CHAPTER 4:

Isolation of compounds from *Elaeodendron transvaalense* extract

4.1 Introduction

Extracts from *Elaeodendron transvaalense* (Burtt Davy) (Celastraceae) have been used in traditional medicine by the Vhavenda people of South Africa (Limpopo province) to treat coughs, diarrhoea, stomach ailments, herpes and sexually associated diseases. Stem bark is mostly used to prepare infusions and decoctions (Mabogo, 1990). Other medicinal uses of *E. transvaalense* are shown in Table 4.1. Traditional healers prescribe it presently to people who are suffering from HIV/AIDS (Bessong *et al*., 2005). Dimethyl–1,3,8,10–tetrahydroxy–9- methoxypeltogynan and three pentacyclic triterpenes have been isolated from its bark which is also reported to contain 13.4 % catechol tannin (Hutchings,1996).

Other species belonging to the same family (Celastraceae) have been used in the Amazonian region against cancer, rheumatism and inflammation (Nakagawa *et al*., 2004). Previous reports have shown that species from the Celastraceae family contain biologically active metabolites with antimicrobial and cytotoxic activities (Sansores-Peraza *et al*., 2000). The aim of this part of study was to isolate compounds from *E. transvaalense* .

4.1.1 Plant description

E. transvaalense is a shrub or small tree (about 5 m high) occurring in forests and quite often on rocky outcrops in mountainous regions. The bark is generally smooth and has a grey colour. Leaves are often clustered on reduced lateral shoots, oblong in shape, about 50 mm long and 20 mm wide. The leaf margin is sometimes toothed. The flowers are greenish in colour and produce oblong, yellow to dark orange, berry-like fruits, which are edible (Figure. 4.1).

a **b**

Figure 4.1 (**a**) Bark and (**b**) branches of *E. transvaalense* (Van Wyk *et al.*, 1997).

The species is widely distributed in the north-eastern parts of South Africa. It also occurs along the coastal parts of KwaZulu-Natal and in Mpumalanga, Gauteng and the Limpopo province (Van Wyk, 2000).

4.1.2 Medicinal uses

E. transvaalense is used as remedy for several diseases (Table 4.11). The bark is extensively used for cleaning of the stomach and used as an enema for stomach ache, diarrhoea and fever (Mabberley, 1981).

Table 4.1: Other medicinal uses of *E. transvaalense*

Disease	Place	Preparation	References
Menorrhagia	Zimbabwe	Infusion	Gelfand, 1985
Haemorrhoids	South Africa	Unspecified	Hutchings , 1996
			Van Wyk & Gericke, 2000
Stomach cramps	South Africa	Decoction	Pujol, 1988
Diarrhoea	South Africa	Decoction	Mabberley, 1981
			Van Wyk, 1997
Herpes simplex	South Africa	Decoction	Felhabert, 1997
			Mabogo, 1990
Herpes zoster	South Africa	Decoction	Felhabert, 1997
Stroke	South Africa	Decoction	Felhabert, 1997
Gout	South Africa	Decoction	Felhabert, 1997
Rash	Southern Africa	Infusion	Van Wyk, 2000
HIV/AIDS	South Africa	Infusion	Bessong *et al.*, 2005

4.1.3 Chemistry

Elaeodendron species are rich in gallotannins, proanthocyanidins and a few other phenolic compounds like elaeocyanidin (Figure 4.2) have been isolated from this species (Van Wyk *et al.*, 1997).

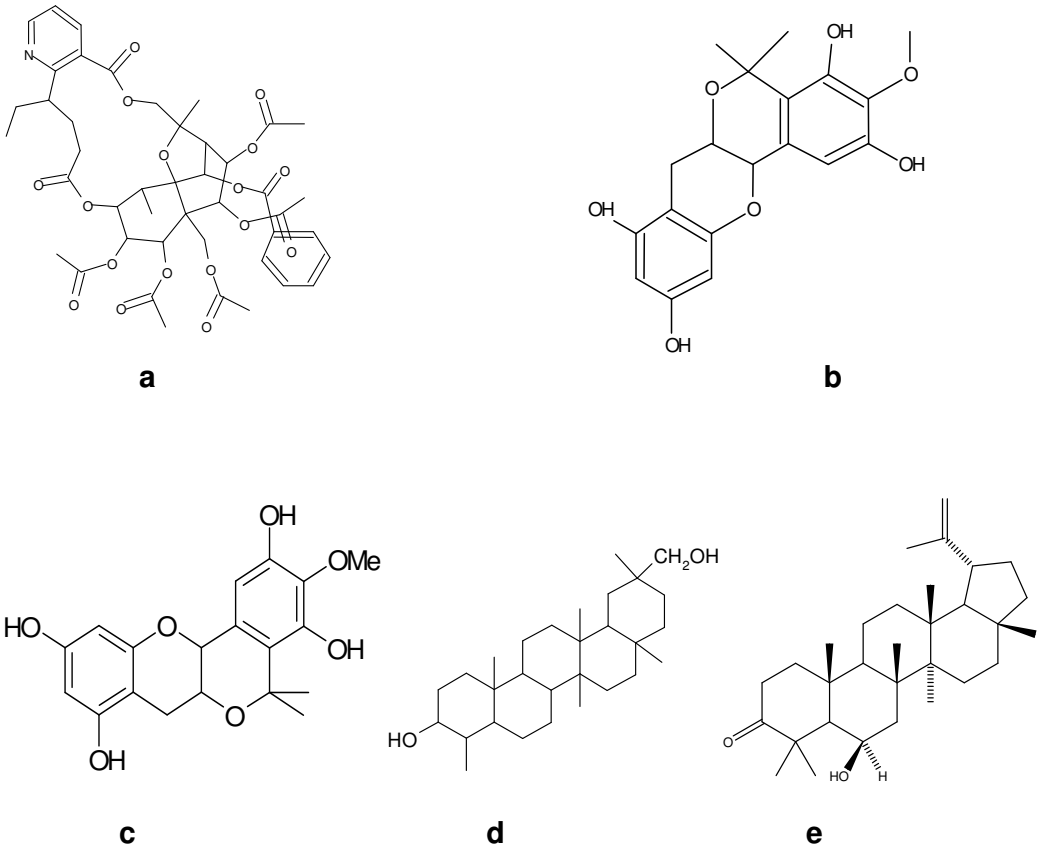


Figure 4.2 Compounds isolated from *E. transvaalense*: (**a**) cassinine, (**b**) elaeocyanidin, (**c**) 6R, 13R-11, 11-dimethyl-1, 3, 10-tetra-hydroxy-9-methoxypeltogynan, (**d**) canophyllol and (**e**) 6-β-hydroxy-lup-20(30)-en-3-one (Drewes & Mashimbye, 1993, Drewes *et al.*, 1991).

4.2 Materials and methods

4.2.1 Plant material

Stem bark of *E. transvaalense* was collected in Venda (Northern Limpopo). A voucher specimen is preserved in the HGWJ Schweickerdt herbarium at the University of Pretoria (Tshikalange 092524).

4.2.2 Preparation of extracts

In unreported results, different fresh extracts (acetone, chloroform, ethyl acetate and ethanol) of *E. transvaalense* were tested for their ability to inhibit NF-κB and Tat proteins. The ethanol extract exhibited the best activity and led us to isolate compounds from this extract.

Stem bark of *E. transvaalense* was collected and left to dry at room temperature for two weeks. The dried powder stem bark was placed in a container and soaked in ethanol. The container was closed and left in a dark cupboard for three days at room temperature before the extract was filtered and concentrated to dryness under reduced pressure (40 ^{0}C). The residue was soaked again in ethanol and filtered. The filtrates were dried with a rotary evaporator to give a total mass of 150 g (extract).

4.2.3 Isolation and identification of compounds

A 10 cm diameter glass column (Figures 4.3 and 4.4) was filled with 1.5 kg silica gel. The extract (120 g) was dissolved in a minimal amount of solvent

and mixed with 200 g silical gel. The column was eluted with a solvent gradient of hexane: ethyl acetate in 100:0 to 0:100 ratios. The column was then washed with ethyl acetate:methanol (9:1) and 100 % methanol. 45 fractions of 50 ml each were collected; fractions containing the same compounds as determined by TLC plates were combined and concentrated to dryness under reduced pressure. TLC plates of 11 pooled fractions (A-K) were developed with hexane: ethyl acetate 9:1, 7:3 and 3:7. Fraction I yielded a pure compound (**1**). Other fractions were crystallized and yielded 5 pure compounds (**2**, **3**, **5, 6** & **7**). TLC plates were examined under UV light (254 and 366 nm) after development and also dipped in vanillin (15 g vanillin, 500 ml ethanol and 10 ml concentrated 98 % sulphuric acid) and heated to detect compounds not absorbing UV. Fractions G and H were combined and subjected to a second column eluted with a solvent gradient of hexane: ethyl acetate in 100:0 to 0:100 ratios which resulted in isolation of compound **4.**

4.3 Results and discussion

The column chromatography (Figure 4.3) yielded 45 fractions (Figure 4.5) which were pooled together according to their TLC profile and resulted in 11 pooled fractions (Figure 4.6). From these pooled fractions seven compounds were isolated (Figure 4.7).

