C H A P T E R 6

6. ISOLATION OF BIOACTIVE COMPOUNDS FROM *D. VISCOSA*

6.1 Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is one of the most powerful tools available for determining the structure of both organic and inorganic species. NMR in conjunction with other analytical instruments such as ultraviolet (UV) and mass spectrometer (MS) can provide useful information for elucidating the structure of the isolated pure compounds. MS provides information in terms of molecular mass of the structural components of isolated compound; as such an accurate measure of the molecular weight of the compound can be obtained (Skoog and West, 1980). In this study, the crude leaf extract of *D. viscosa* was fractionated and purified by column chromatography on variety of supports and the structure of isolated compounds elucidated using NMR and MS.

6.2 Methodology

6.2.1 Chromatographic purification of the extracts.

Initially, 5 kg of dried material of *D. viscosa* was serially extracted with 3 x 5L of the following solvents: hexane, DCM, acetone and methanol respectively. The extracts were filtered with Whatman no. 1 filter paper and dried using rotary evaporator at a reduced pressure and a temperature below 40 °C. The residues were allowed to dry under a stream of air. The TLC analysis and bioautography were determined in order to select the extracts with a number of components of good bacterial and antioxidant activities.

Briefly, 30.1 g of DCM extract was loaded onto vacuum liquid chromatography (VLC) (95 X 480 mm) packed with silica gel 60 particle size 0.063-0.2 mm (70-230 mesh) (Fluka Chemika) . The column was eluted with 750 ml at a gradient system beginning with pure hexane (100%) through hexane-EtOAc (90:10, 80:20, 70:30, 50:50, 30:70, 20:80, 10:90, 0:100) and finally with EtOAc: MeOH (95:5, 90:10, 80:20, 70:30, 0:100). About 150 ml were collected in test tubes and eluents from test tubes that exhibited similar Rf values as indicated by TLC analysis upon spraying with vanillin-sulphuric acid and visualized under UV, were pooled together and a total of 15 fractions were obtained and dried. TLC analysis and bioautography were performed and fractions with good antibacterial activity were subjected to subsequent column chromatography (11 cm height x 10 cm diameter) over silica gel 60 (17cm x 4.9 cm) with 320 ml eluent (EtOAc: MeOH as outlined above). Fractions that were found to have similar R_f values as revealed by TLC chromatogram pattern were pooled together. The following fractions (fractions 3, 4+5, 6+ 7+8) were selected for further purification by additional chromatographic procedures. Some of these fractions contained only one compound by TLC. After removing the solvent structures were elucidated by NMR and MS. After the structure of the pure compounds was obtained the samples were recovered and used for biological assays. The

above procedure was applied to further purify 3.240 g of acetone crude extract. Fractions from tubes 24-26 were combined and further purified using Sephadex LH 20 pre-swollen with DCM:MeOH ratio (2:1) and the column was eluted with 50 ml of the DCM:MeOH mixtures and 10 ml of eluent was collected and air dried.

6.3 Results and Discussion

6.3.1 Crude extract obtained by VLC

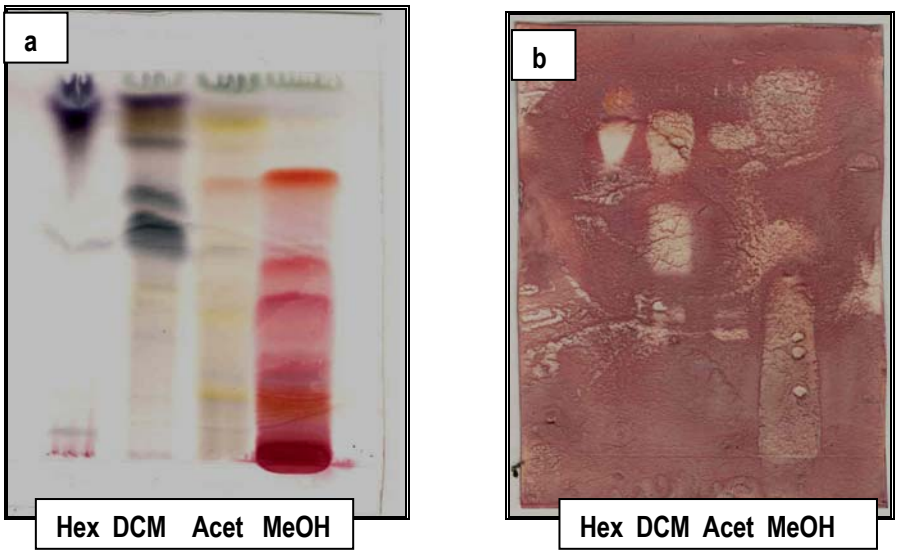


Figure 6. 1: TLC chromatograms separated by CEF eluent system: (a) shows the TLC profile of compounds separated by CEF solvent system followed by spraying with vanillin in sulphuric acid. (b) indicates bioautography of extracts against *S. aureus*.

