Chapter 3

Extract preparation and isolation and identification of active compounds from *Elaeodendron croceum*

3.1 Introduction

From the twelve extracts tested against HIV, the extract of *Elaeodendron croceum* was identified as most active. The extract was prepared according to a specific preparation procedure proposed by the University of Cordoba, Spain.

After determining the activity of the extract, bio-guided fractionation was used to isolate and identify the active compound from the extract. To isolate the single compound from the extract, several chromatographic techniques such as thin layer chromatography (TLC), column chromatography and liquid-liquid fractionation were used. Several columns were needed to obtain a pure compound. Precipitation of the compound from the semi-purified fractions made it easier to isolate the compound.

After isolation, analysis such as one-dimensional and two-dimensional nuclear magnetic resonance (NMR) was used to determine the structure of the compound. Two-dimensional NMR analyses proved to be most useful, as several of these analyses were needed to confirm the structure. It was determined that the active compound is digitoxigenin-glucoside, a compound that belongs to the cardiac glycosides.

3.2 Materials and Methods

3.2.1 Selection of plants

Several South African plants were tested against HIV. The selection was done on previous antibacterial, antifungal and antiviral activity found in these plants. For each

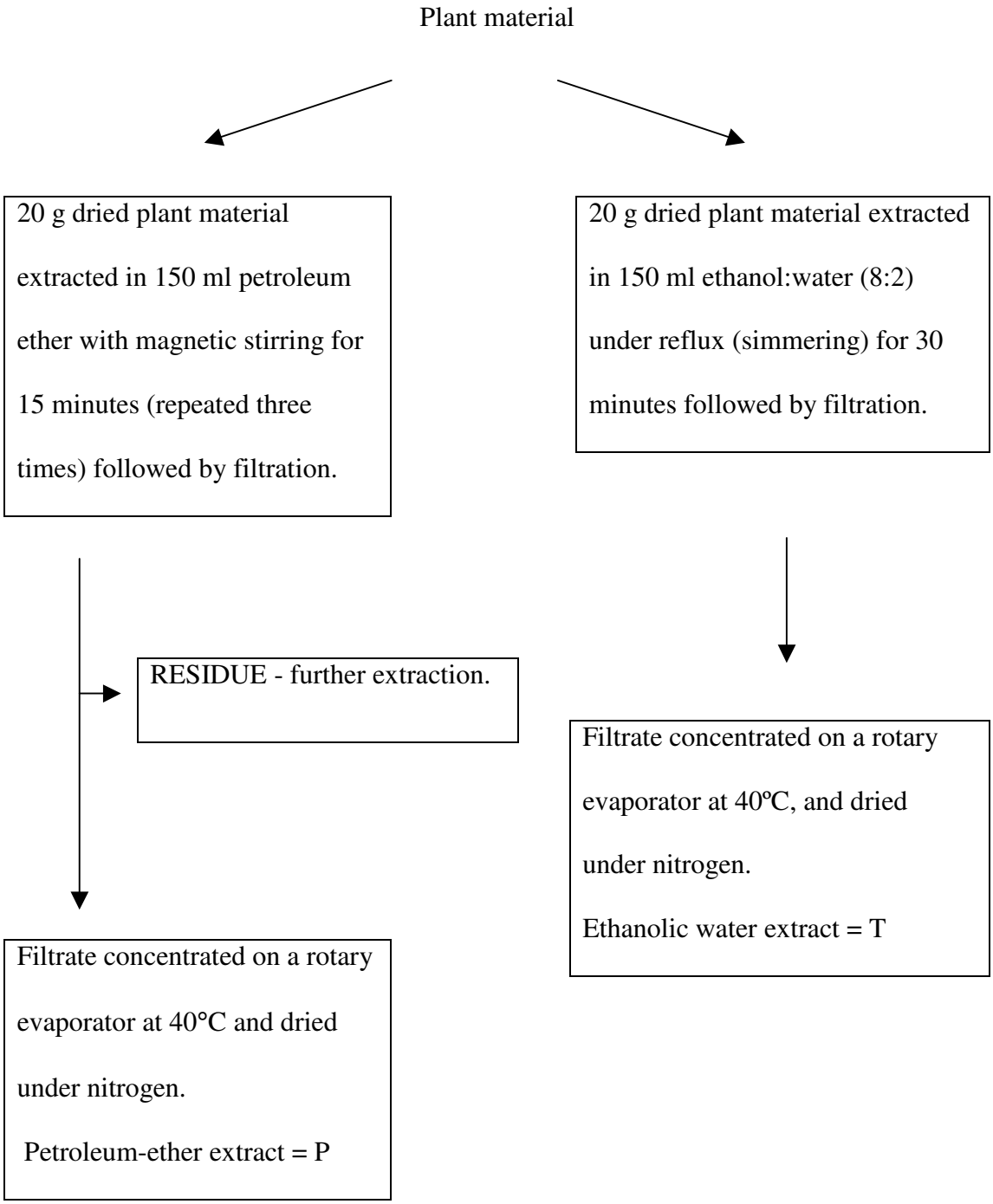
plant a herbarium specimen had been deposited in the H.G.W.J. Schweickerdt Herbarium (PRU) at the University of Pretoria. A total of four extracts were prepared for each plant, to maximise the extraction of different polarity compounds from the plant. Extracts were prepared by using petroleum ether, ethanolic water (8:2), ethyl acetate and methanol. The twelve plants selected are given in Table 3.1. For each plant the herbarium specimen number and the type of extracts prepared are given. For some of the extracts the residue was only a small amount and were therefore not used further in the bioassays.

Table 3.1 The twelve species analysed for anti-HIV activity, their herbarium specimen numbers and the type of extracts prepared (P = Petroleum ether, T = Ethanolic water, E = Ethyl acetate and M = Methanol).