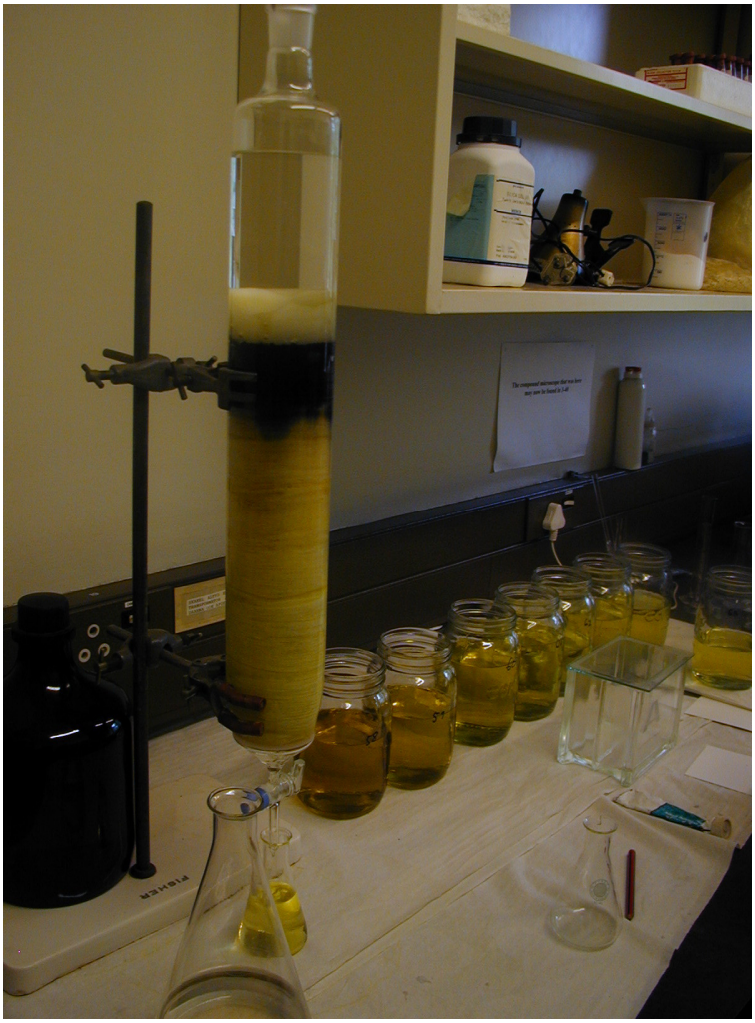


Figure 4.3 Column chromatography

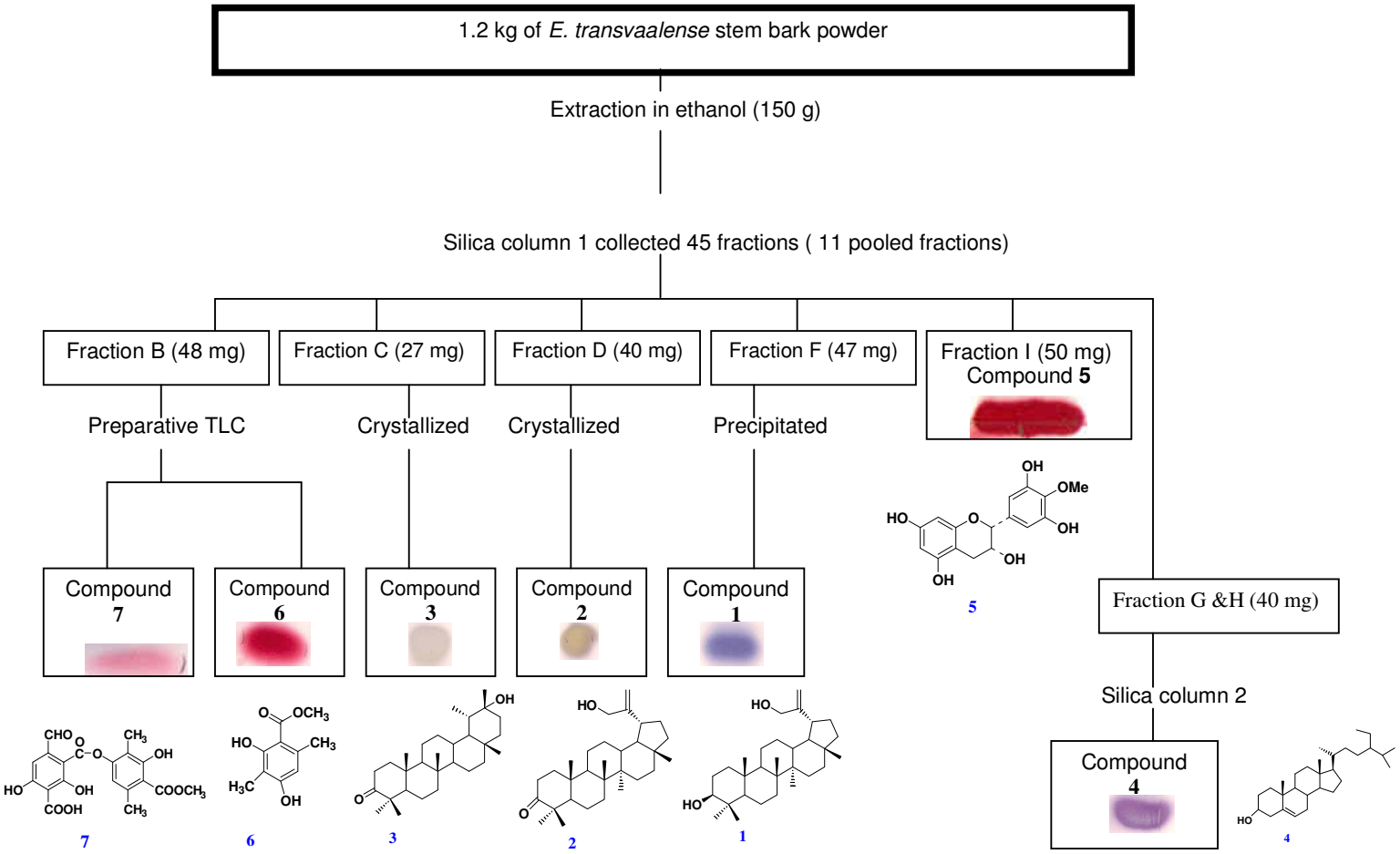


Figure 4.4: Schematic presentation of isolation steps followed.

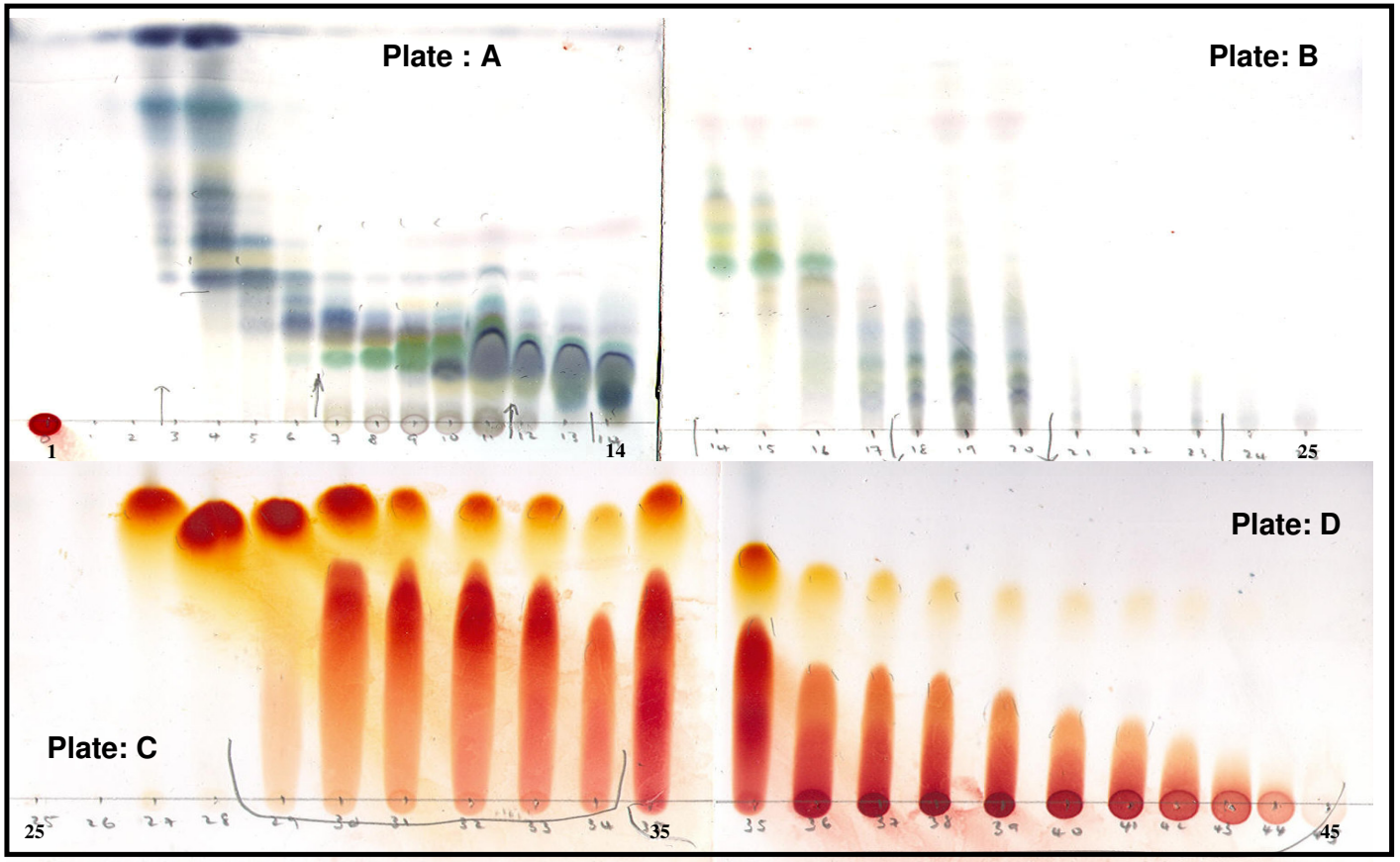


Figure 4.5 Fractions from the first silica column on TLC plates sprayed with Vanillin reagent. Plate A and B developed with hexane: ethyl acetate (9:1 and 7:3), Plate C and D fractions developed with hexane: ethyl acetate (1:9).

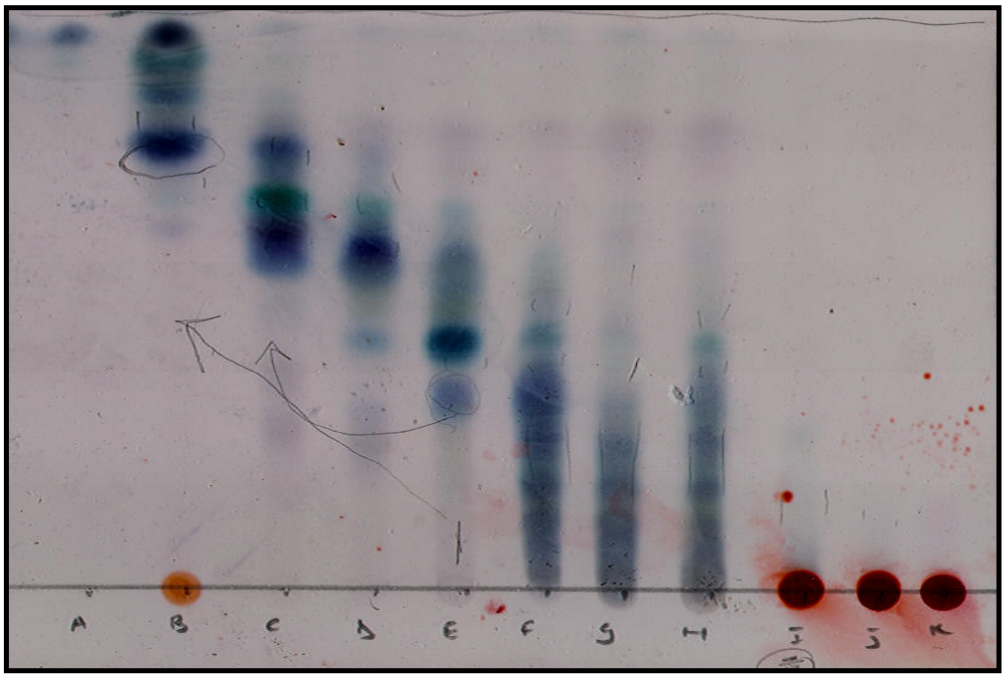


Figure 4.6 The 11 pooled fractions (silica column 1) TLC plates sprayed with Vanillin reagent.