6.3.2 Chromatographic purification

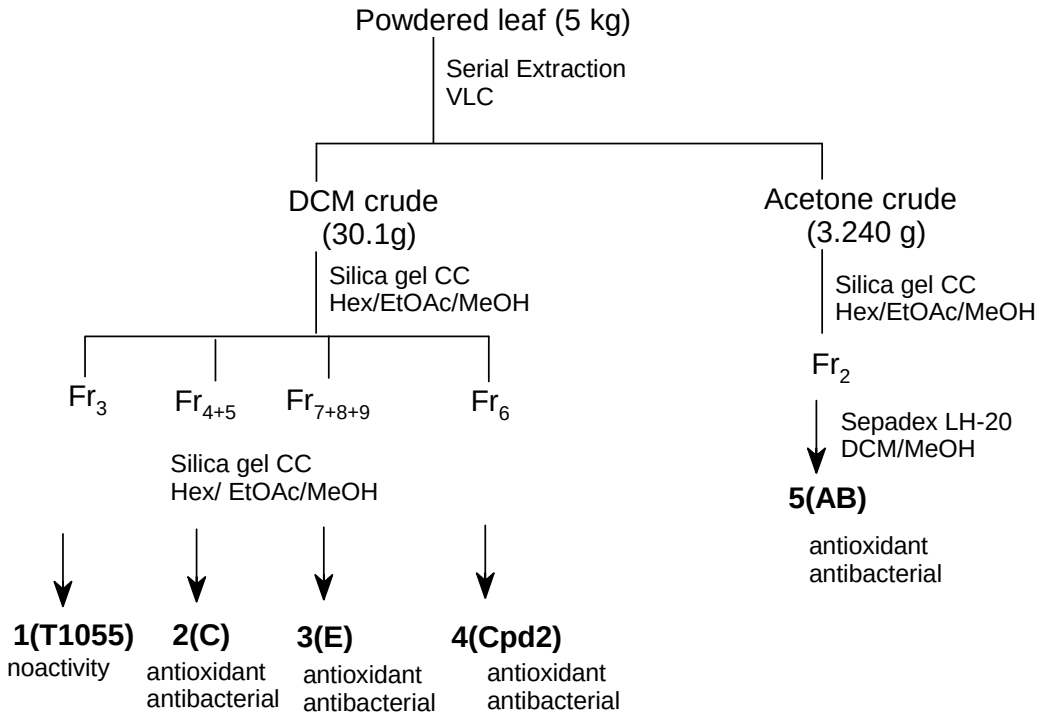


Figure 6.2: Schematic representation of isolation and purification of compounds from *D. viscosa* crude leaf extract.

6.3.1.1 Purification of DCM crude extracts

From serial extraction of *D. viscosa* crude material, four extracts were obtained, hexane, DCM, acetone and methanol (Fig 6.1**)**. Only DCM and acetone extracts were selected for further purifications because they showed more compounds with good activity against *S. aureus.* Fifteen fractions were obtained from the DCM extracts (Fig. 6.3) and to obtain pure compounds, fractions of similar TLC profile and activity were pooled together, evaporated and rechromatographed. Four column chromatography experiments were run separately and fractions (3; 4+5; 6; 7+8+9) were subjected to separate column chromatography packed with silica gel 60 and eluted with Hexane:EtOAc with the following increasing polarity (100:0; 90:10; 80:20; 70:30; 50:50; 30:70; 20:80; 10:90; 0:100). The effluents collected in test tubes (50 mL) were allowed to dry and upon drying some compounds crystallized forming pure compound whereas some were further purified using Sephadex LH20. The Sephadex LH20 column was eluted with DCM: MeOH at ratio of 2:1.