Plant	**Herbarium specimen number**	**Extracts prepared**
Croton pseudopulchellus	(E A Prozesky 91)	P, T
Helichrysum melanacme	(N.L. 16)	P, T
Nidorella anomala	(N.L. 28)	P, T
Nidorella auriculata	(N.L. 29)	P, T
Ekebergia capensis	(G. Prinsloo 10)	P, T
Rapanea melanophloes	(G. Prinsloo 12)	P, T, E
Euclea natalensis	(G. Prinsloo 13)	P, T, E, M
Polygala myrtifolia	(G. Prinsloo 5)	P, T, E
Elaeodendron croceum	(G. Prinsloo 11)	P, T
Bidens pilosa	(G. Prinsloo 4)	P, T
Cryptocarya latifolia	(G. Prinsloo 14)	P, T
Protasparagus africanus	(G. Prinsloo 15)	P, T

3.2.2 Extract preparation

The plant extracts were prepared according to the procedure in Figure 3.1.



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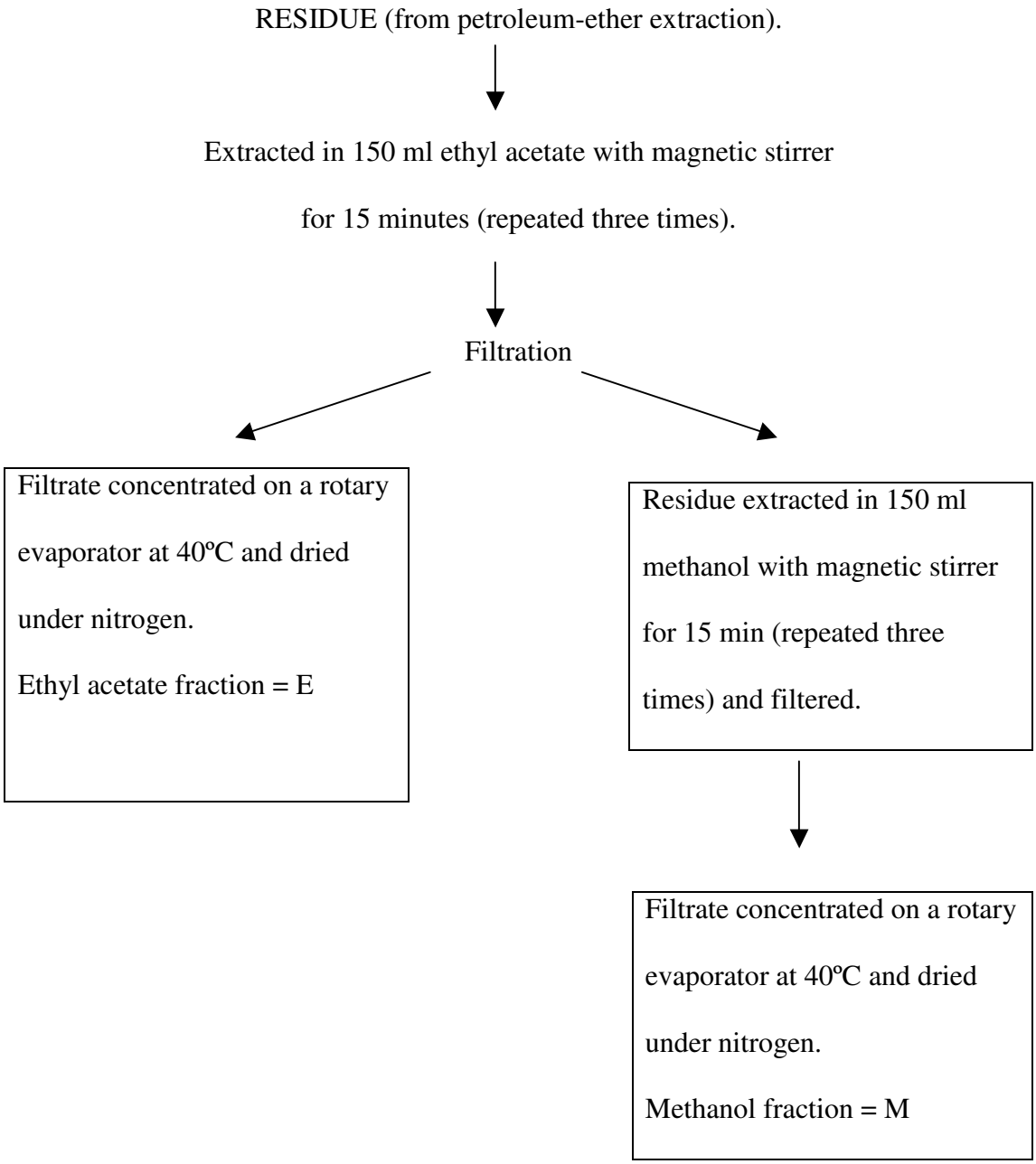

Figure 3.1 Extraction procedure.

After preparation of these extracts, it was analysed at the University of Cordoba in Spain for activity against HIV. The *E. croceum* ethanolic extract was selected for the isolation of active compounds, because it had the best anti-HIV activity. The HeLa-Tat-Luc and MT-2 VSV pseudotype assays and results will be discussed in Chapter 4.

3.2.3 Isolation of active compounds

Liquid-liquid fractionation was used to remove a large quantity unwanted compounds from the fraction. The extract volume was measured and an equal amount of chloroform was added for fractionation, and the process was repeated three times. The green chloroform layer that was below the red water layer was removed. The green chloroform extract was combined and concentrated on a rotary evaporator. This extract was then separated on a silica gel column.