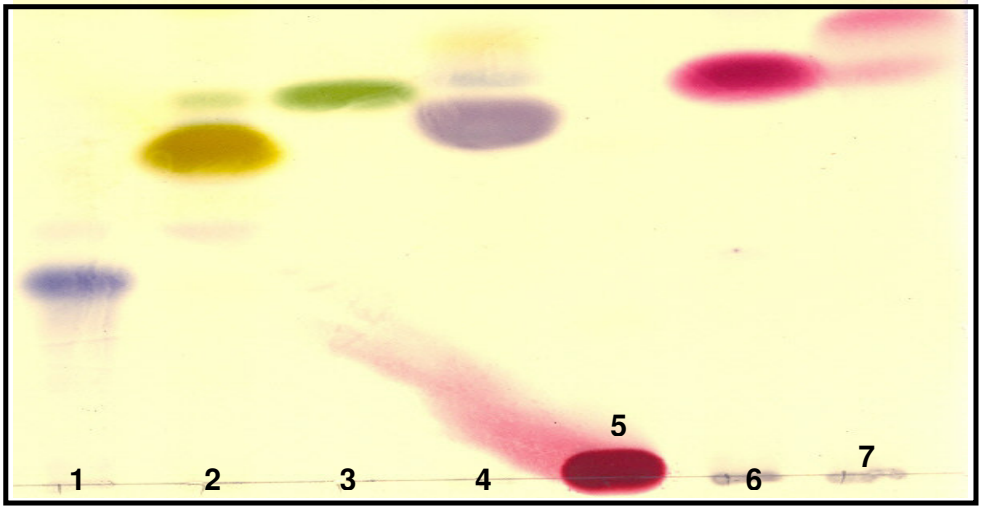
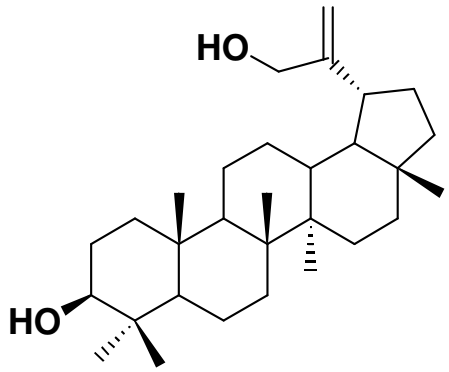


Figure 4.7 Isolated compounds as seen on TLC plates sprayed with Vanillin reagent.

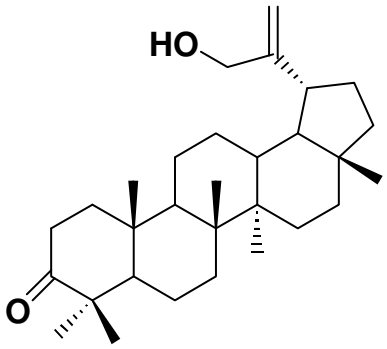
4.3.1 Triterpenoids isolated

Fraction F contained a white precipitate that was washed with ethyl acetate to give one pure compound **1** (Figure 4.7) as determined by TLC and other spectroscopic methods (Table. 4.2).



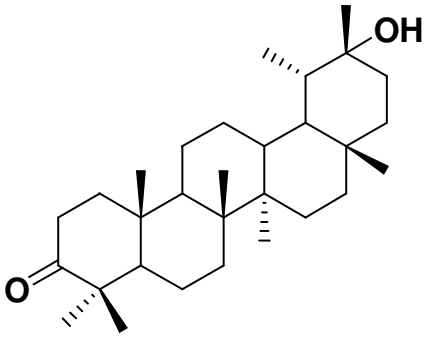
1

lup-20(30)-ene-3,29-diol, (3α)-(9CI)



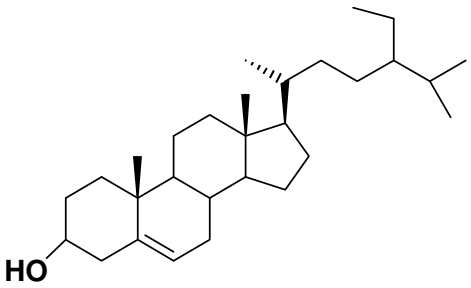
2

lup-20(29)-ene-30-hydroxy-(9CI)



3

Ψ – taraxastanonol



4

β-sitosterol

Figure 4.8 Structures of isolated triterpenes**.**

Compound **1** showed in its ^{1}H-NMR (Figure 4.8) spectrum a triterpenoid pattern with six methyl singlets at δ_H 0.76 , 0.78 , 0.83 , 0.95, 0.97 and 1.03, two olefinic protons at δ_H 4.95 (d 1.4 H), 4.88 (s) attached to carbon at δ_C 106.9, both protons correlated in HMBC (Figure 4.10) with the carbon at δ_C 65.1, another two protons attached to a oxygen bearing carbon at $^{\delta}$H 4.11 (d, J=14.8 Hz), **406** (d, J=14.8 Hz), and a proton δ_H 3.16 (dd, J=10.4, 5.7 Hz) on carbon at δ_C 79.0. The ^{13}C – NMR spectrum of compound 1 is shown in Figure 4.9. The data obtained with 2D NMR experiments HMBC, HMQC, COSY and NOESY (Figures 4.10 & 4.11) supported the structure for compound **1** (Abdel-Mogib, 1999). This compound has been previously isolated from the whole plant extract of *Daphne oleoides*, which is used as a purgative and the infusion of the leaves is used to treat gonorrhoea and applied to abscesses (Ullah *et al*., 1999).

Fractions C and D were crystallized to give compounds **2** and **3** as shown previously in Figure 4.3. Both compounds were obtained as white crystals, but showed different colours on TLC plates. Compound **2** showed similar signals in ^{1}H-NMR (Figure 4.12) with the previous compound except for the disappearance of the C-3 proton and the appearance of a carbonyl carbon in ^{13} C-NMR (Figure 4.13) at δ_C 218.2, which indicate the enzymatic oxidation of the hydroxyl group into the corresponding ketone group in compound **2**. This was supported by the HMBC correlation between C-3 and the H-23 and H-24 methyls and previously reported data (Fang et al., 1984). Compound **3**'s structure was supported by data published by Anjaneyulu *et al*. (1999) and Hinge *et al*. (1966). This compound was also isolated from resin of *Protium*

heptaphyllum and has shown analgesic effects (Susunaga *et al*., 2001 & Rudiger *et al*., 2007).

Fractions G and H were combined and subjected to a second silica gel column 2 and one pure compound (**4**) was obtained. This compound was identified as β - sitosterol when spectra were compared to published data (Prozesky, 2004). ^{13}C – NMR data (Table 4.2) of all the triterpenes isolated form *E. transvaalense* was also supported by other published data (Scleich *et al*., 2006, Sasunaga *et al*., 2001, Mahato & Kundu, 1994, Ullah *et al*., 1999 & Burns *et al*., 2000).

Table 4.2: ^{13}C – NMR data of tritepernoids isolated compounds (1-3).

C	**1** δC	**2** δC	**3** δC
1	38.7 t	39.6	39.6
2	27.4 t	34.1	33.8
3	79.0 d	218.3	218.0
4	38.9 s	47.3	47.4
5	55.3 d	54.9	54.8
6	18.3 t	19.7	19.8
7	34.3 t	33.6	34.1
8	40.9 s	40.8	41.4
9	50.4 d	49.7	49.1
10	37.1 s	36.9	36.7
11	21.0 t	21.6	22.2
12	26.7 t	26.7	26.7
13	38.0 d	31.8	39.0
14	43.0 s	42.9	43.2
15	27.4	27.4	26.8
16	35.5 t	35.4	38.1
17	43.0 s	43.0	35.6
18	49.0 d	48.8	47.3
19	43.8 d	43.8	38.7
20	154.8 s	154.7	73.5
21	31.8 t	31.8	35.4
22	39.9 t	39.8	37.7
23	28.0 q	26.7	26.8
24	15.4 q	21.0	21.1
25	16.1 q	16.0	16.2
26	16.0 q	15.8	16.1
27	14.6 q	14.5	14.8
28	17.7 q	17.7	17.8
29	106.9 t	106.8	17.8
30	65.1 t	65.0	30.3