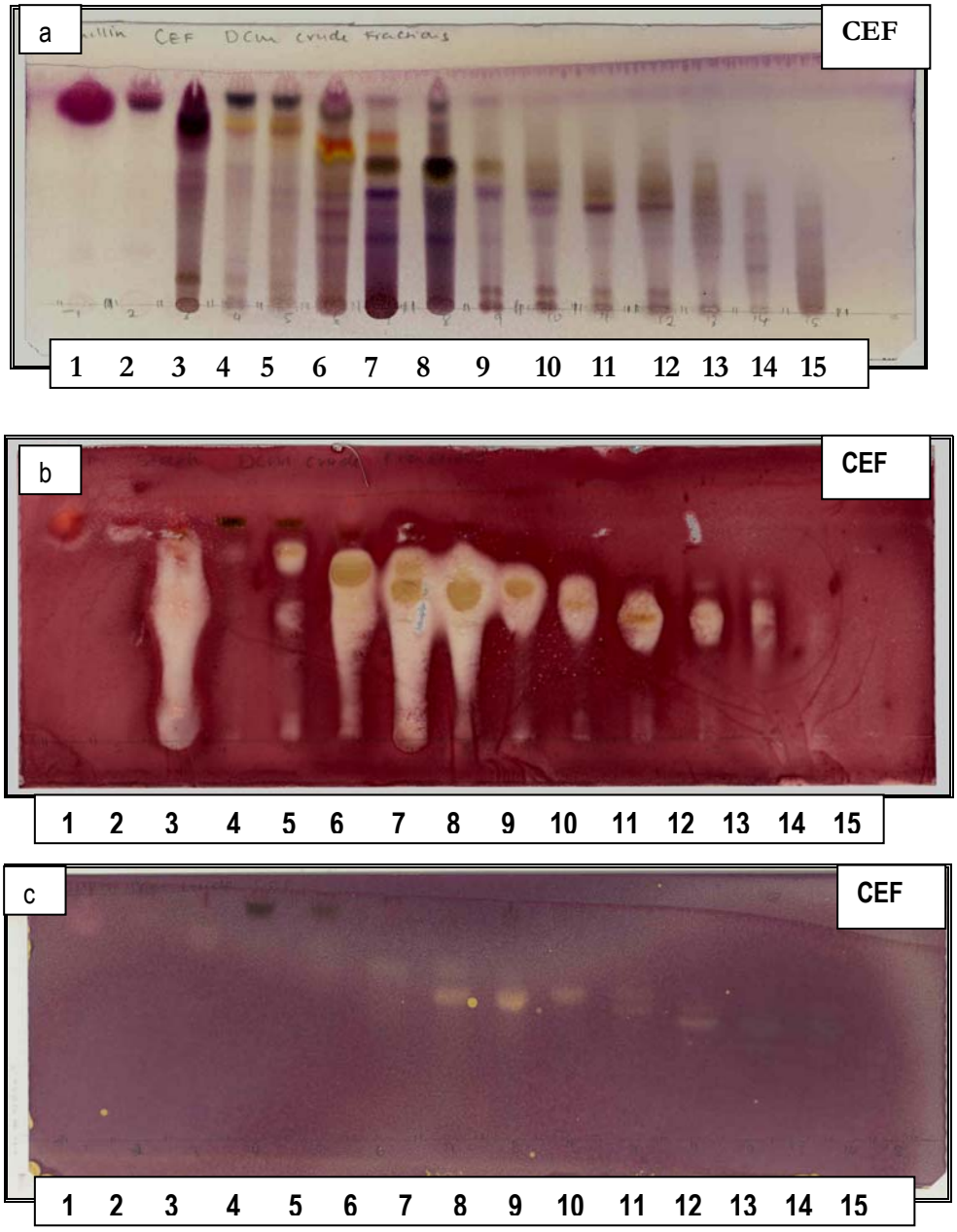


Figure 6.3: TLC chromatograms (a) TLC pattern of fractions obtained from DCM VLC and the extracts were separated by CEF solvent system followed by spraying with vanillin-sulphuric acid. (b) bioautography of VLC fractions against *S. aureus*, (c) TLC chromatogram of antioxidant activity after spraying with 0.2% DPPH in methanol.

Purification of fraction 3

Fraction 3 (0.89 g) was further purified by using column chromatography silica gel 60 and various fractions were collected into 50 ml test tubes and allowed to dry. The fractions that had similar TLC pattern were pooled together

resulting in 5 fractions (Fig. 6.4). Sample in test tube 3 formed yellow crystals (9 mg) upon drying and the pure compound was coded T1055 (Compound 1), which had R_f of 0.84 when separated with CEF eluent system.

Figure 6.4: TLC profiles of pooled fractions from column chromatography of fraction 3 eluted with CEF. Sample from test tube 3 formed a pure Compound 1.

Purification of combined fractions 4+5

Column chromatography on silica gel of combined fractions 4+5 (2.106 g) gave fractions of various TLC profiles **(**Fig. 6.5). Fractions from test tubes with similar TLC patterns were pooled together and allowed to dry under a stream of air. A pure compound in test tubes 48-60 formed yellow crystals (8 mg) upon drying and was coded C (Compound 2) with R_f of 0.79 when developed in CEF solvent system.

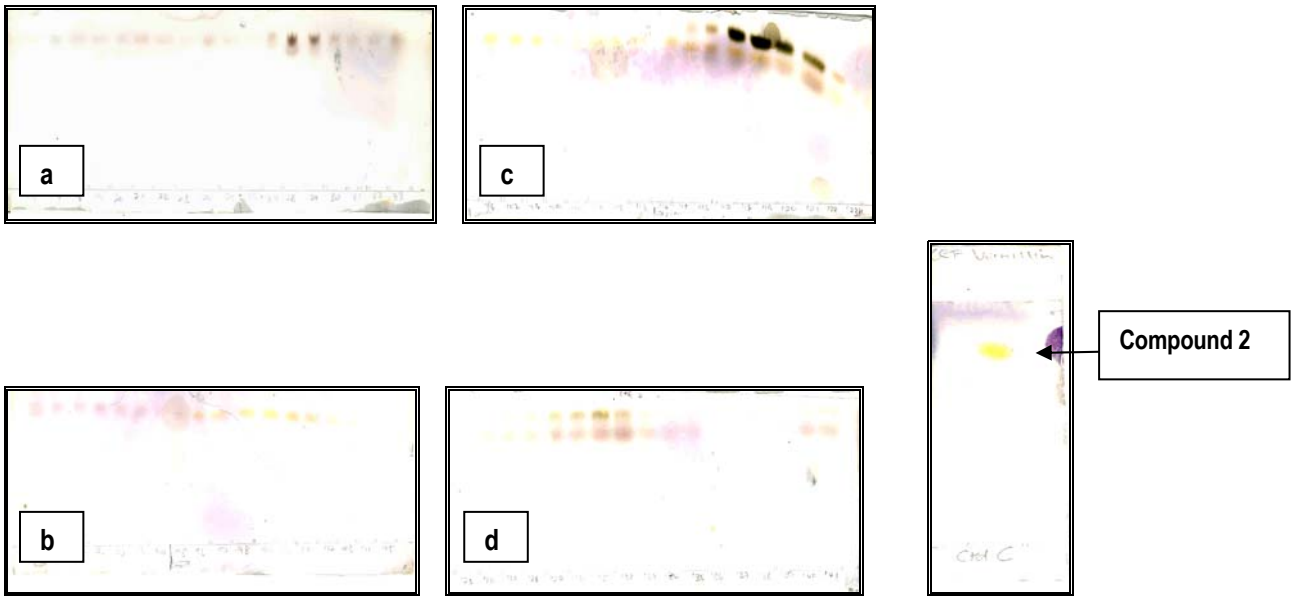


Figure 6.5: TLC chromatogram obtained from fractions 4+5 column chromatography separated by CEF. Fractions were collected from (a) test tube 1-30, (b) test tube 31-50, (c) test tubes 51-66, (d) test tube 67-78.

Purification of combined fractions 7+8+9

Combined fractions 7+8+9 (1.033 g) was subjected to column chromatography silica gel 60 (Fig. 6.6) and eluted with Hex: EtOAc to produce various fractions and the ones with similar TLC profiles were pooled together producing pure compounds upon drying. Eluents in test tubes 72-78 upon drying, formed pure compound (yellow powder) (25 mg) and was coded E (Compound 3) with R_f of 0.61 when developed in CEF solvent system.

