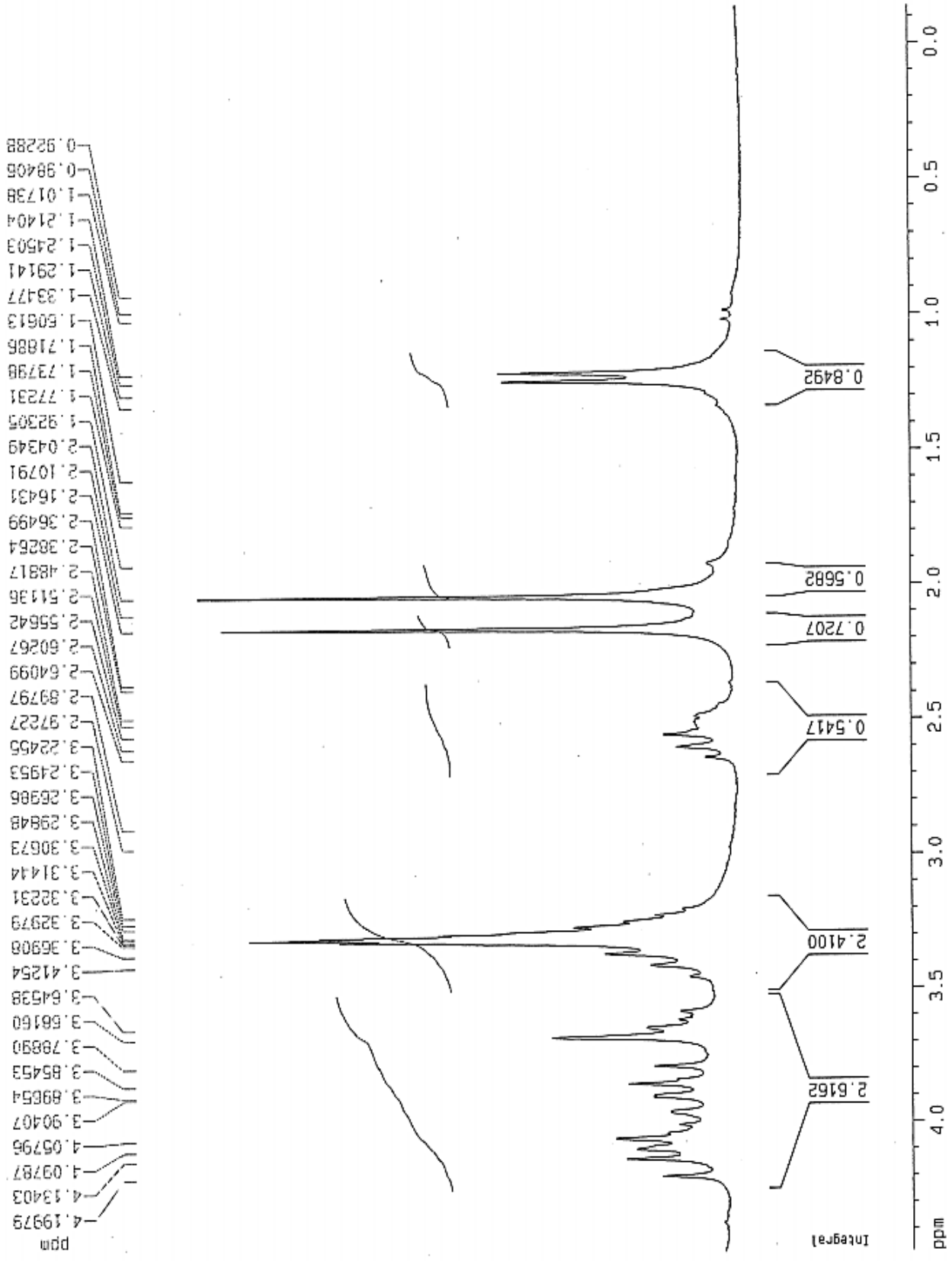

DR.A.MAJEED/S.AMP.60.9/CDCL3+CD3OD
HMBC/
ppm
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
ppm
5.0
5.5
6.0
6.5