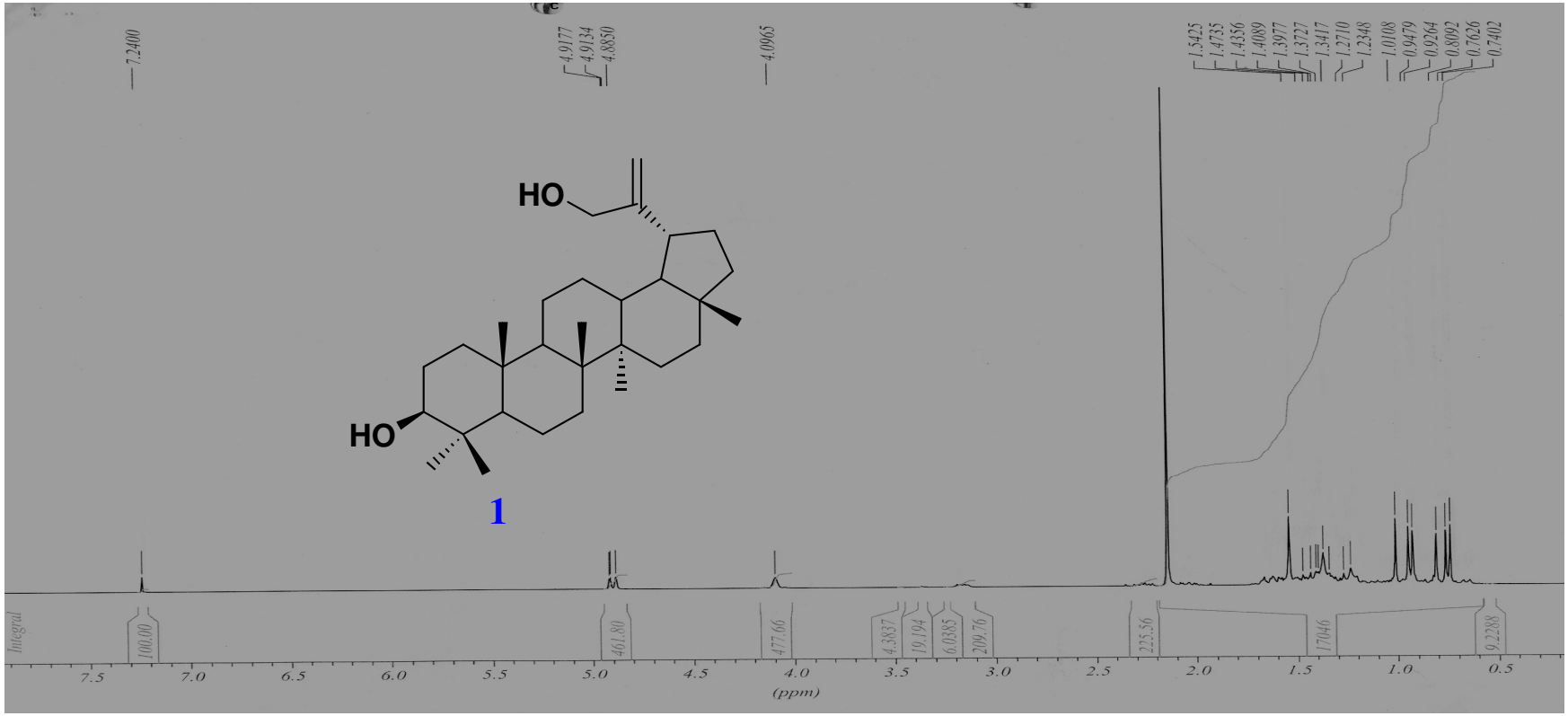


Figure 4.9 ^{1}H – NMR spectrum of Compound **1**

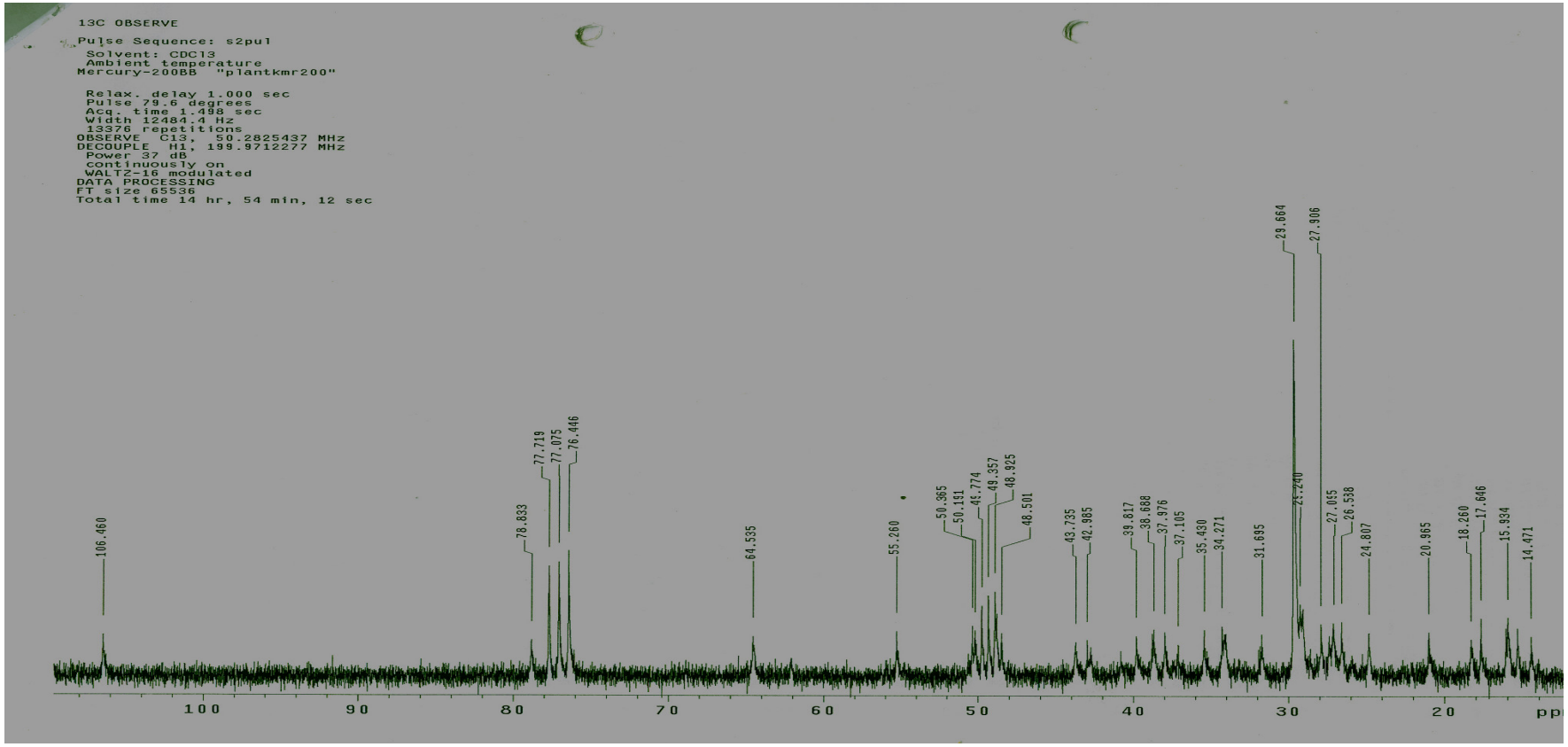


Figure 4.10 ^{13}C – NMR spectrum of Compound **1**

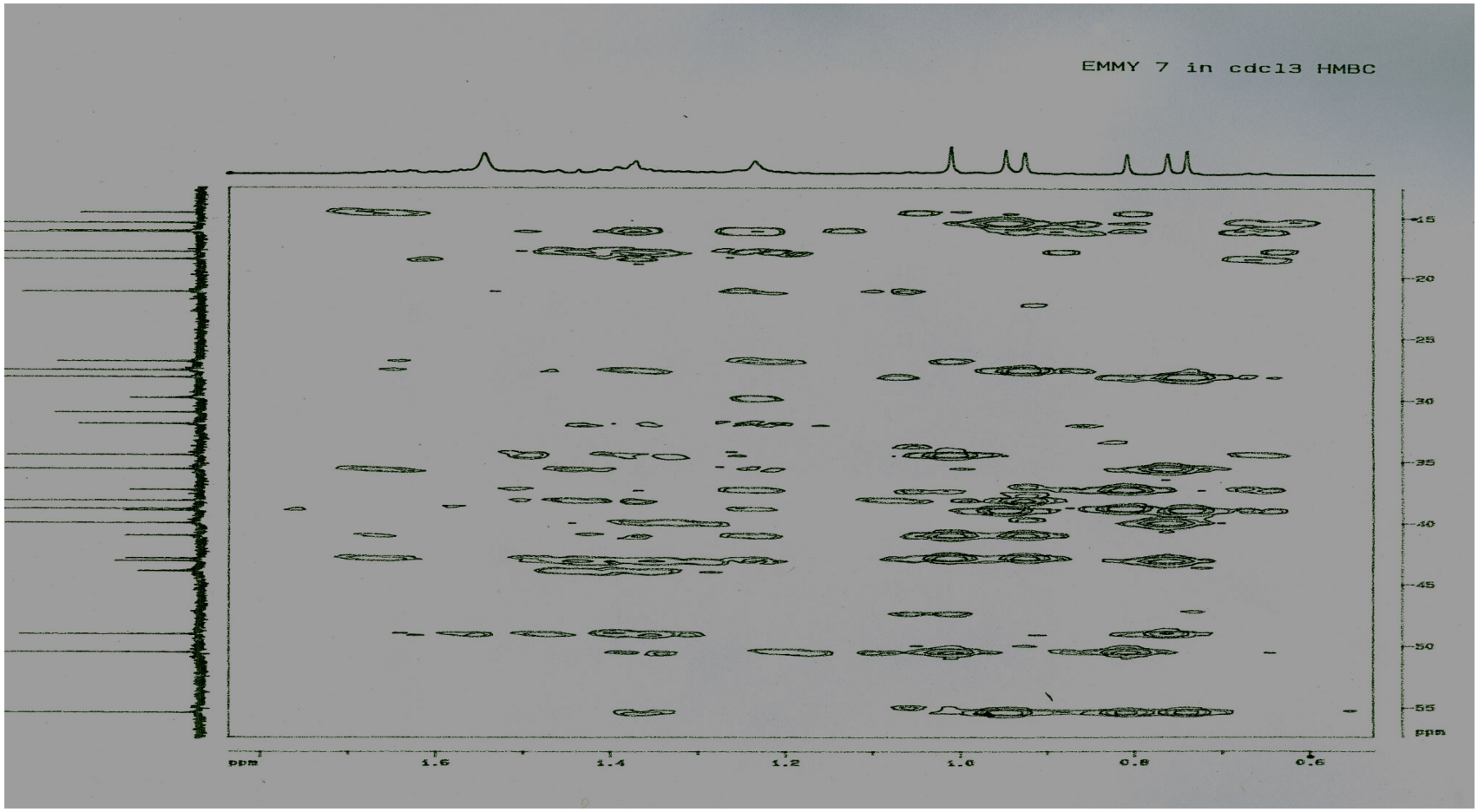


Figure 4.11 HMBC spectrum of Compound **1**

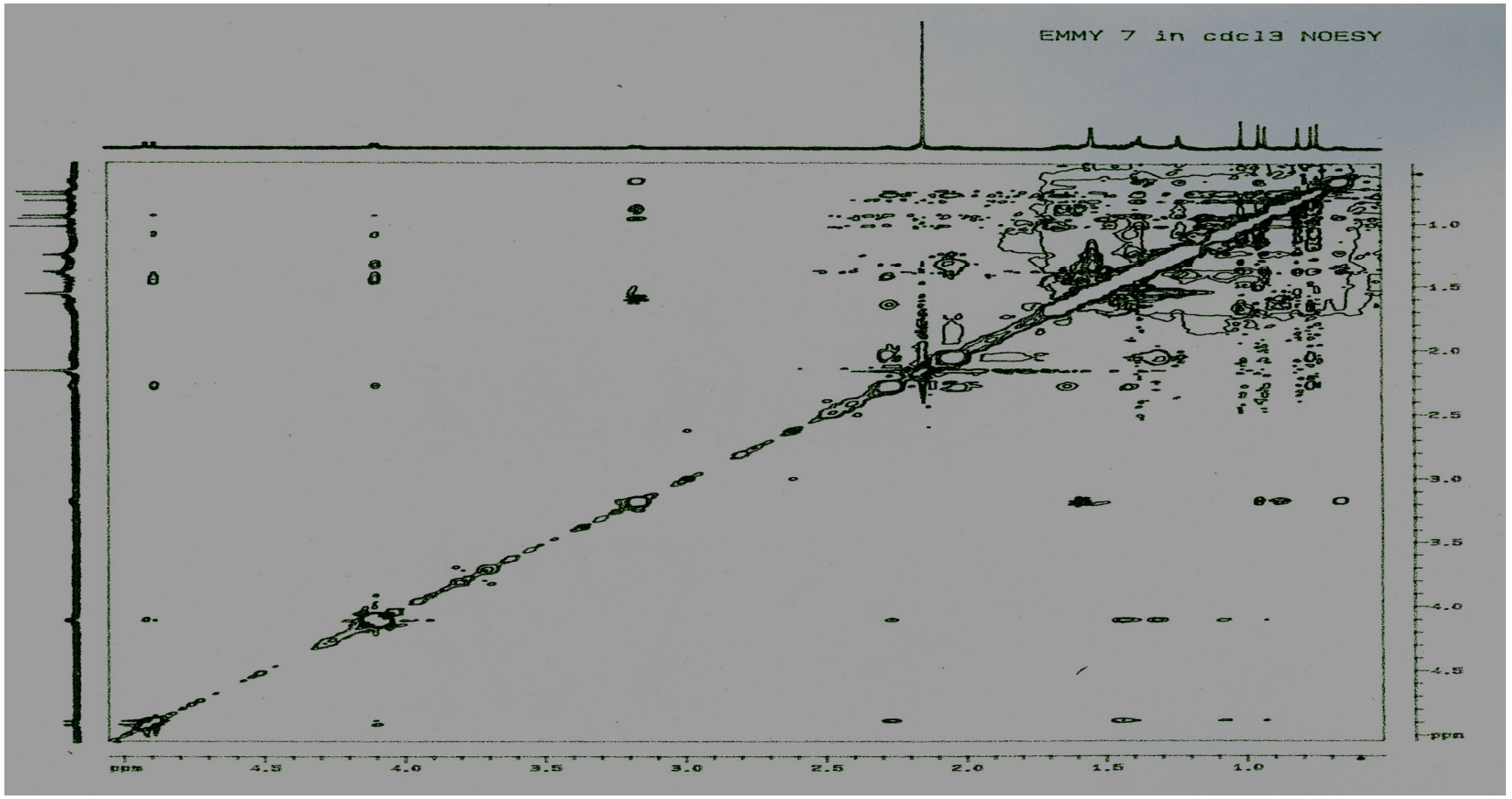


Figure 4.12 NOESY spectrum of Compound **1**

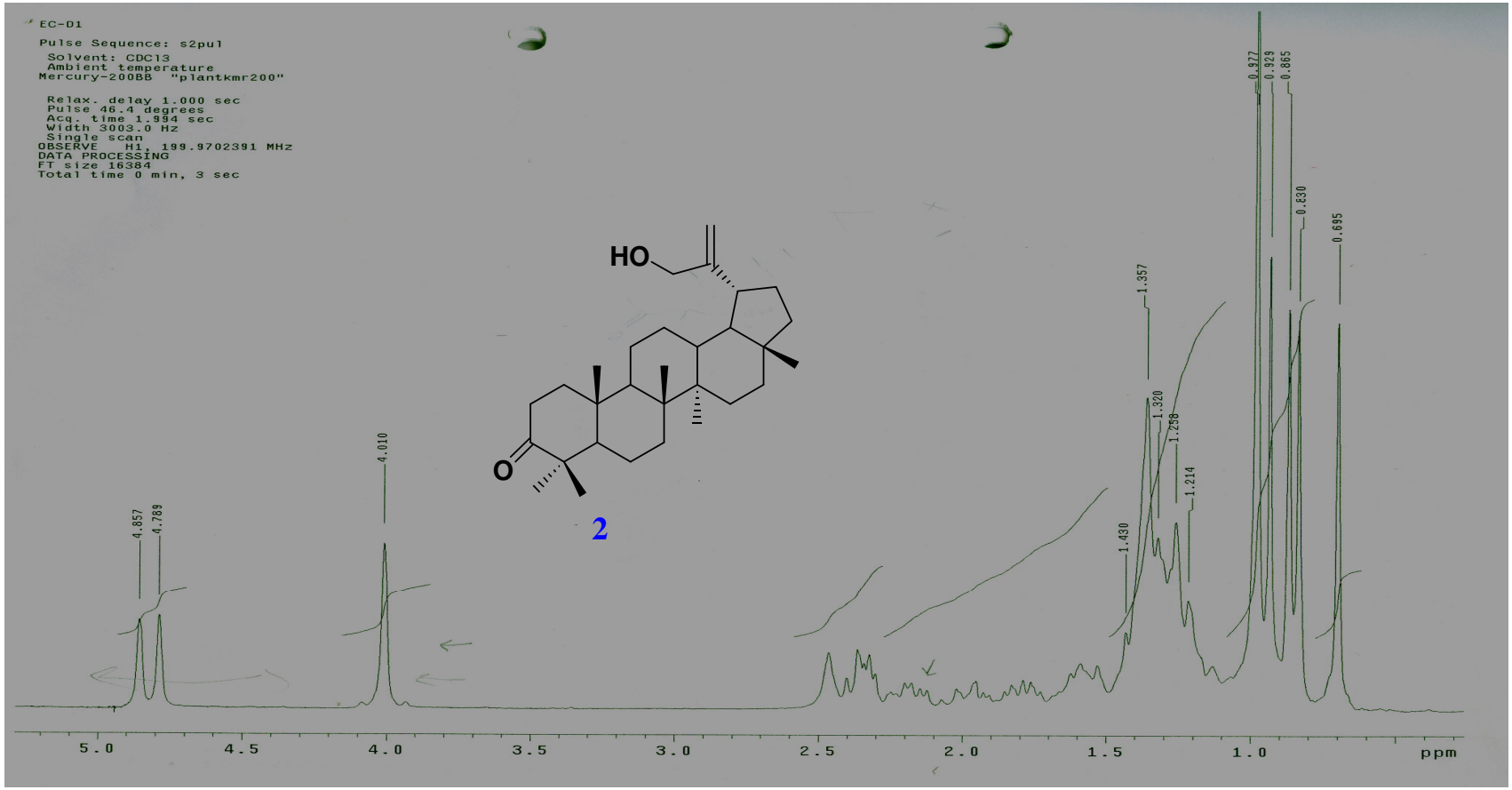


Figure 4.13 ^{1}H – NMR spectrum of Compound **2**

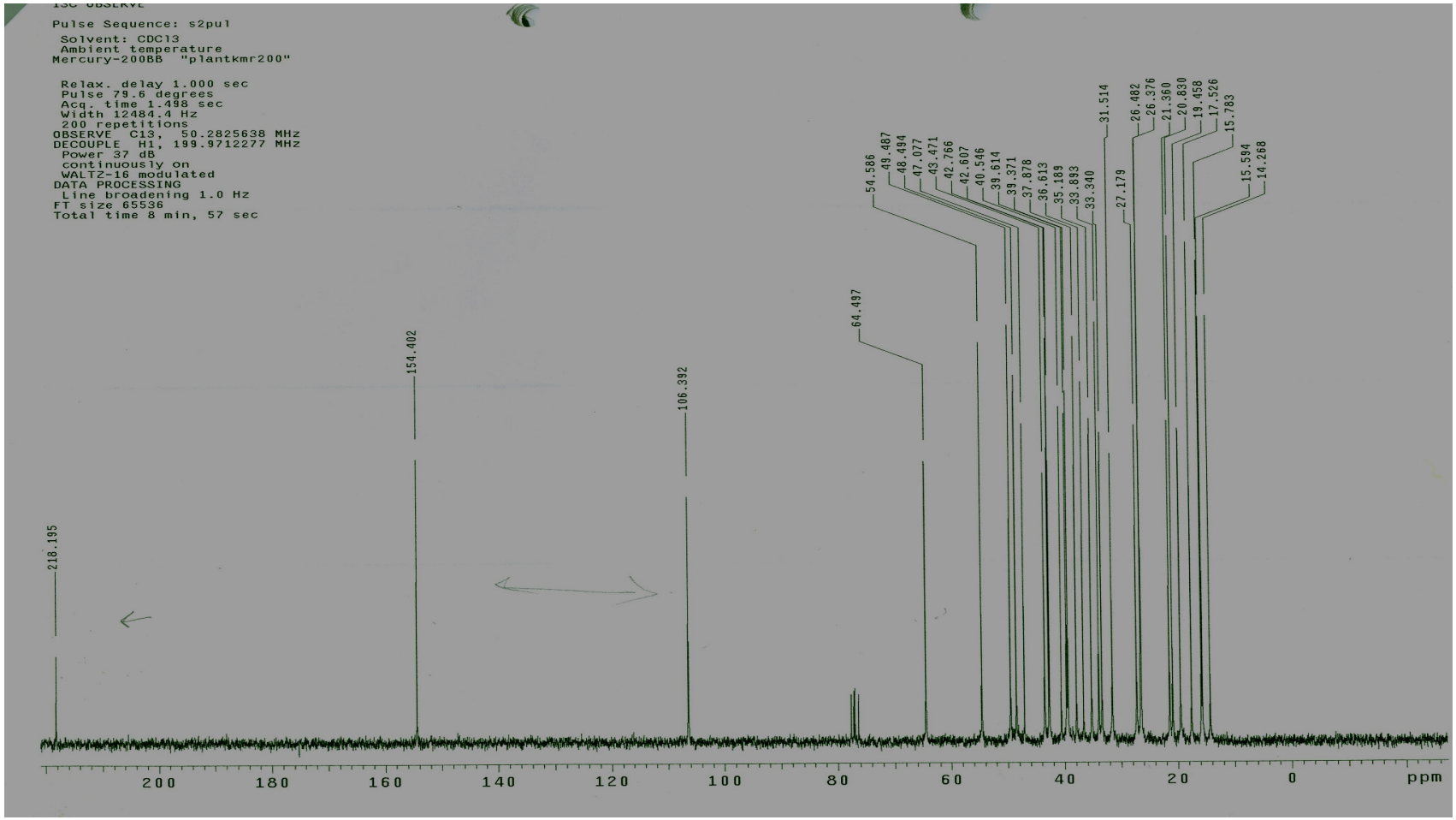


Figure 4.14 ^{13}C – NMR spectrum of Compound **2**

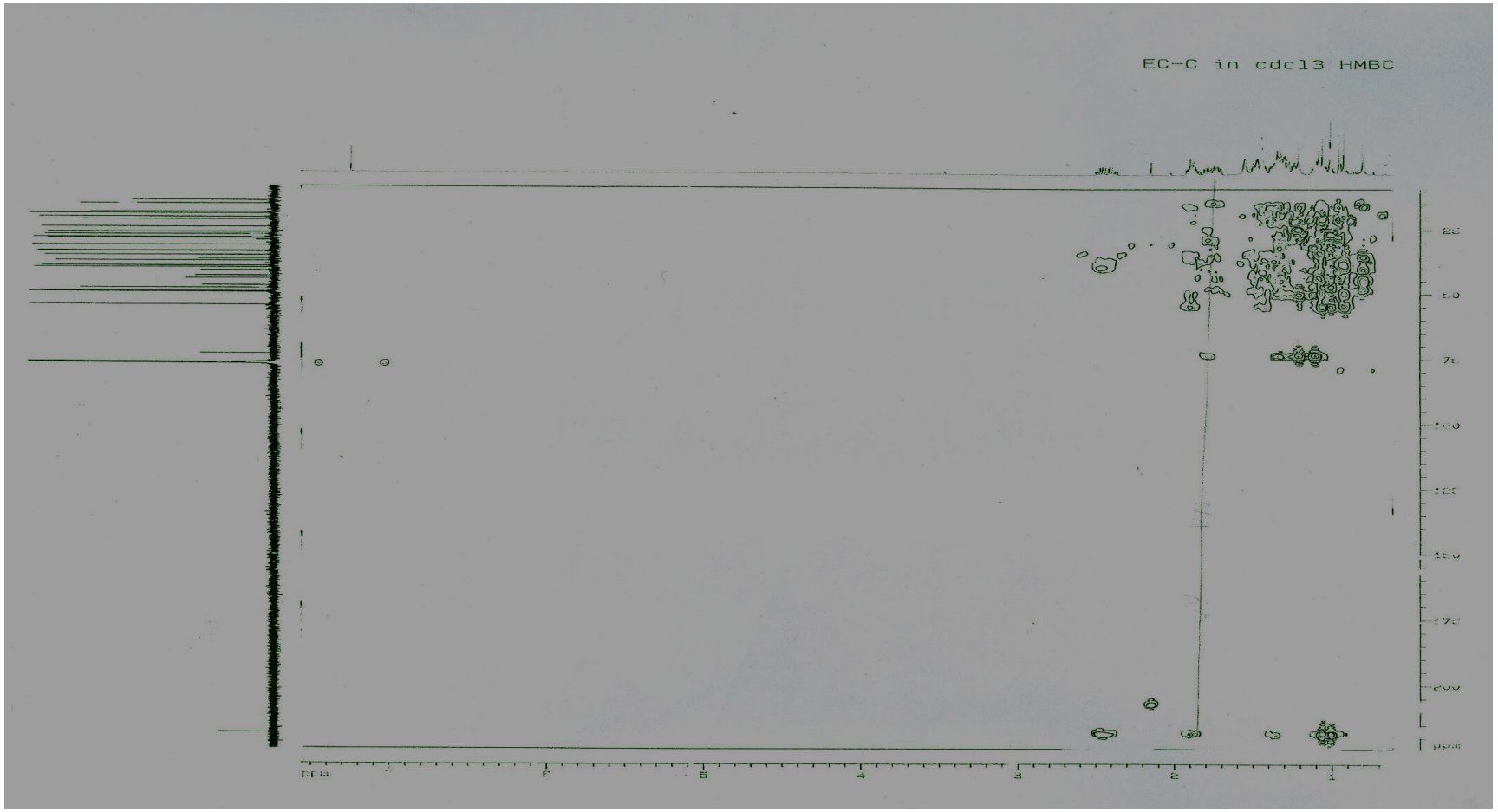


Figure 4.15 HMBC spectrum of Compound **3**

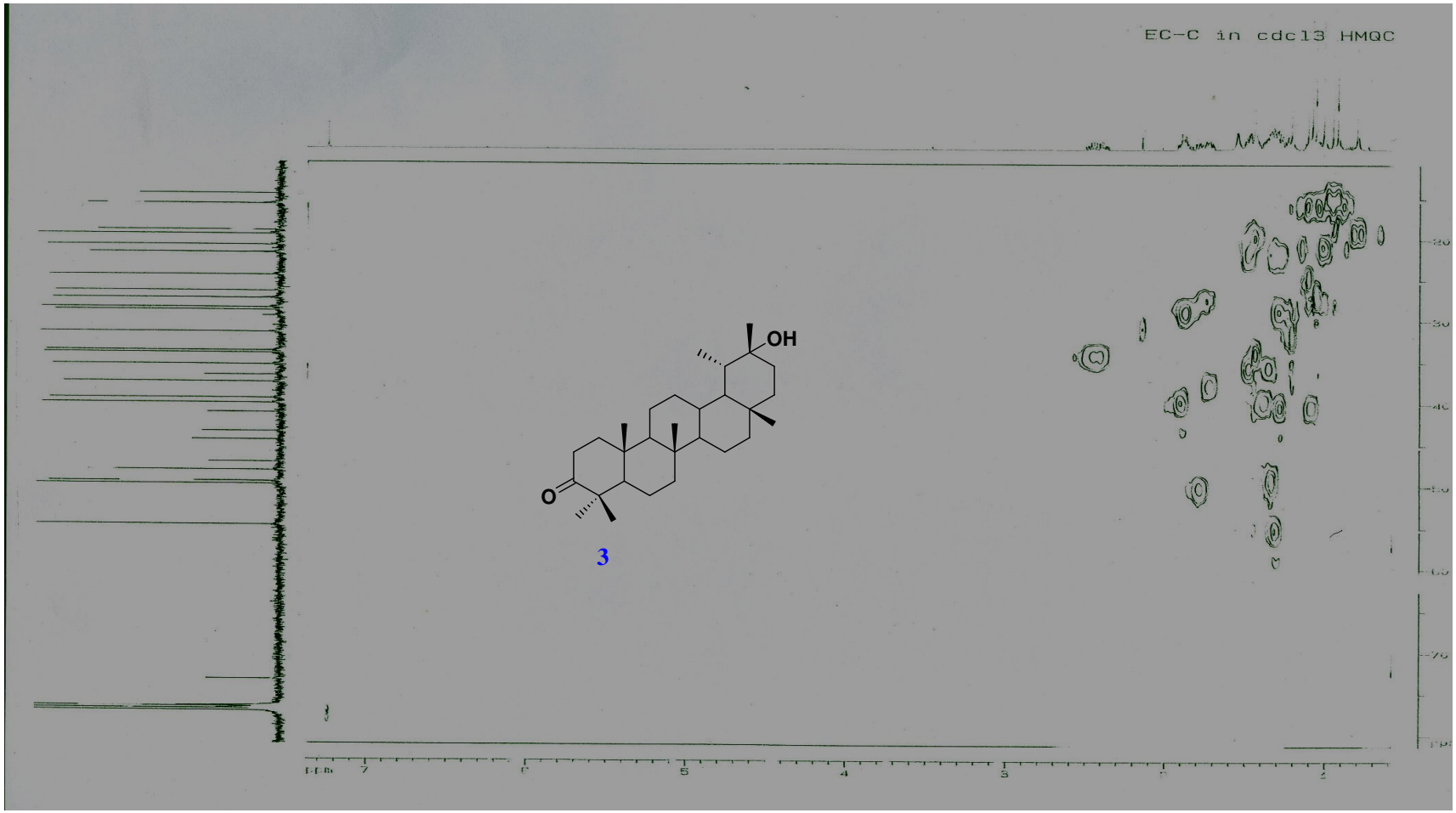
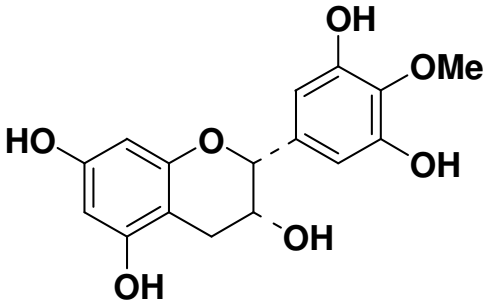


Figure 4.16 HMQC spectrum of compound **3**

4.3.2 Methylepigallocatechin