The *E. croceum* extract was separated on a dry silica column (silica gel 60). The concentrated extract was mixed with a small amount of dry silica gel powder. The extract and silica gel powder mixture was allowed to dry overnight, to allow complete evaporation of the solvent. The extract and silica gel mixture was then added to the prepared dry silica gel column, and covered with a layer of clean dry silica gel powder. A variety of solvents from apolar to polar were used, starting with the apolar solvents. The following solvents were used during the separation process: hexane, ethyl acetate, acetone, ethanol and methanol. The concentrations were changed from 100% solvent to 70:30, 50:50, 30:70 ratios with the next solvent. Hexane removed most of the non-polar compounds from the column, and was used until all the yellow and orange compounds eluted from the column. As the coloured bands eluted from the column, the concentration of hexane was decreased and the concentration of ethyl acetate slowly increased. As the ethyl acetate was increased, a yellow and later a dark green band eluted form the column. Most of the green colour eluted from the column as the ethyl acetate reached a 100% concentration in the column. After all the colour bands had eluted from the ethyl acetate solvent, the acetone concentration was

increased very slowly. After acetone, ethanol and methanol were used as solvents. The concentration of ethanol and methanol was increased very slowly as to allow all the compounds to elute from the column before the polarity changed too much. After the ethanol concentration was increased it seemed as if most of the compounds were already eluted from the column. Methanol was used to clear compounds that were still trapped in the column. Very few compounds eluted from the column at this stage.

The fractions were concentrated and evaluated by means of thin layer chromatography (TLC) on silica gel 60 aluminium plates. The best separation of the compounds on the TLC plate was obtained by using a mobile phase of chloroform: methanol (95:5).

The TLC plates were evaluated and similar fractions were combined and the combined fractions were tested again for anti-HIV activity. After selection of the active fractions, these fractions were separated on a silica column again. A dry silica column had been prepared exactly as described with the separation of the crude extract. A smaller column had to be used, as the fraction quantity was much less than the original extract. The same solvents were used as with the previous separation method, although smaller quantities were used as most of the unwanted compounds had been removed from the fractions with the previous column.

The smaller fractions contained a maximum of five compounds, and some of the fractions were already purified to contain only a single pure compound. All these fractions were tested against HIV to determine which fractions or compounds inhibited the virus. The fraction containing the active compound was dried by rotary

evaporation, until only a small volume of the solvent was left. The concentrated fraction was then exposed to air to allow evaporation of the last solvents from the fraction. As the fraction was allowed to dry, a white precipitate formed. The precipitate was then removed and purified further by firstly adding a small amount of ethyl acetate to it to dissolve the impurities. The precipitate did not dissolve in ethyl acetate but did in methanol. The purified compound was identified as the active compound and analysed by spectroscopic methods to identify it.

The figure below (Figure 3.2) is a summary of the isolation process of the active compound.

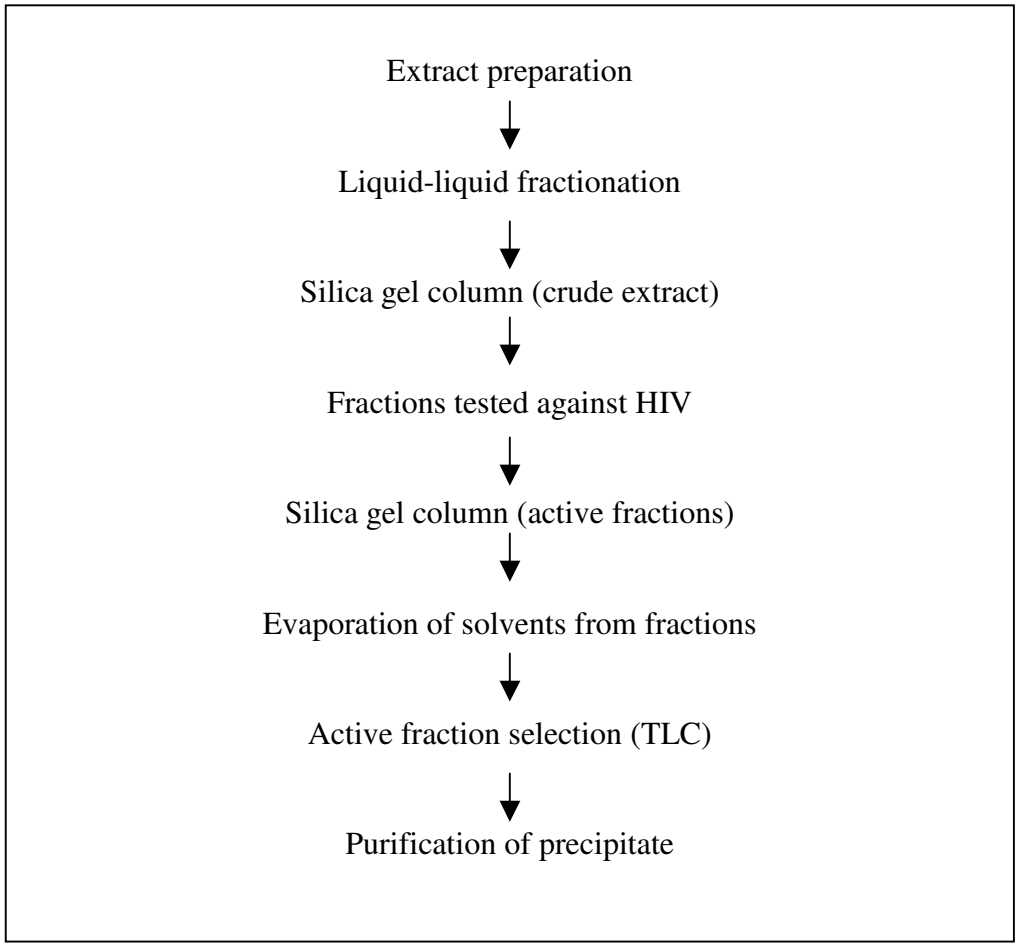

Figure 3.2 Diagrammatic representation of the isolation process.

3.2.4 Identification of the active compound

To identify and determine the structure of a compound can be a lengthy and difficult process, as several methods are used to determine the structure. The purified compound was identified by using ^{1}H-NMR, ^{13}C-NMR, HMQC, HMBC, COSY and NOESY analyses.

One-dimensional proton and carbon nuclear magnetic resonance (NMR) spectra were obtained first to get an idea of the type of compound. These analyses were performed in deuterated methanol on a 300 MHz Varian NMR apparatus. The two-dimensional NMR analyses were obtained on a 500 MHz Brücker apparatus. The results were compared with published data to determine the structure, and to obtain information on the specific compound that had been isolated.