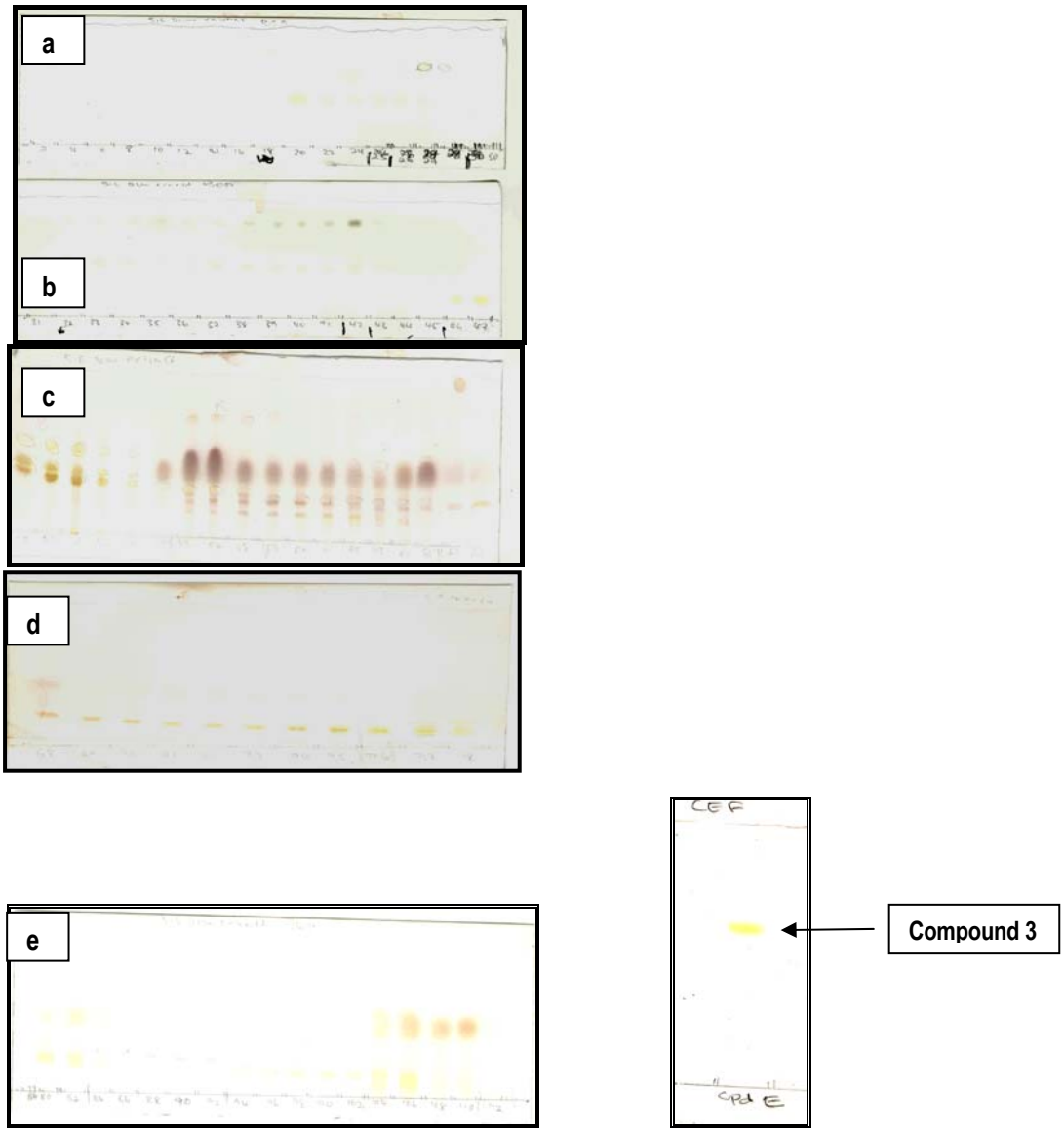


Figure 6.6: TLC chromatogram of fractions obtained from combined fractions 7+8+9 column chromatography separated by BEA eluent system. Fractions were collected from (a) test tube 1-28, (b) test tube 30-48, (c) test tubes 50-66, (d) test tubes 68-78, (e) test tubes 80-112.

Purification of fraction 6

Fraction 6 (1.8 g) was subjected to a small column and several fractions were obtained. Constituents of test tubes 23-25 upon drying crystallized; the compound was cleaned with Sephadex LH20 eluted with DCM: MeOH (2:1). The pure compound, a yellow powder (7mg) was coded Cpd2 (Compound 4) with R_f of 0.61 when developed in CE eluent system (Fig 6.7).