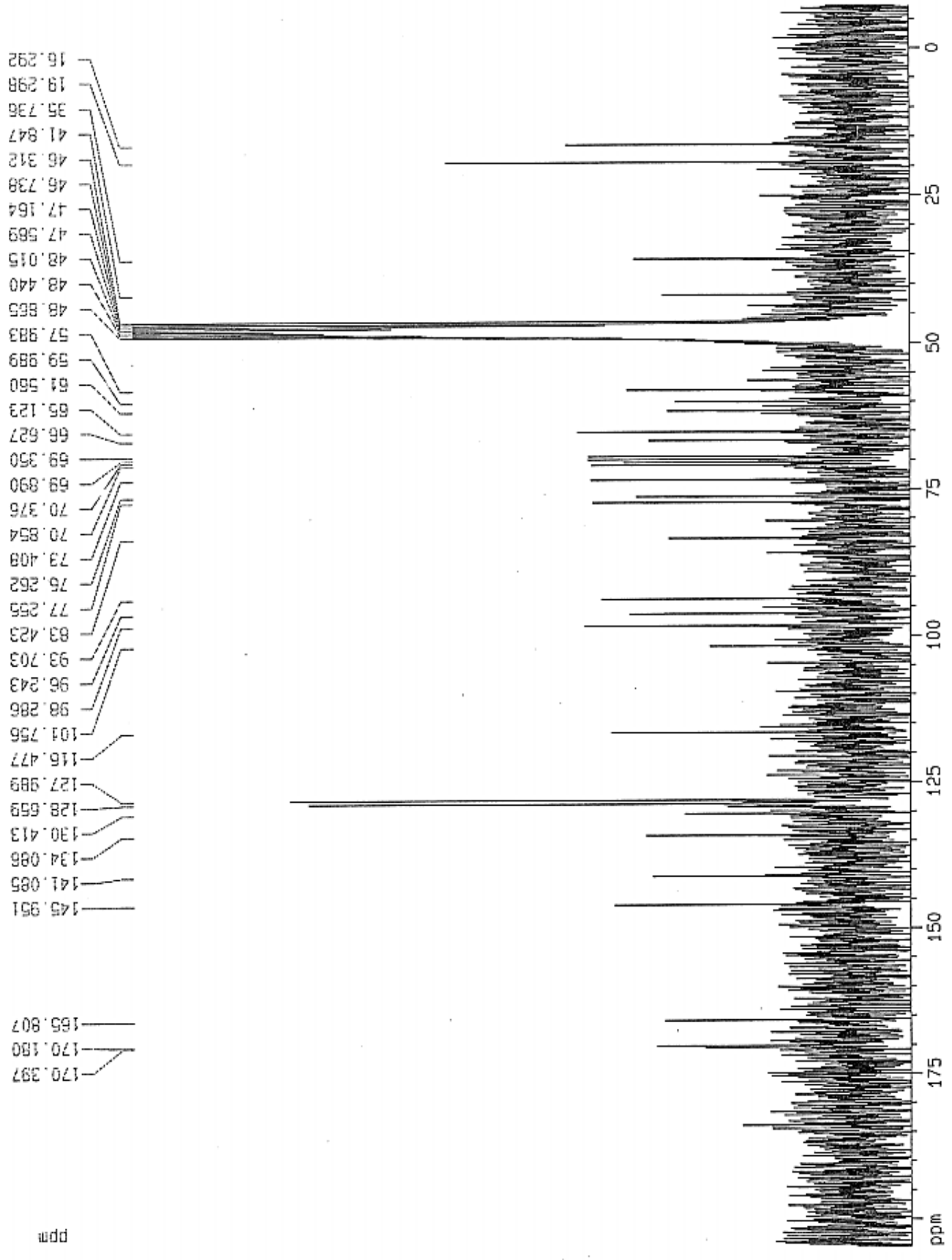

DR.A.MAJEED/$.AMP.60.9/CDCL3+CD3OD
HMBC/
ppm
20
30
40
50
60
70
80
90
100
ppm
1.0
1.5
2.0
2.5
3.0
3.5
4.0