3.3 Results

The anti-HIV activity results of the plant extracts and isolated compound will be discussed in Chapter 4.

Figure 3.3 shows a TLC plate with the fractions obtained from the first silica gel column using a solvent system of chloroform: methanol (95:5).

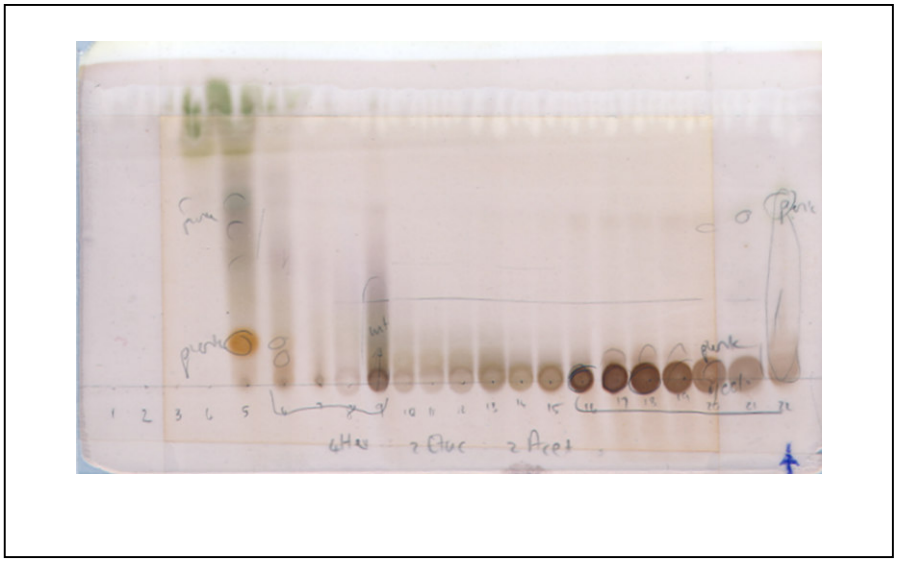

Figure 3.3 TLC (silica gel 60) plate showing column separation of the ethanolic water extract. The TLC was developed with a solvent system of chloroform: methanol (95:5).

The presence of the compound was confirmed by using TLC plates. After spraying the plates with vanillin colour regent, the characteristic blue colour could be used to identify the compound. Figure 3.4 shows the purified compound on a TLC plate. The compound proved to be a large molecular mass compound with an aglycone and a sugar moiety which is responsible for the striped effect on the TLC plate. It was eventually identified as digitoxigenin-glucoside (Figure 3.5). The structure consists of a terpenoid with an attached glucose molecule and a lactone ring. The compound forms part of the well-known group of cardiac glycosides.

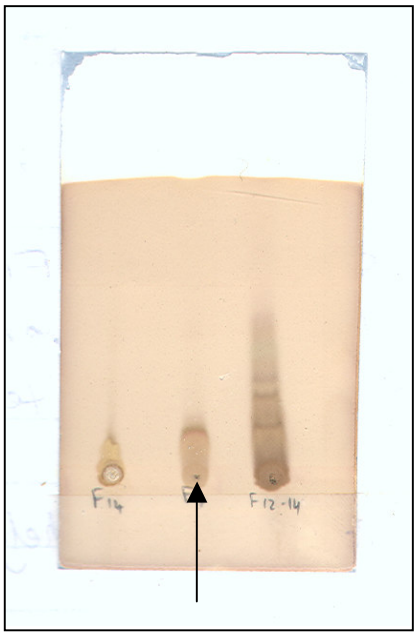

Figure 3.4 TLC plate showing the purified compound digitoxigenin-glucoside, isolated from the active fraction.

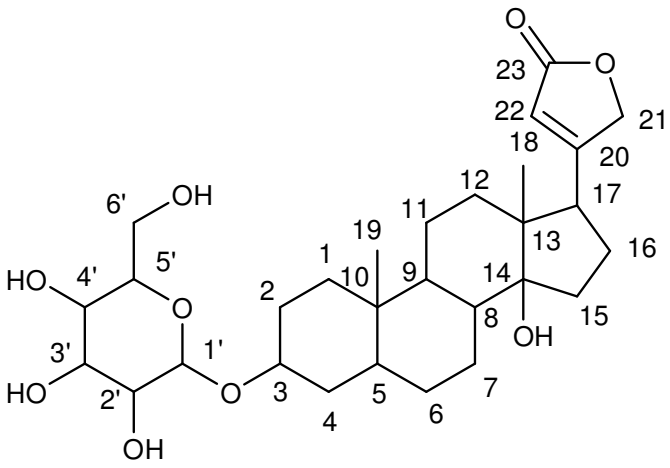

Figure 3.5 Structure of digitoxigenin – 3-O-glucoside.

Figure 3.6 shows the one-dimensional ^{1}H-NMR of the compound, with the chemical shift values given in Table 3.2 together with the ^{13}C-NMR results.