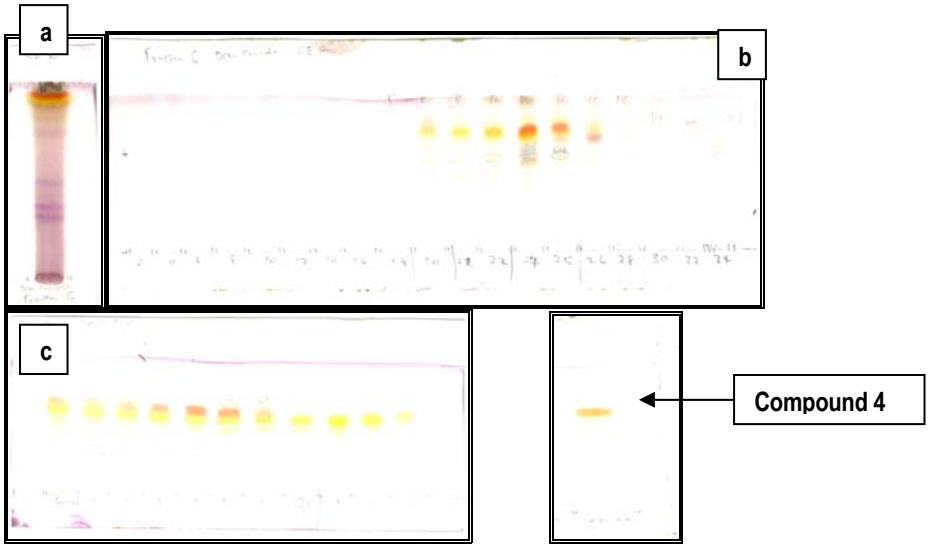


Figure 6.7: TLC chromatograms of fractions collected from silica gel column chromatography separated by CEF eluent system. (a) DCM crude extract; (b) fractions collected from test tubes 1-34 (c) further purification of fractions from tubes 23-25 using Sephadex LH20; (d) pure compound Cpd2 (compound 4).

6.3.1.2 Purification of acetone crude extract.

Acetone crude extract (3.240 g) was fractionated with column chromatography on silica gel 60 and tubes 24-26 were combined as they had the same TLC profile. The extract from tubes 24-26 was further subjected to a Sephadex LH20 chromatography eluted with 50 ml of DCM: EtOAc ratio (2:1). A pure compound (yellow powder) was obtained and coded AB (Compound 5) (Fig. 6.8) at R_f of 0.41 when developed in CE eluent system.

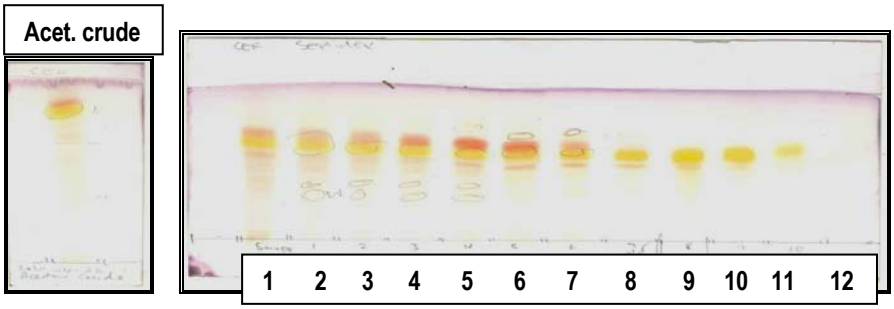


Figure 6.8: TLC profiles of acetone crude eluted on Sephadex LH20 column chromatography, fractions from test tubes 1-12 was obtained and pure Compound AB (compound 5) was obtained from test tube 8-11 by crystallization.

6.3.2 Structure Elucidation of the Isolated Compounds

6.3.2.1 Compound 1

The mass spectrum showed molecular ion peak at m/z = 329, [MH^+, 100%] base peak, corresponding to the molecular formula $C_{18}H_{16}O_6$ and also an intense peak at 285 (M-43) (Fig 6.10) corresponding to the standard flavonol C-ring contraction (Harborne,1994) for 3-methyl ether flavone. The ^1H NMR ($CDCl_3$) spectrum showed three methoxy signals at δ 3.84, 3.86 and 3.88 (3H each, s, OMe-3, 7 and 4'. There was presence of AA'BB' system due to ring B at δ 7.03 (2H, d, J= 9.0 Hz, H-3', H-5') and δ 8.08 (2H, d, J=8.7, H-2', H-6'). The presence of free 5-OH group was confirmed by chelated -OH signal at δ 12.66. ^{13}C NMR data are presented in Table 6.1. The spectral data are in close agreement with those reported in the literature (Table 6.1), the compound is therefore 5-hydroxyl- 3, 7, 4'-trimethoxyflavone (Fig. 6.9). This compound had previously been reported from many plant species e.g. *Siparuna apiosyce* (family Monimiaceae) (Leitao *et al*., 2000) and Aniba species (Rossi *et al*., 1997). There was a discrepancy in C-2 value and this was resolved by comparing our ^{13}C NMR data with several 3- OMe flavones (Horie e*t al*., 1998) and kaempferol (Markham, 1982). C-2 of 3- OMe flavone shifted down field to ≈ 155.

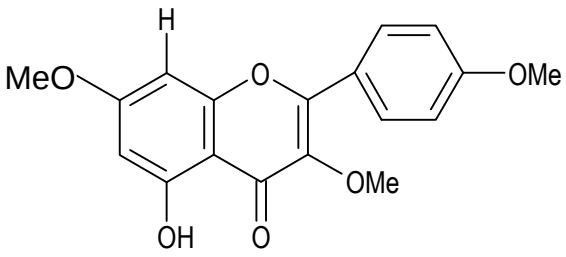
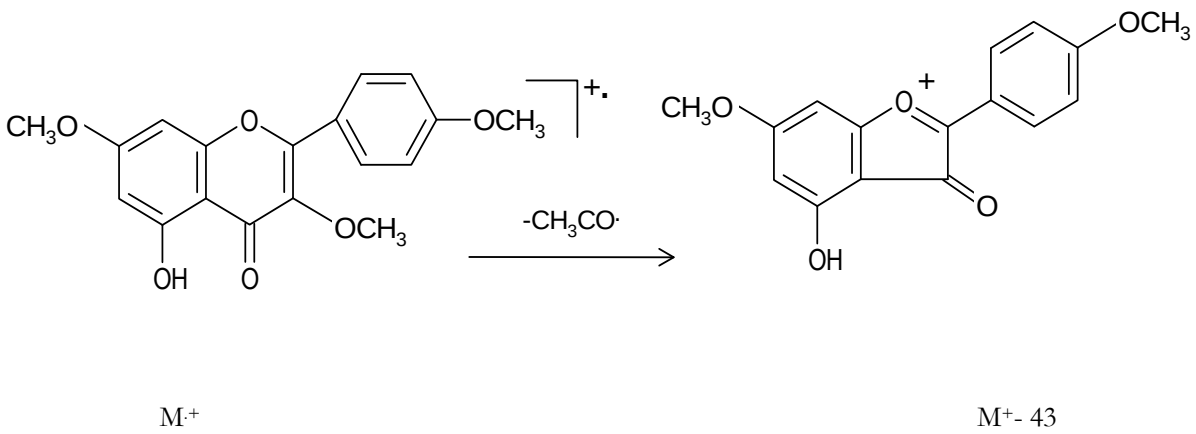