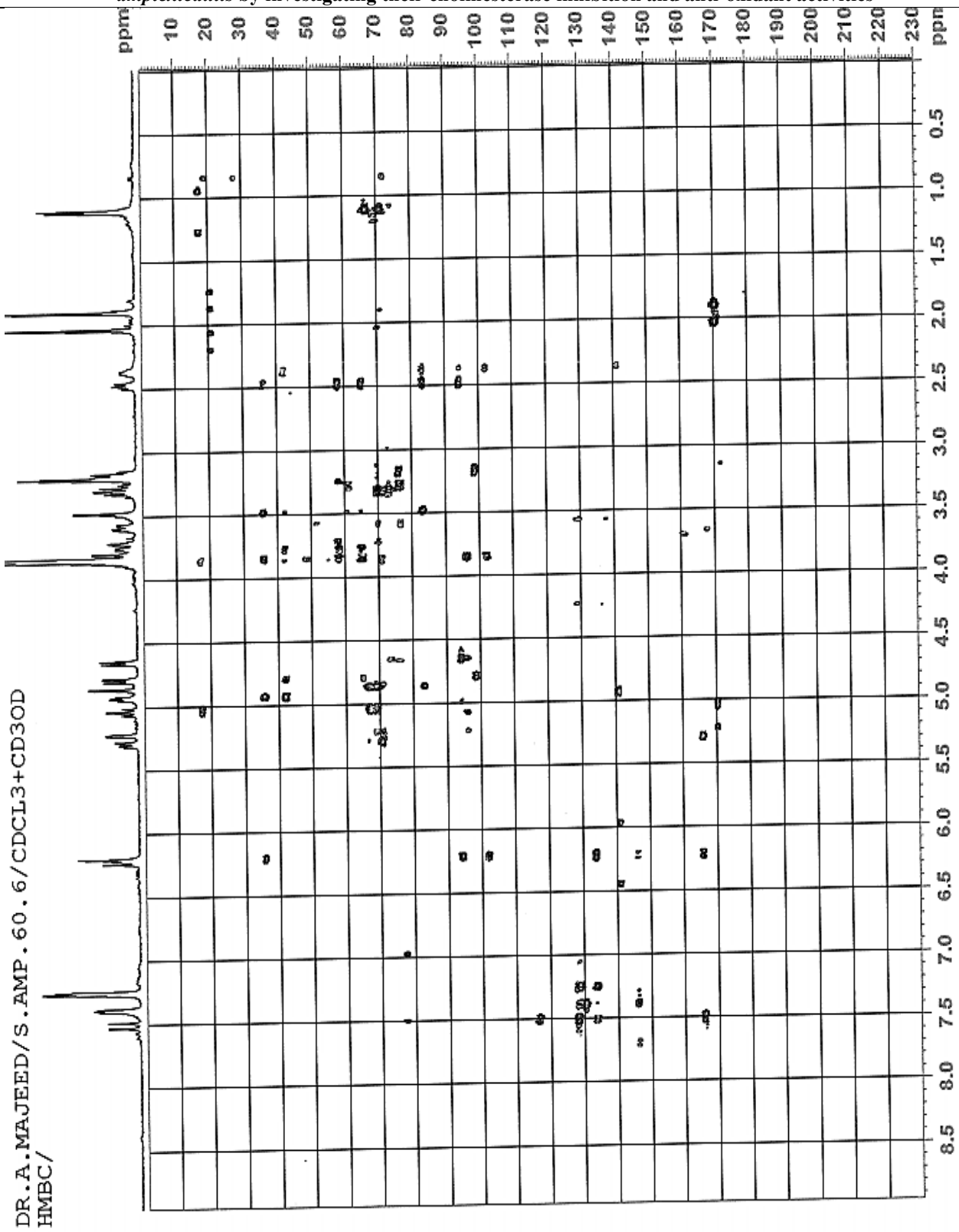
Compound ***3***. ^{1}H-NMR, ^{13}C-NMR, and HMBC spectrum of scropolioside D (***3***).