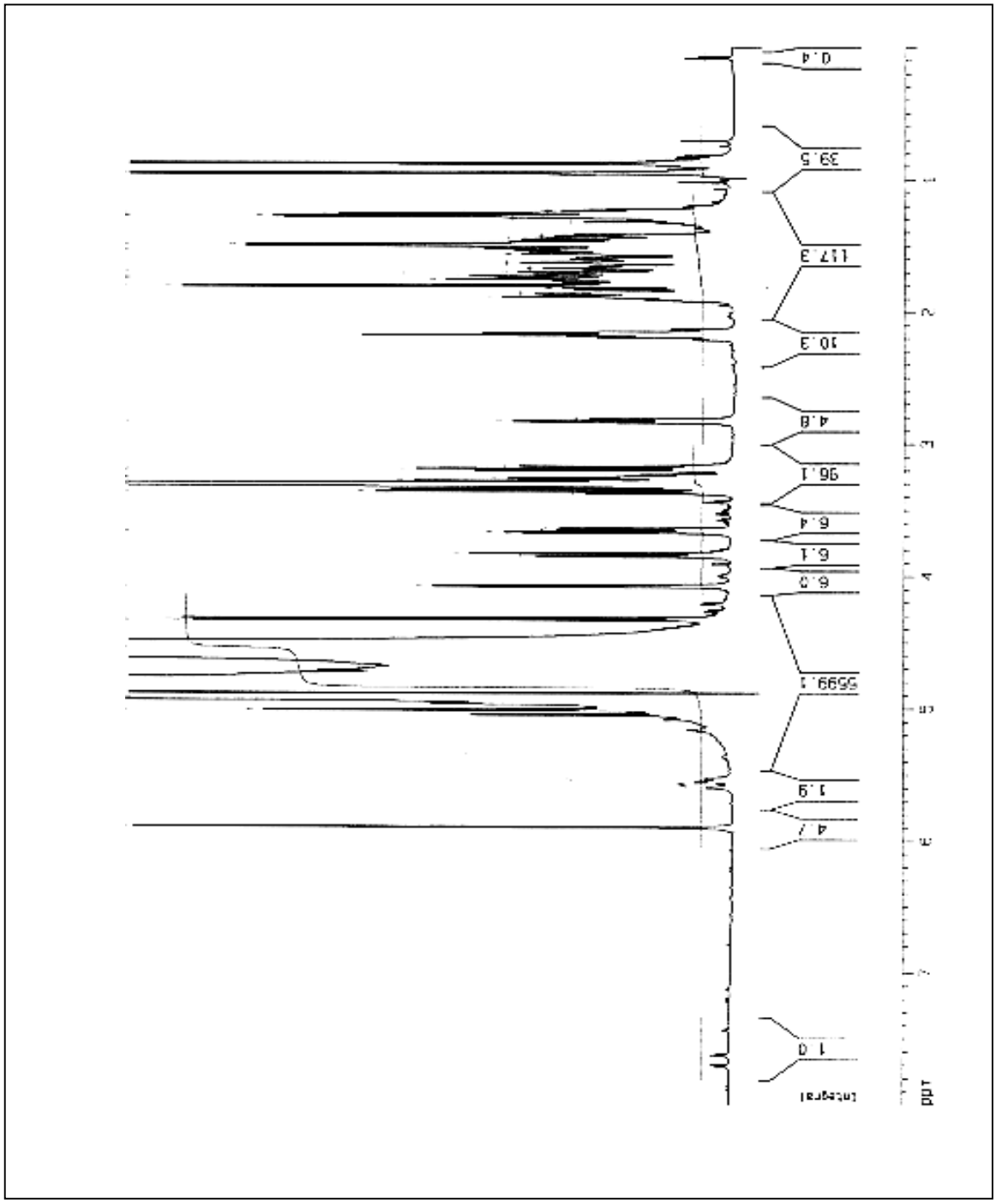

Figure 3.6 ^{1} H-NMR of purified digitoxigenin-glucoside in deuterated methanol.

Figure 3.7 shows the ^{13}C-NMR spectrum of digitoxigenin-glucoside ranging from 0-200ppm. The ^{13}C-NMR of the isolated compound correlates well with values published by Rathore *et al.* (1985) and by Cheung *et al.* (1981). The ^{13}C-NMR, HPLC and MS values have been published by Kawaguchi *et al.* (1989).

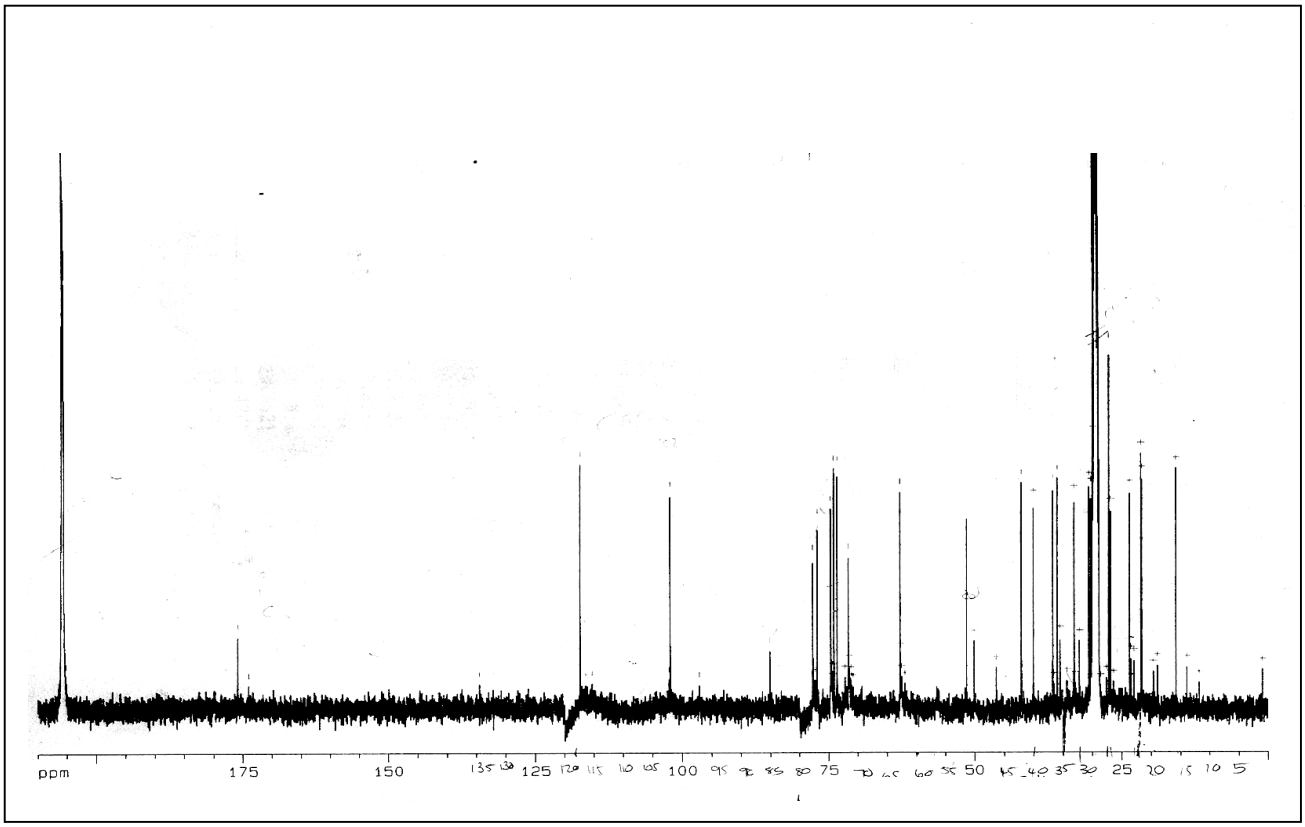

Figure 3.7 ^{13}C-NMR of digitoxigenin-glucoside in deuterated methanol.