Figure 6.9: Structure of 5-hydroxy -3, 7, 4'-trimethoxyflavone.



M·+ M+- 43

Figure 6.10: Mass Spectrum Fragmentation of 5-hydroxyl-3, 7, 4'-trimethoxyflavone

Table 6.1: ^{13}C NMR data of Compound 1

^{13}C Assignment	Compound 1 ($CDCl_3$)	5-hydroxy-3,7,4' trimethoxyflavone ($CDCl_3$) (Rossi *et al.*,1997)
2	156.0	148.0*
3	138.8	138.9
4	178.8	178.9
5	162.0	161.7
6	97.8	97.8
7	165.4	165.4
8	92.1	92.2
9	156.7	156.8
10	106.0	106.0
1'	122.8	122.8
2', 6'	130.1	130.0
3', 5'	114.0	114.0
4'	161.6	162.0
OMe -4'	55.8*	55.8
OMe-7	55.4*	55.4
OMe-3	60.1	60.1

*Interchangeable

6.3.2.2 Compound 2

The mass spectrum showed molecular ion peak at m/z = 344, [$M^{+\cdot}$ 100%] base peak, corresponding to the molecular formula $C_{18}H_{16}O_7$ and also intense peaks at 329 [M-CH_3] and 301 [M-43] (Fig. 6.12) corresponding to 6-OCH_3 flavonol fragmentation (Markham, 1982) and standard flavonol C-ring contraction (Harborne, 1994) for 3-methyl ether flavone respectively. The ^{1}H NMR (acetone-d_6) spectrum showed three methoxy signals at δ 3.87, 3.88 and 3.90 (3H each, s, OMe-3, 6 and 4′) and there was presence of AA'BB' system due to ring B at δ 7.12 (2H, d, J= 9.6 Hz, H-3', H-5') and δ 8.11 (2H, d, J=9.3, H-2', H-6'). The presence of free 5-OH group was confirmed by chelated -OH signal at δ 12.97. ^{13}C NMR data are presented in Table 6.2. The spectral data are in close agreement with those reported in the literature (Table 6.2). The compound is therefore 5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone (santin) (Fig. 6.11). Santin had earlier been reported from the leaf extract of *D. viscosa* (Sachdev and kulshreshtha, 1982; Abdel-Mogib *et al*., 2001).

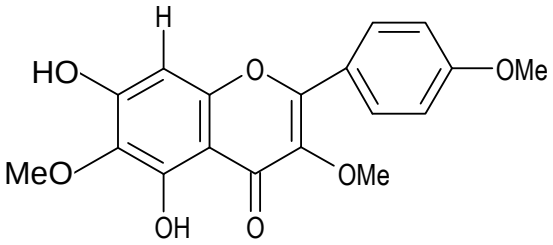


Figure 6.11: Structure of 5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone

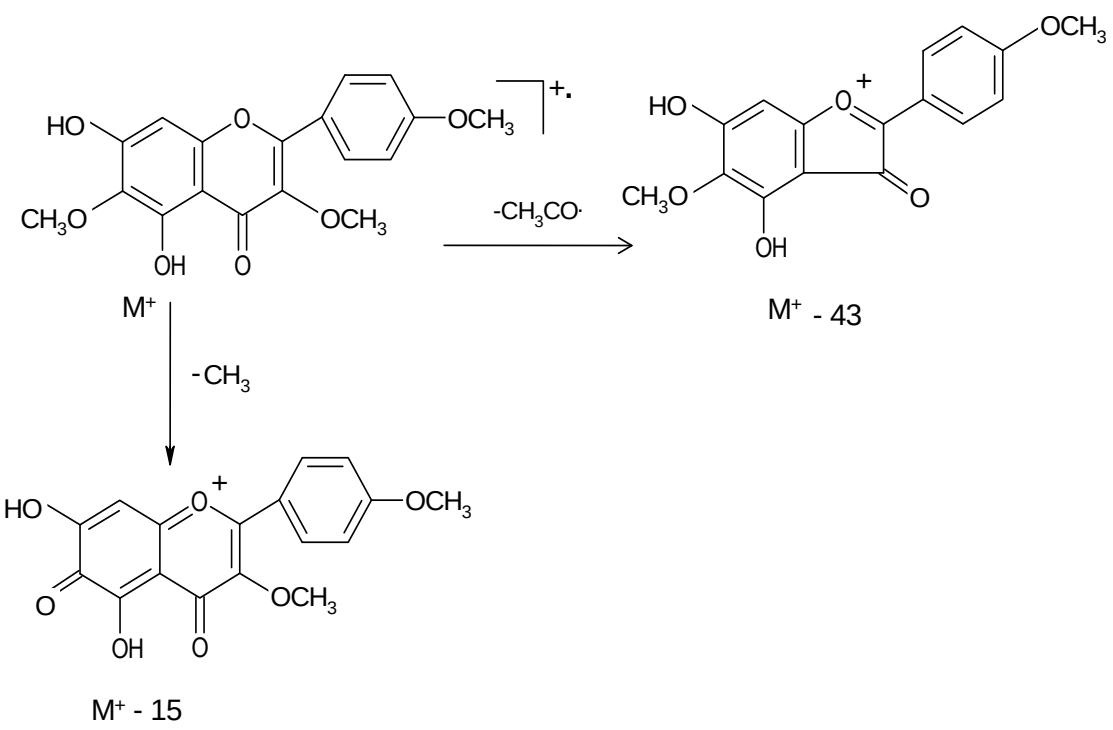


Figure 6.12: Mass Spectrum fragmentation of santin.