Fraction I contained one pure compound **5,** (Figure 4.16) as determined by TLC and other spectroscopic methods. It was obtained as a brown powder (50 mg). The ^{1}H-NMR (Table 4.3 and Figure 4.17) spectrum showed four aromatic protons at δ_H 5.71 (d, J=2.3), 5.89 (d, J=2.3), 6.40 (2H, d, J= 0.6), two methane protons at δ_H 4.68 (d, J=0.8) and 3.45 (s br), methylene protons at δH 2.45 (J=4.4) and 2.70 (J=3.2), and one methoxyl group at δ_H 3.65 (s). The ^{13}C-NMR spectra (Figure 4.18) indicated the presence of two methine carbons attached to an oxygen function (δ_C 78.6, 65.6), a methylene carbon (δ_C 28.8 t), 12 aromatic carbons δ_C 156.3 (s), 95.8 (d), 157.2 (s), 94.8 (d), 156.9 (s), 99.2 (s), 135.6 (s), 106.8 (x2C, d each), 150.7 (x2c, s each), 135.2 (s), and a methoxyl carbon (δ_C 60.3 q). The coupling constant between protons at δ_H 4.68 and 3.45 is 2.3=Hz which indicated ββ relative configuration. The above spectroscopic data indicated that compound **5** is (-)4'-*O*-methylepigallocatechin which have been isolated from the same genus previously (Drewes & Mashimbywe, 1993). Hussein *et al.* (1999) reported anti-HIV -1-protease activity for this compound. .



(-)4'-*O*-methylepigallocatechin (**5**)

Figure 4.17 Structure of compound **5**

Table 4.3 ^{1}H – NMR and ^{13}C – NMR data of compound **5.**

Position	$^{\delta}H$	$^{\delta}C$
2	4.68 d (J= 0.8)	78.6 d
3	3.45 (J= 3.9)	65.6 d
4 a 4 b	2.73 dd (J=3.2) 2.87 dd (J= 4.4	28.8 t
5		156.3 s
6	5.71 d (J=2.3)	95.8 d
7		157.2 s
8	5.89 d (J= 2.3)	94.8 d
9		156.9 s
10		99.2 s
1´		135.6 s
2', 6'	6.40 d (J= 0.6)	106.8 d
3',5'		150.7 s
4'	3.65	135.2 s
OMe		60.3 q