Table 3.2 ^{1}H-NMR and ^{13}C-NMR values for digitoxigenin-glucoside in deuterated methanol.

C-atom	^{13}C-chemical shift	^{13}C-chemical shift Rathore *et al.*,1985	^{1}H-chemical shift
1	30.9	30.06	2.83, 2.6t
2	28.0	29.61	2.23, 1.84t
3	72.0	70.54	3.3d
4	34.0	35.20	2.23, 1.78t
5	36.8	36.64	1.7d
6	27.7	26.56	1.9, 1.27t
7	22.4	21.21	1.45, 1.28t
8	42.4	41.79	1.67d
9	36.9	35.74	1.8d
10	37.5	35.20	
11	22.5	21.28	1.81, 1.28t
12	41.0	40.03	1.5, 1.5t
13	52.0	49.63	
14	86.0	85.34	
15	31.3	33.08	1.45, 1.58t
16	27.8	26.92	1.9, 2.23t
17	50.0	51.03	2.85d
18	16.4	15.74	0.75q
19	24.1	23.62	0.95q
20	172.0	174.78	
21	75.8	73.44	4.9, 5.0t
22	117.7	117.50	
23	172.1	174.78	5.9d
1'	102.6	95.01	4.3d
2'	75.7	90.49	4.08d
3'	77	81.96	3.32d
4'	75.5	75.57	3.15d
5'	77	78.18	3.22d
6'	63.1	68.95	3.58, 3.79d

COSY is a two-dimensional NMR analysis showing the hydrogen atoms that are attached to specific carbon atoms. Hydrogen atoms in close proximity will be indicated for each carbon atom. The COSY spectrum of the isolated compound is given in Figure 3.8.

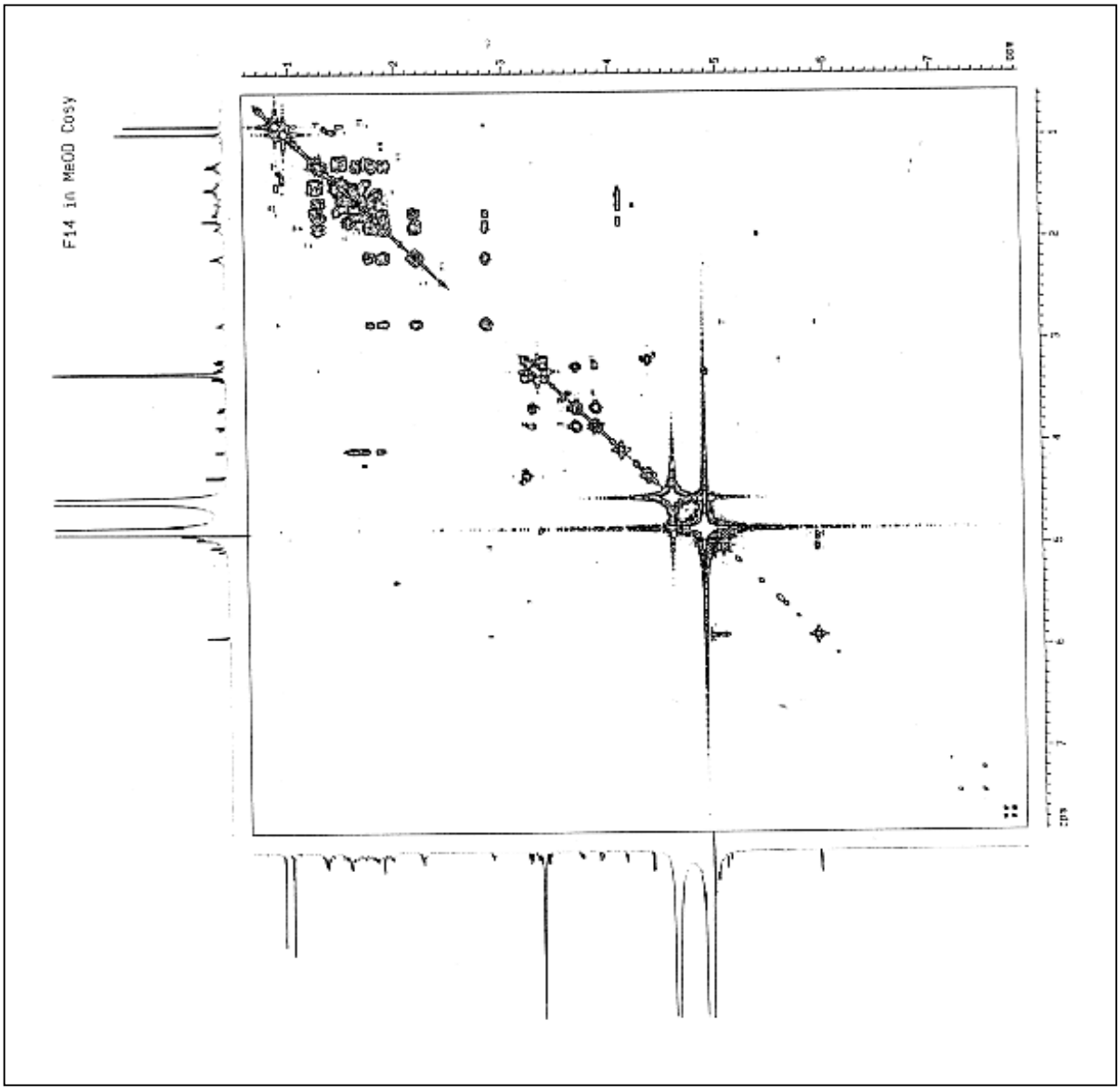

Figure 3.8 COSY analysis for digitoxigenin-glucoside in deuterated methanol.