Table 6. 2: ^{13}C NMR data of Compound 2

^{13}C Assignment	Compound 2 (acetone-d_6)	Santin ($CDCl_3$) (Rashid *et al*., 1992)
2	157.1	156.2
3	138.8	138.7
4	179.2	179.6
5	152.5	151.8
6	131.0	130.1
7	153.0	152.2
8	93.83	93.1
9	156.0	155.9
10	105.0	106.5
1'	123.0	123.1
2', 6'	130.4	130.2
3', 5'	114.3	114.1
4'	162.1	160.2
OMe -4'	55.2	55.4
OMe-6	60.1	61.8
OMe-3	59.6	60.1

6.3.2.3: Compound 3

The mass spectrum showed molecular ion peak at m/z = 330 [M^+, 100%] base peak, corresponding to the molecular formula $C_{17}H_{14}O_7$ and an intense peaks at 315 (M-CH_3) and 287 (M-43) (Fig. 6.14) corresponding to the 6-OCH_3 flavonol fragmentation (Markham, 1982) and standard flavonol C-ring contraction (Harborne, 1994) for 3-methyl ether flavone respectively. The ^{1}H NMR (acetone-d_6) spectrum showed two methoxy signals at δ 3.86 and 3.87 (3H each, s, OMe-3 and OMe-6). There was presence of AA'BB' system due to ring B at δ 7.02 (2H, d, J= 9.0 Hz, H-3', H-5') and δ 8.04 (2H, d, J=9.0, H-2', H-6'). ^{13}C NMR spectral data were compared with that of santin in which rings A and C are intact and kaempferol in which ring C is intact. The presence of free 5-OH group was confirmed by chelated -OH signal at δ 12.98. The spectral data are in close agreement with those reported in Table 6.3. The compound is therefore 5, 7, 4'-trihydroxy-3, 6-dimethoxyflavone (Fig. 6.13). This compound was previously reported as a constituent from the genus *Dodonaea* such as from *D. viscosa* (Sachdev and Kulshreshtha, 1983), *D. attenuata* (Payne and Jefferies, 1973) and *D. angustifolia* (van Heerden *et al*., 2000).

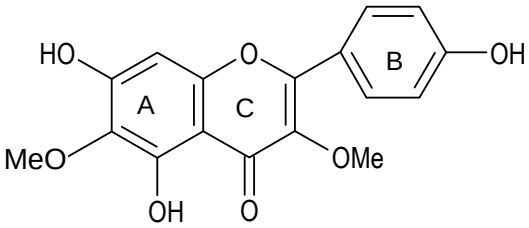


Figure 6.13: Structure of 5, 7, 4'-trihydroxy-3, 6-dimethoxyflavone.