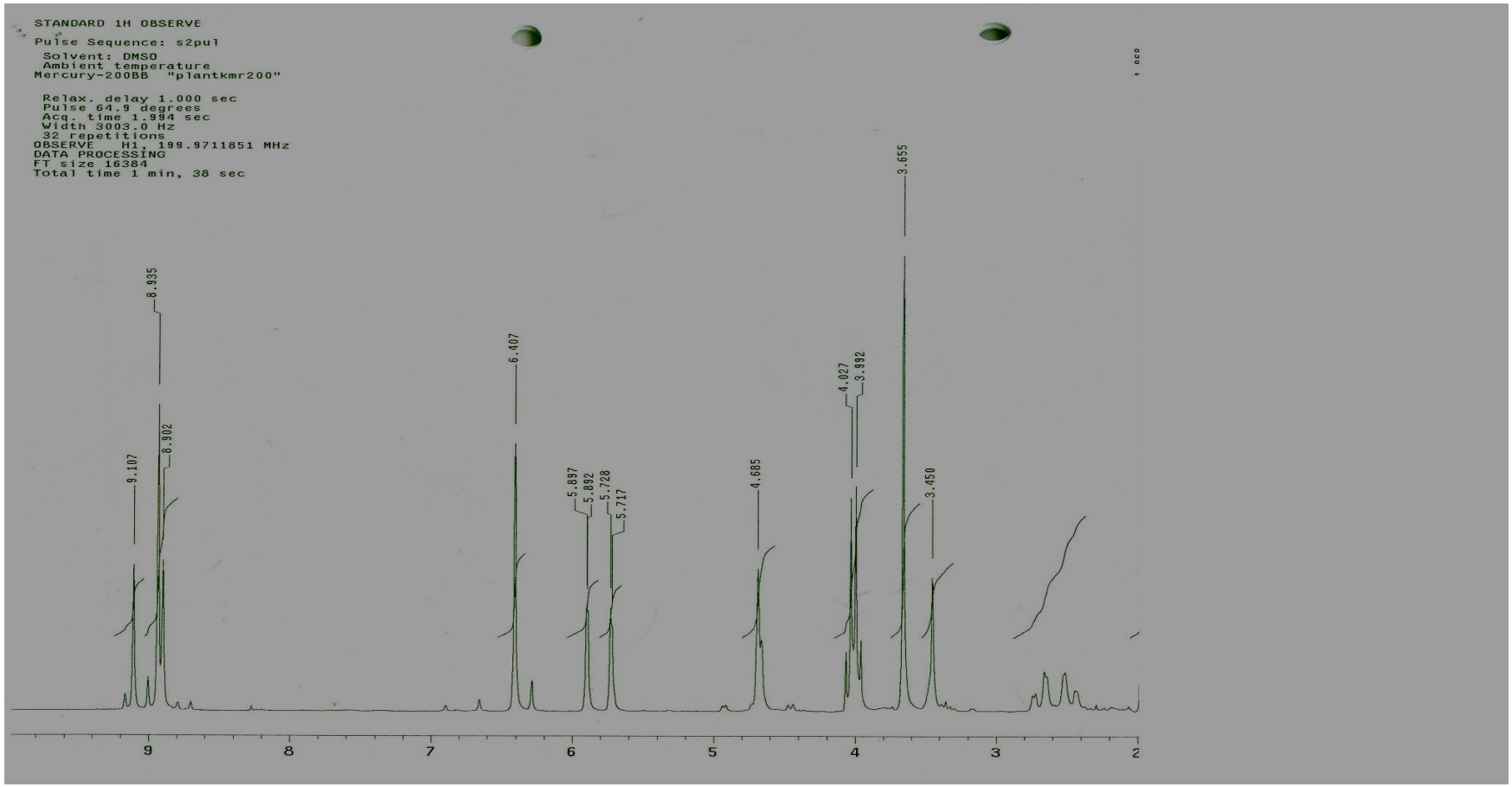


Figure 4.18 ^{1}H – NMR spectrum of Compound **5**

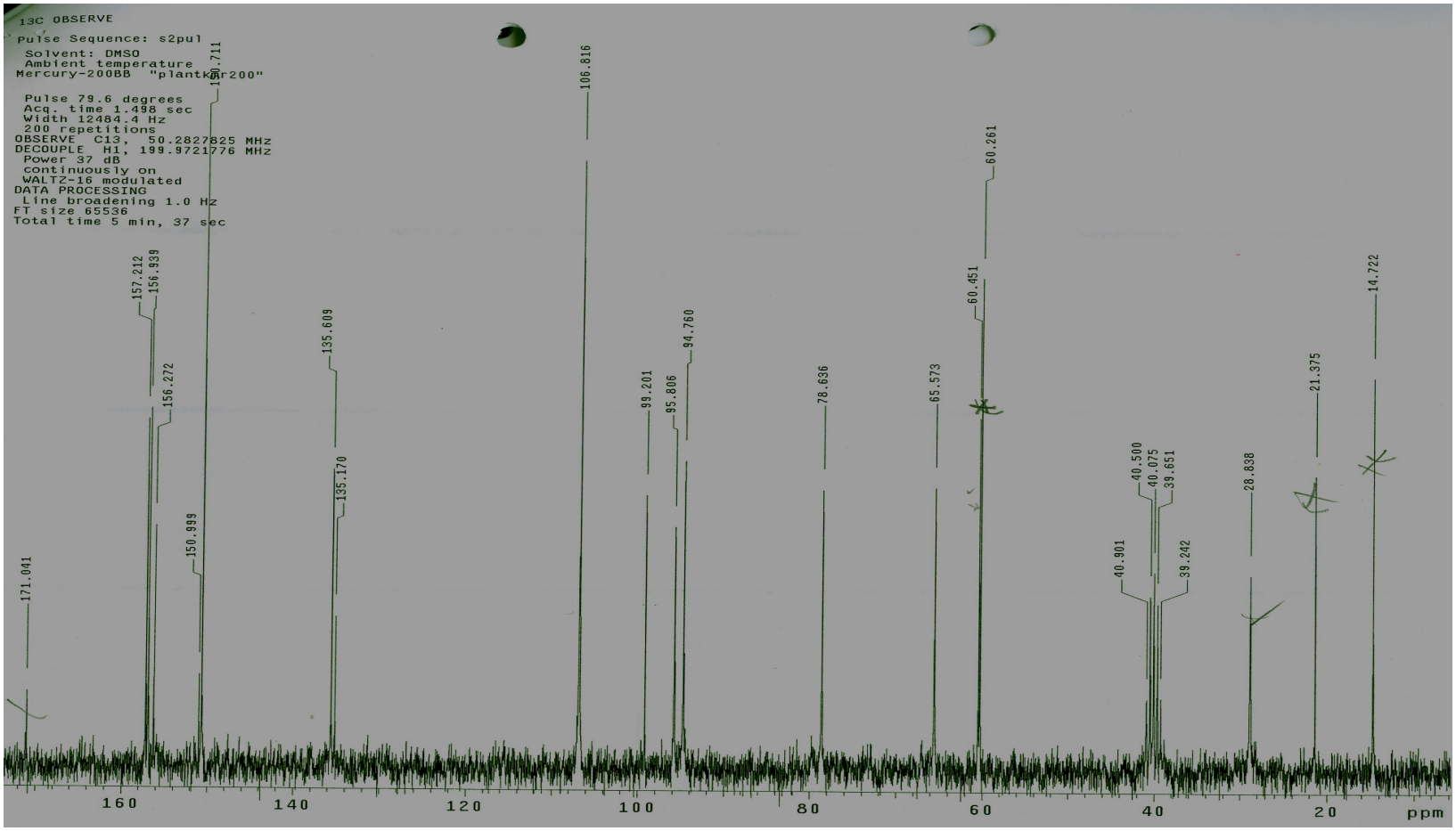
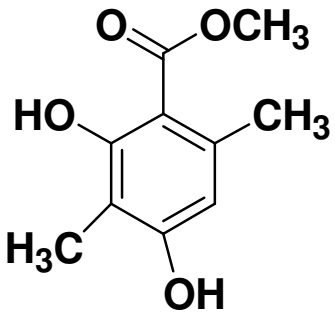


Figure 4.19 ^{13}C – NMR spectrum of Compound **5**

4.3.3 Phenolic derivative and depside

Compounds **6** and **7** (Figures 4.19 and 4.20) were obtained through preparative TLC of fraction B which was developed in hexane:chloroform:water (10:4:1). The plate was half developed, dried and redeveloped fully.



Atraric acid **(6)**

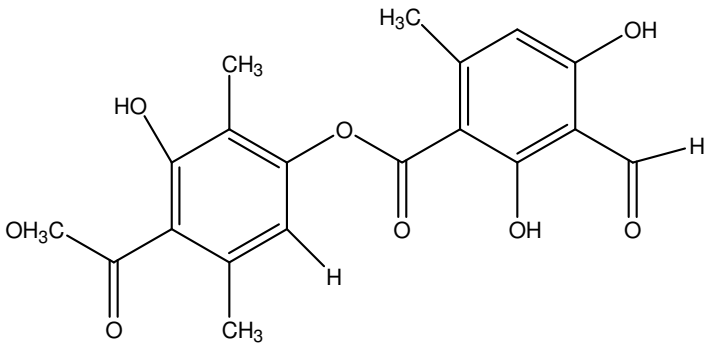
Figure 4.20 Structure of compound **6**

Compound **6** was formed as crystals. The ^{13}C-NMR (Figure 4.22) revealed the presence of 10 carbons, including six of which five where substituted on a benzene ring, including two hydroxyls (δ_C 163.1, 158.2) and one methylated (δ_C 51.8) carboxyl group (δ_C 172.6), in addition to two aromatic methyl groups (δ_C 24.1, 7.6) ^{1}H-NMR (Figure 4.21) spectral data showed two aromatic methyls δ_H 2.43, 2.07 singlets and an aromatic proton at δ_H 6.19.

The foregoing data indicated that the isolated compound is methyl 2,4-dihydroxy-3,6-dimethylbenzoate a phenolic derivative (atraric acid). This was confirmed by 2D-NMR spectra e.g. HMBC, HMQC, COSY. The NMR spectral data are in agreement with those previously reported (Gormann *et al.*, 2003; Lee *et al.*, 2001).The compound was isolated for the first time from lichens (Cooke & Down,1971), however it was reported to be a constituent in higher

plants such as *Alseodaphne andersonni*, *Acer nikoense*, *Dianella revolute*, *Frullania brasiliensis*, *Pygeum africanum* and *Xylosma velutina* (Bardon *et al*., 2002 and Schleich *et al*., 2006). It is difficult to say whether this compound is a secondary metabolite of *E. transvaalense* or is the result of lichens that colonized the stem bark, producing polyketides through the acetate-polymalonate pathway.

The LR MS for compound **7** (Figure 4.20) showed a peak at J 75 (MH^+) corresponding to $C_{19}H_{18}O_8$, this was supported by ^{13}C and DEPT NMR analysis of the compound.



Atranorin (**7**)

Figure 4.21 Structure of compound **7**

^{1}H-NMR data showed four singlets signals at δ 3.97, 2,67,2.53 and 2.07 corresponding to four methyl groups (Figure 4.23), one of them (3.97) esterified to the carboxyl group at C-4 and the others attached to the positions C-6, 2, 5. The spectra also showed three singlet signals, each intergrated for one proton, at δ 6.39, 6.50, 10.30 corresponding to two aromatic protons at C-5 and 6, the third (10.30) is belonging to the aldehydic group at C-8 (δ_c 193.8) (Figure 4.24).

This data indicated the compound to be the depside, atranorin as depicted in Figure 4.20, and is in agreement with those data reported for the same compound. Also this was supported by 2D NMR data, COSY, HSPC, HMBC and NOESY (Figures 4. 23 and 4.24). Compound **7** was also isolated originally from lichens (Santos *et al*., 2004) but recent literature reported it's presence in higher plants as well (Athukoralage *et al*., 2001).