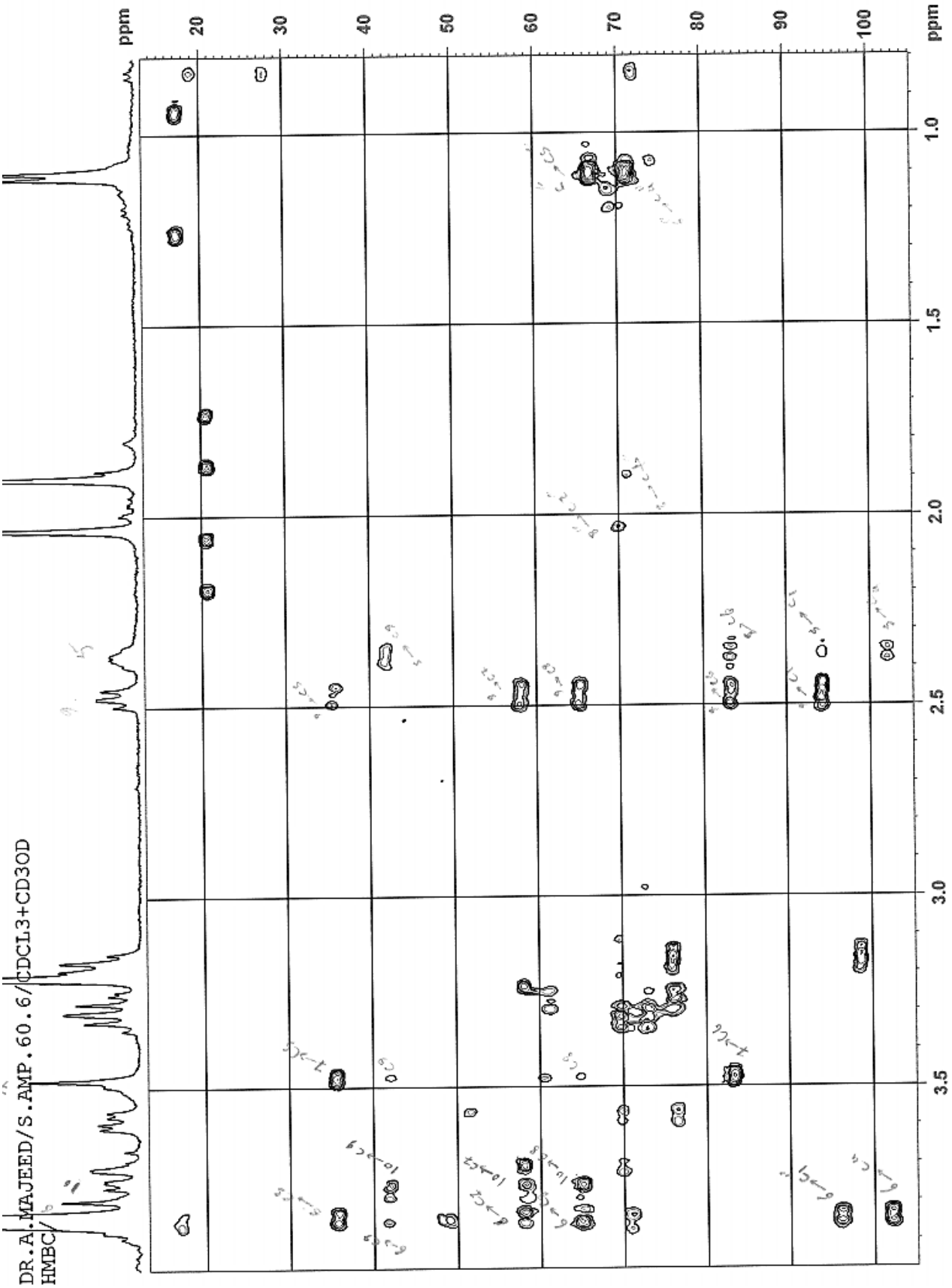


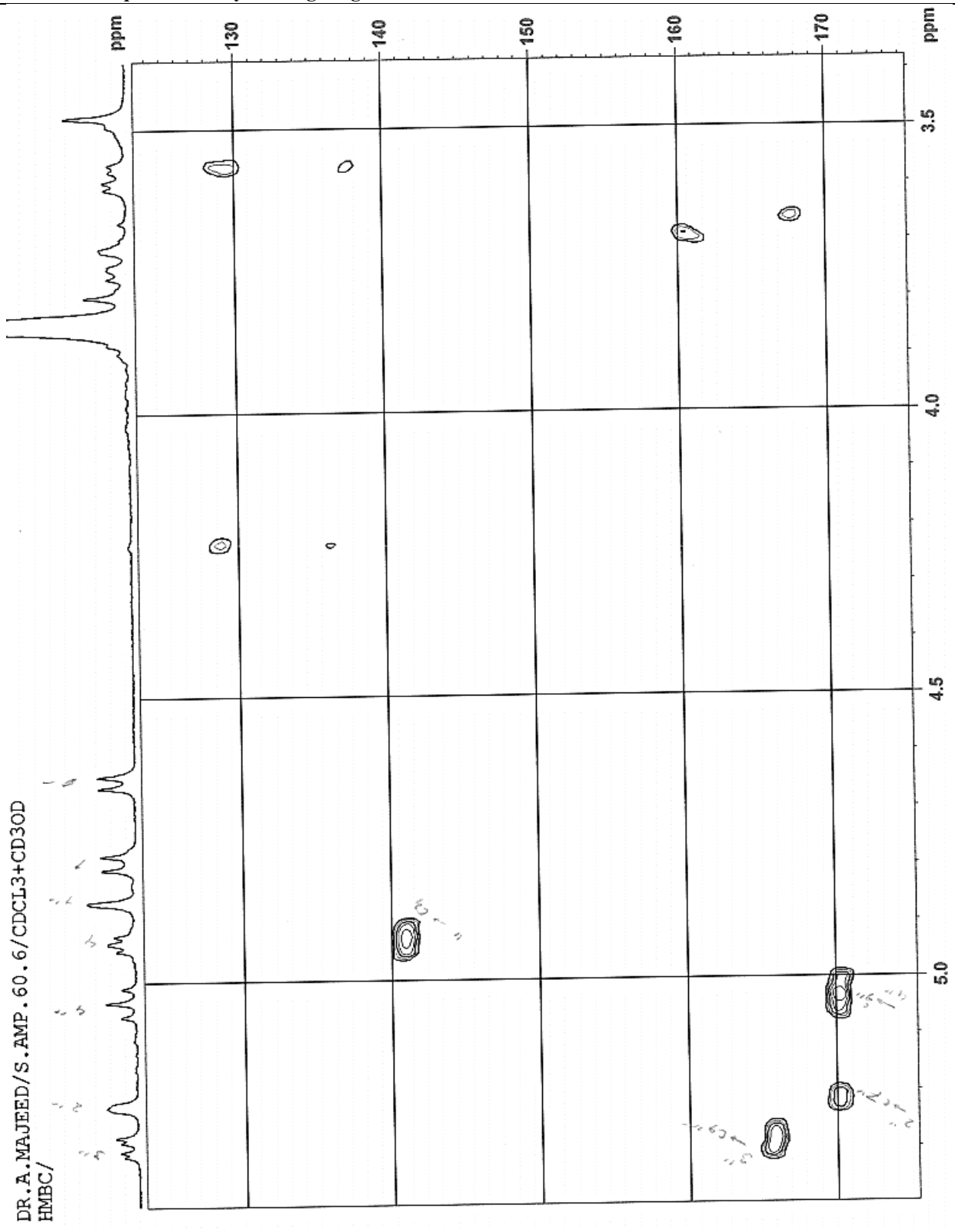

4.76376
4.80271
4.87951
5.00460
5.09128
5.11441
5.13514
5.15273
5.20120
5.36300
5.40539
5.42558
6.39438
6.40960
6.48942
6.57437
6.90790
6.93765
6.98513
7.00759
7.03109
7.07083
7.13140
7.33403
7.40145
7.41857
7.43249
7.58236
7.60007
7.61975
7.70034
8.01315
0.5515
1.2130
0.5272
0.4711
1.0000
0.8312
Integral
ppm
5.0
5.5
6.0
6.5
7.0
7.5
8.0
ppm