HMBC spectra show the linked hydrogen and carbon atoms in the structure over short distances of one or two C-H bonds (Figure 3.9).

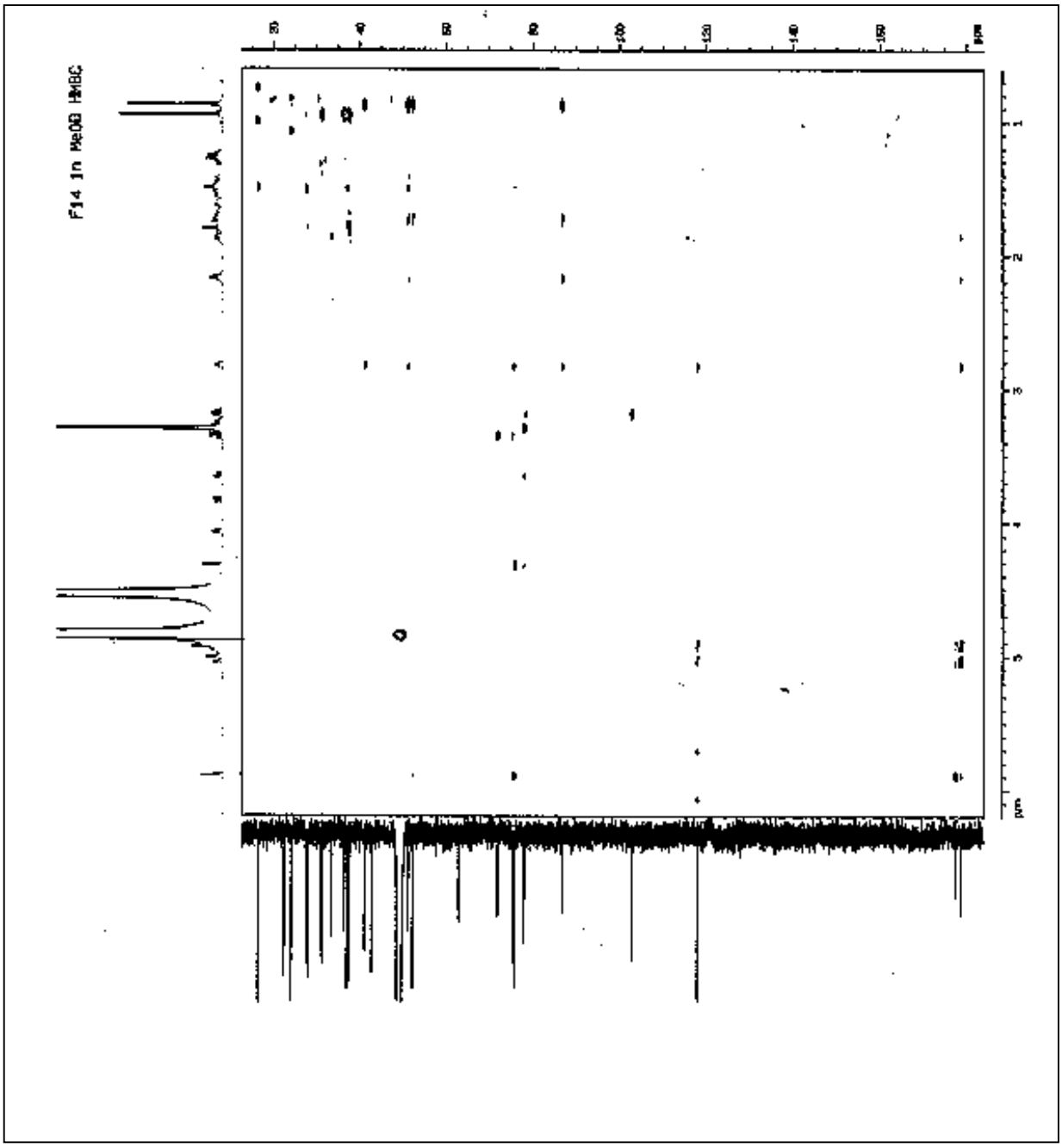

Figure 3.9 HMBC for digitoxigenin-glucoside in deuterated methanol.

The HMBC spectrum showed cross-peak connectivity between Me-18/C-17, C-13, C-12, C-14; Me-19/C-1, C-5, C-9, C-10; H-17/C-13, C-21, C-14, C-20, C-23; H-1`/C-3; CH_2-17/C-22, C-20, C-23; H-23/C-21, C-20, C-23. The cross-peak connectivity for digitoxigenin-glucoside is shown in Figure 3.10.

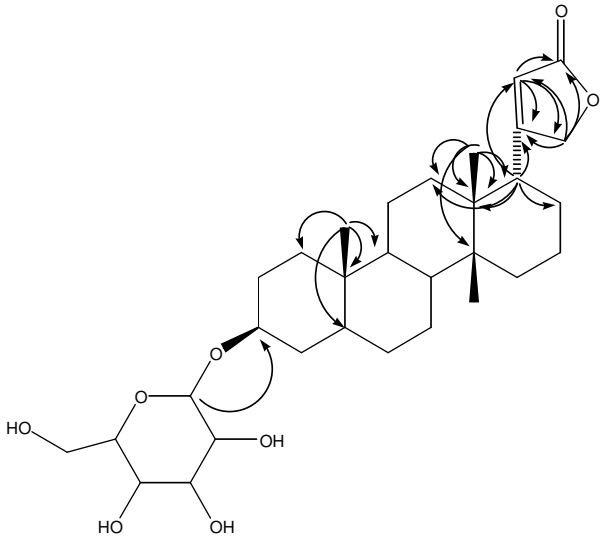

Figure 3.10HMBC cross-peak connectivity for digitoxigenin-glucoside.