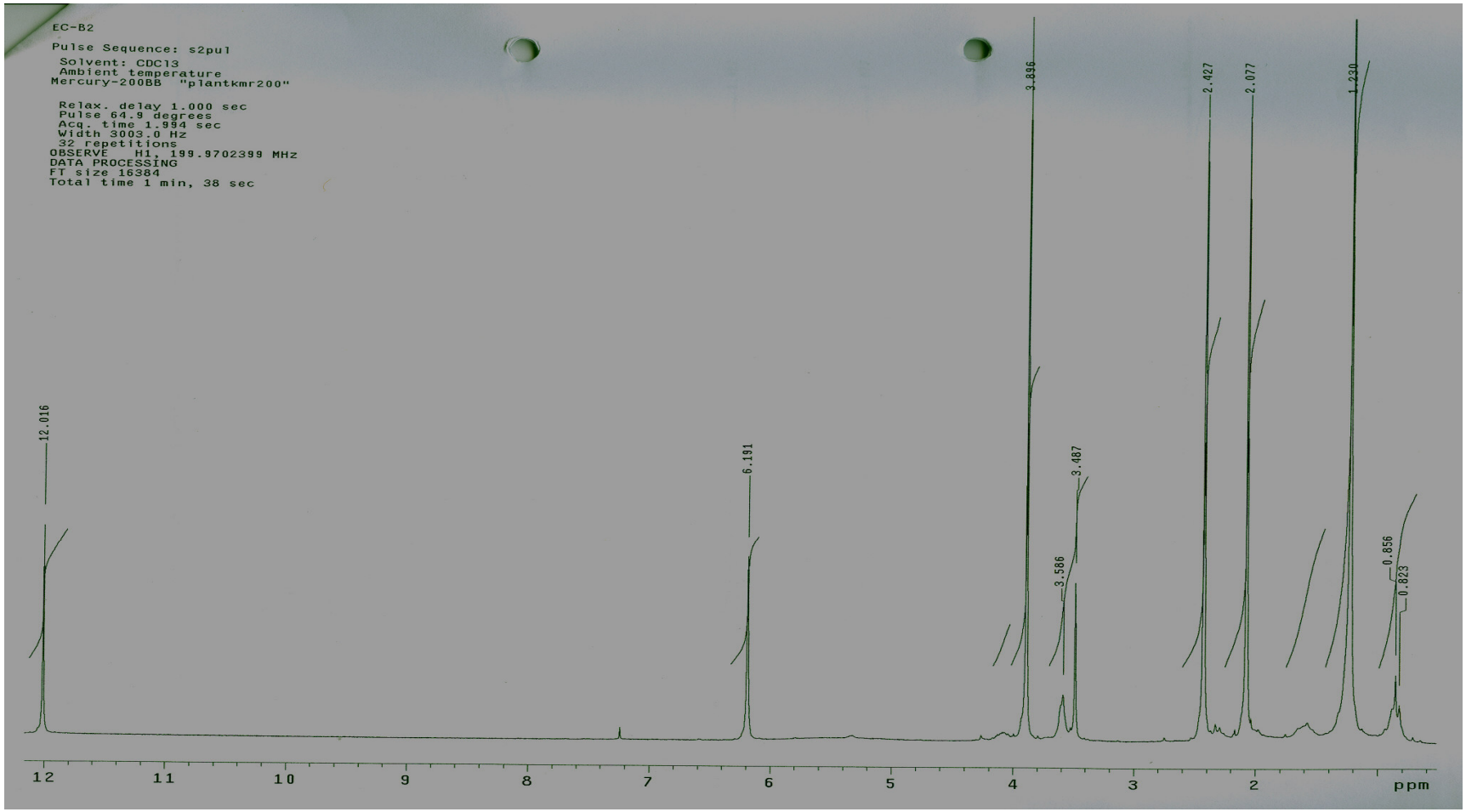


Figure 4.22 ^{1}H – NMR spectrum of Compound **6**

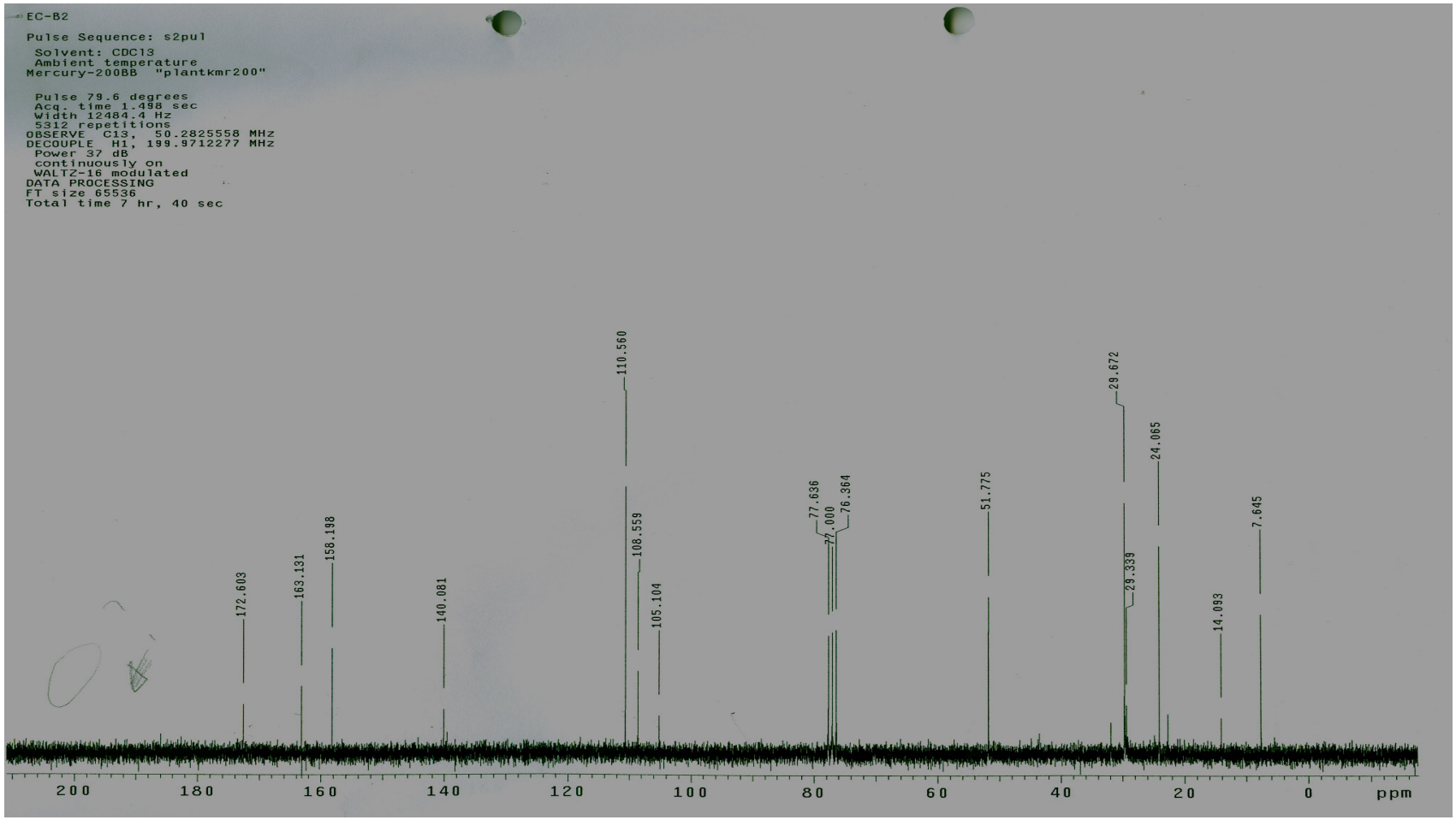


Figure 4.23 ^{13}C – NMR spectrum of Compound **6**

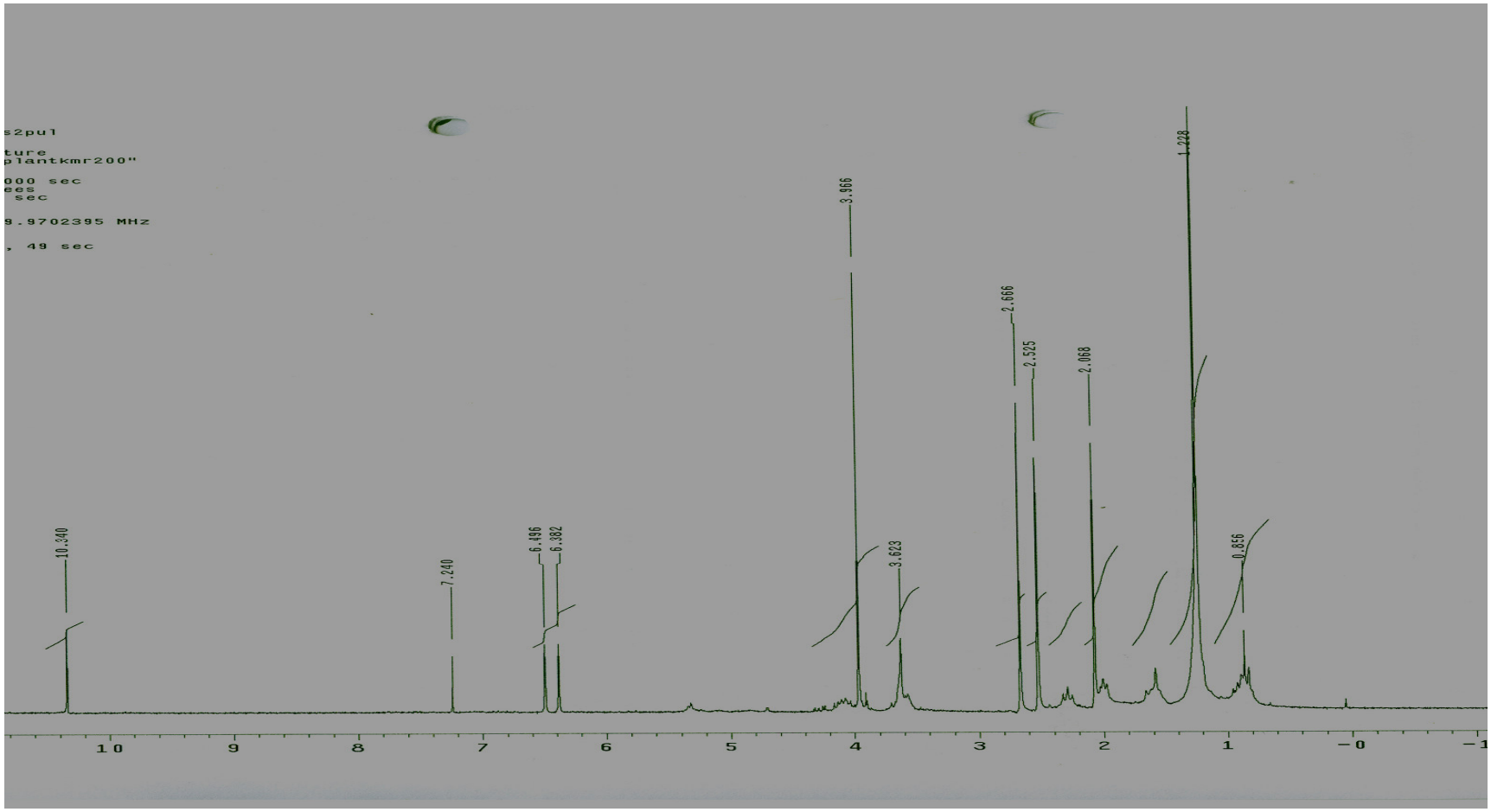


Figure 4.24 ^{1}H – NMR spectrum of Compound **7**

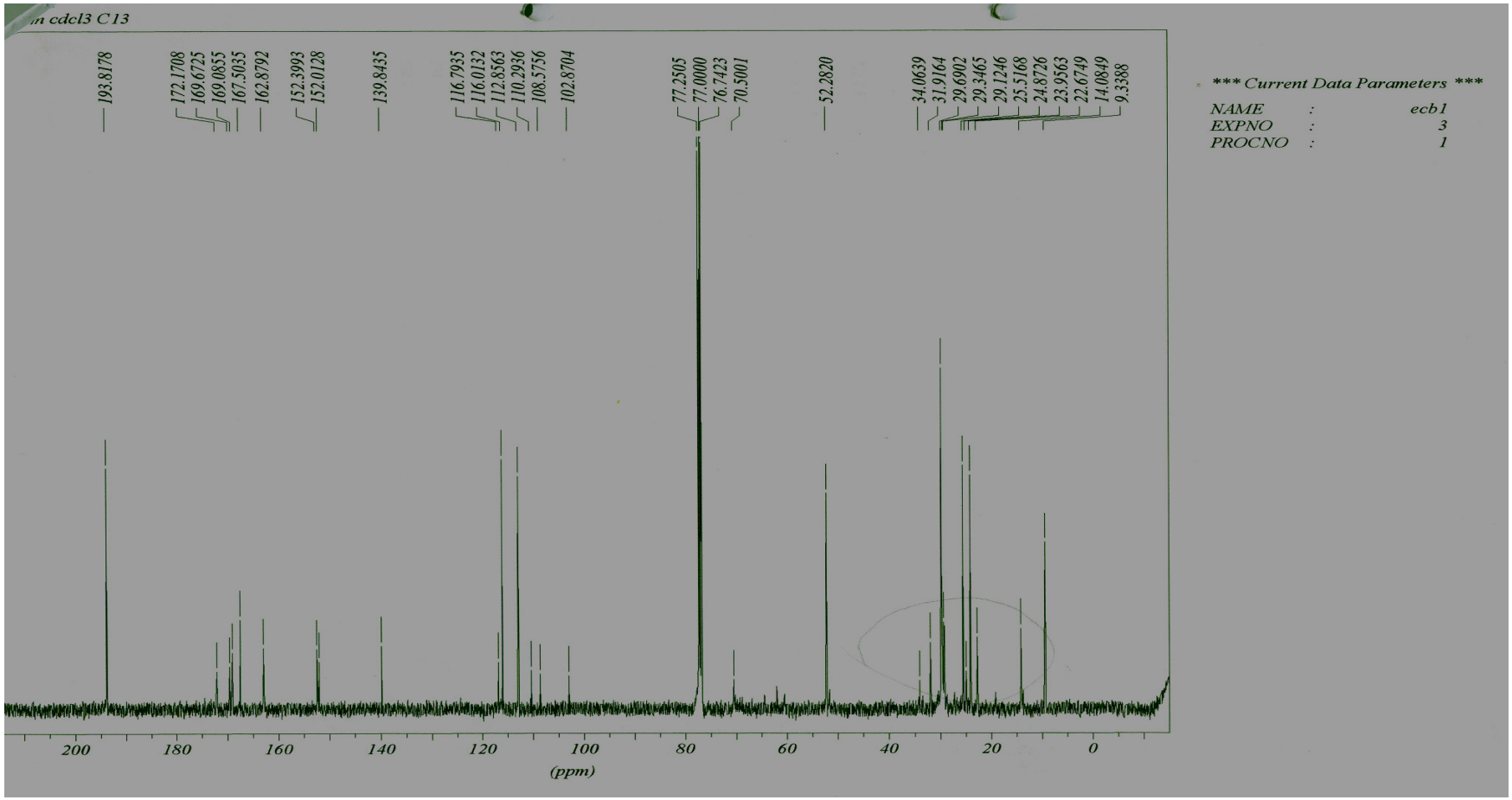


Figure 4.25 ^{13}C – NMR spectrum of Compound **7**

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