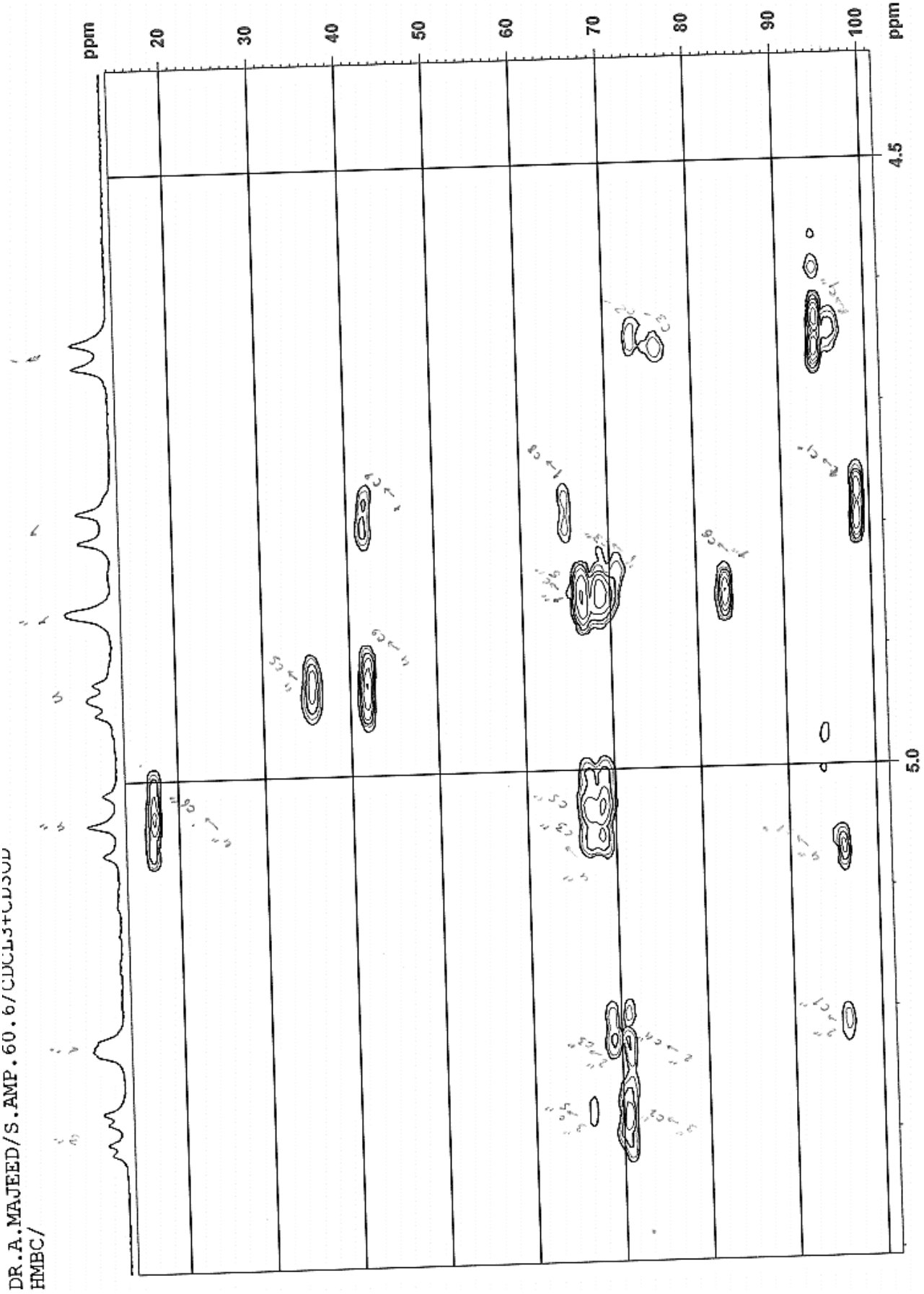

0.8492
0.5682
0.7207
0.5417
2.4100
2.6162
Integral
ppm
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0


16.292
19.298
35.736
41.847
46.312
46.738
47.164
47.589
48.015
48.440
48.865
57.983
59.989
61.560
65.123
66.627
69.350
69.890
70.376
70.854
73.408
76.262
77.255
83.423
93.703
96.243
98.286
101.756
116.477
127.989
128.659
130.413
134.086
141.085
145.951
165.807
170.180
170.397
0
25
50
75
100
125
150
175
ppm


DR.A.MAJEED/S.AMP.60.6/CDCL3+CD3OD
HMBC/
ppm
ppm


DR.A.MAJEED/S.AMP.60.6/CDCL3+CD3OD
HMBC
ppm
20
30
40
50
60
70
80
90
100
ppm
1.0
1.5
2.0
2.5
3.0
3.5


ppm
130
140
150
160
170
ppm
3.5
4.0
4.5
5.0
DR.A.MAJEED/S.AMP.60.6/CDCL3+CD3OD
HMBC/


ppm
20
30
40
50
60
70
80
90
100
ppm
4.5
5.0
DR.A.MAJEED/S.AMP.60.6/CDCL3+CD3OD
HMBC/