The HMQC spectrum (Figure 3.11) indicates the linked hydrogen and carbon atoms in the structure over longer distances with more than two hydrogen atoms attached to a single carbon atom.

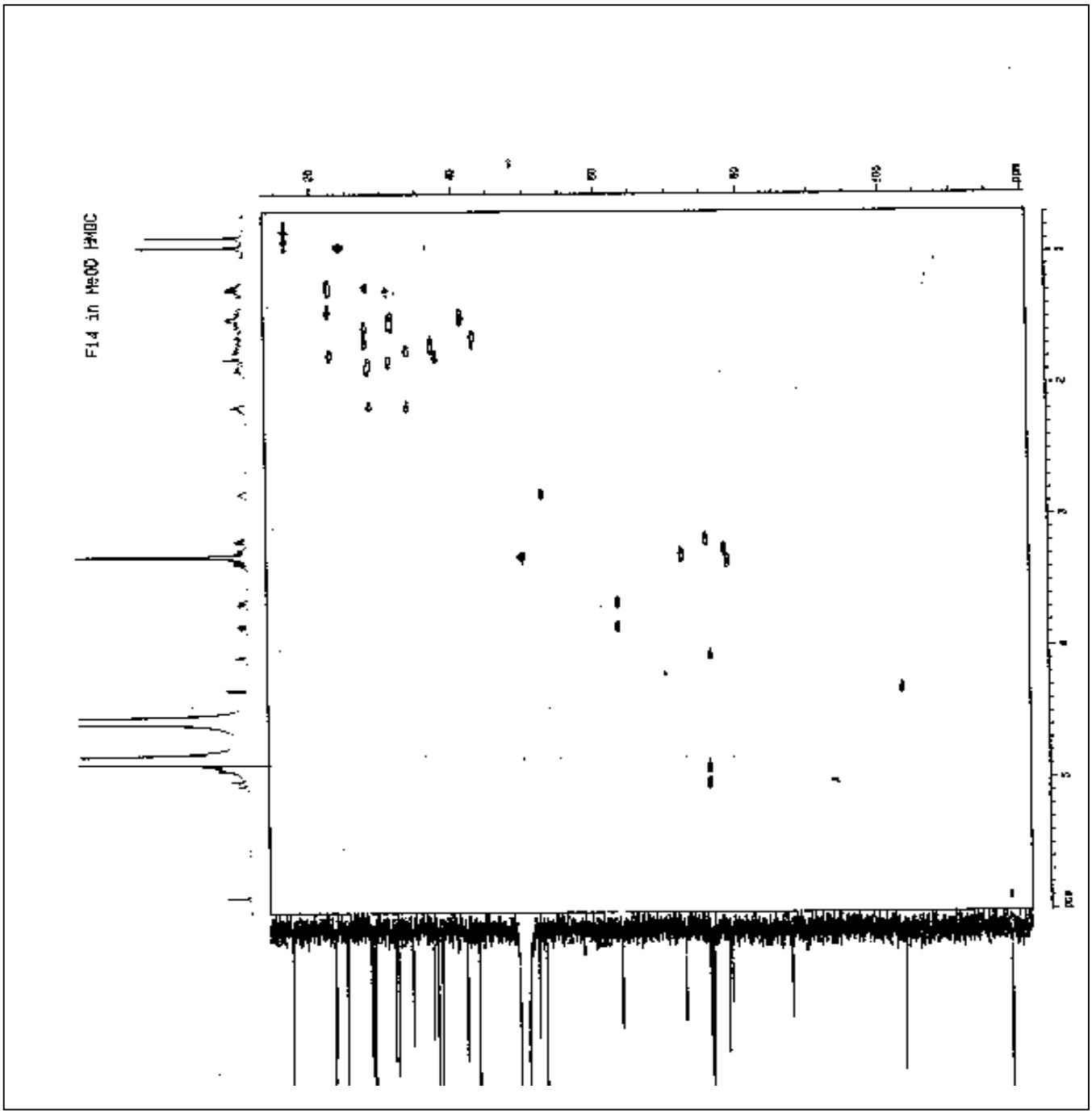

Figure 3.11 HMQC for digitoxigenin-glucoside.

3.4 Discussion

Bio-guided fractionation was used to identify and eventually isolate the active compound from the crude extract. From the twelve plant extracts that were prepared, only the extract from *E. croceum* showed promising results against the anti-HIV assays that have been used in the study.

The elucidation of the chemical structure of digitoxigenin – 3-O-glucoside was carried out using one- and two-dimensional NMR. The ^{1}H-NMR of digitoxigenin – 3-O-glucoside showed the presence of two singlet methyl groups at δ_H 0.89 and 0.92, and signals at δ 5.86 (t, J = 1.8 Hz), 9.83 (dd, J = 18.1, 1.8 Hz), 7.99 (dd, J = 18.1, 1.8 Hz) indicating the presence of an α, β – unsaturated lactone a doublet proton at 4.34 (d, J = 6.6) of β –orientation and an anomeric proton of the attached glucose. ^{13}C-NMR spectrum of digitoxigenin – 3-O-glucoside showed 29 carbons (2 methyls, 11 methylene, 11 methine and 5 quaternary carbons). Signals at δ_C 77.3, 77.0, 75.7, 75.8, 62.1 and 102.6 indicated the presence of a glucose molecule. According to the NMR data (Table 3.2, Figures 3.6-3.11), the compound was identified as digitoxigenin 3-O-glucoside (Figure 3.5), a known compound, which was isolated from *Digitalis lanata* (Singh & Rastogi, 1970) before, but with no medicinal uses related to its antiviral activity.

The compound forms part of the cardiac glycosides, which is known for its potent cardiac activity. These molecules are also known for their toxicity, and might explain the activity of the compound against HIV. It was therefore important to determine the toxicity of the compound and the extract. The toxicity results are discussed in Chapter five.