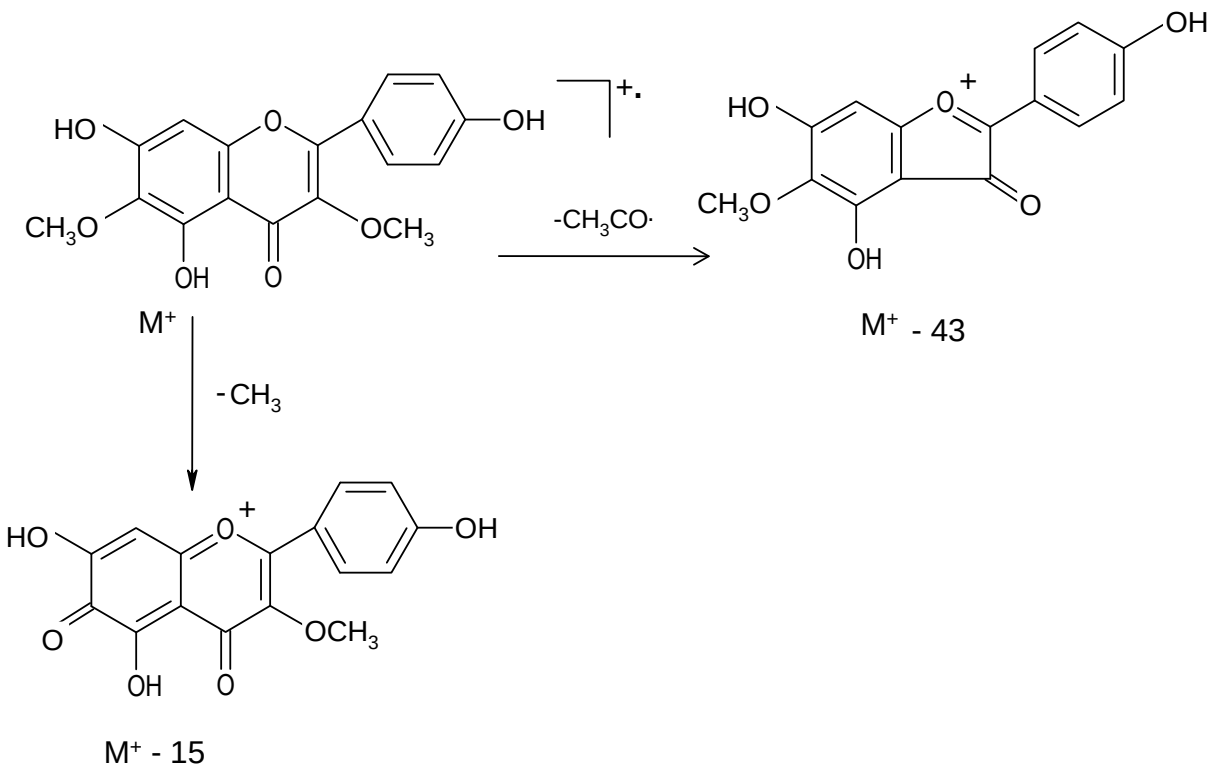


Figure 6.14: Mass Spectrum Fragmentation of 5, 7, 4'-trihydroxy-3, 6-dimethoxyflavone.

Table 6.3: ^{13}C NMR data of Compound 3

^{13}C Assignment	Compound 3 (acetone-d_6)	santin($CDCl_3$) (Rashid *et al*., 1992)	kaempferol (DMSO-d_6) (Markham, 1982)
2	157.6	156.2	146.8
3	138.8	138.7	135.6
4	179.8	179.6	175.9
5	153.6	151.8	160.7
6	131.8	130.1	98.2
7	153.1	152.2	163.9
8	94.4	93.1	93.5
9	156.9	155.9	156.2
10	106.3	106.5	103.1
1'	122.7	123.1	121.7
2', 6'	131.2	130.2	129.5
3', 5'	116.4	114.1	115.4
4'	160.9	160.2	159.2
OMe-6	60.7	61.8	--
OMe-3	60.2	60.1	--
OMe-4'	--	55.4	--

6.3.2.4: Compound 4

The mass spectrum showed molecular ion peak at m/z =301.01 {[M +H]$^{+\cdot}$ 57 %} corresponding to the molecular formula $C_{16}H_{12}O_6$. There was no fragmentation of ions probably due to absence of extensive methoxylation. The ^{1}H NMR (acetone-d_6) spectrum showed one methoxy signal at δ 3.89 (3H, s, OMe-4'). There was presence of AA'BB' system due to ring B at δ 7.10 (2H, d, J= 9.3 Hz, H-3', H-5') and δ 8.02 (2H, d, J=9.6, H-2', H-6'). The presence of free 5-OH group was confirmed by chelated -OH signal at δ 12.17. Also there was a presence of two doublets at 6.26 and 6.54 (1H each, J = 2.4 and 2.1 Hz respectively H-6, H-8). ^{13}C NMR is presented in Table 6.4. The spectral data are in close agreement with those reported in the literature (Table 6.4). The compound is therefore 3, 5, 7-trihydroxy-4'-methoxyflavone (4'-O-methylkaempferol) (Fig. 6.15). We are reporting for the first time isolation of 4'-O-methylkaempferol from *D. viscosa*, however, the compound has been previously isolated from legumes lentil, *Lens culinaris* (Family, Fabaceae) (Latha and Daniel, 2001).

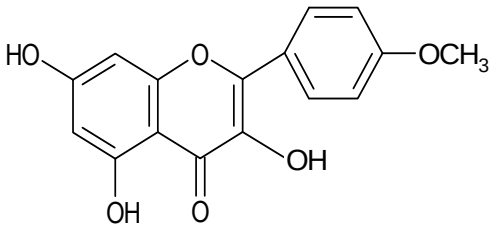
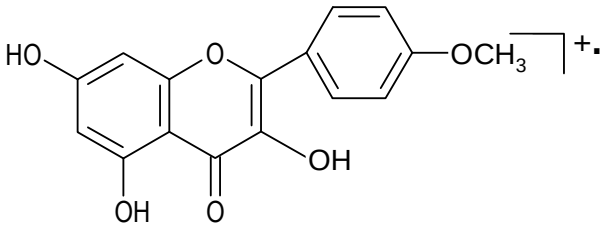


Figure 6.15: Structure of 4'-O-methylkaempferol**.**



$[M+H]^+$ = 301.01, 57%

Figure 6.16: Molecular ion peak of 4'-O-methylkaempferol.

Table 6.4: ^{13}C NMR data of Compound 4

^{13}C Assignment	Compound 4 (acetone-d_6)	4'-O-methylkaempferol (DMSO-d_6) (Harborne, 1982)
2	147.0	146.4
3	136.0	135.8
4	176.0	175.8
5	161.7	160.6
6	98.5	98.2
7	164.5	163.9
8	93.9	93.4
9	157.2	156.2
10	104.0	103.1
1'	123.7	123.2
2', 6'	129.6	129.0
3', 5'	114.2	114.0
4'	161.2	160.6
4'-OCH_3	55.3	55.3

6.3.2.5: Compound 5

The mass spectrum showed molecular ion peak at m/z =287.06 {[M +H]$^{+\cdot}$ 30%} corresponding to the molecular formula $C_{15}H_{10}O_6$. There was no fragmentation of ions probably due to absence of extensive methoxylation. The ^{1}H NMR (acetone-d_6) spectrum showed AA'BB' system due to ring B at δ 7.02 (2H, d, J= 9.0 Hz, H-3', H-5') and δ 8.16 (2H, d, J=9.0, H-2', H-6'). The presence of free 5-OH group was confirmed by chelated -OH signal at δ 12.17. Also there was a presence of two doublets at 6.26 and 6.53 (1H each, J = 2.4 and 2.1 Hz respectively H-6, H-8). ^{13}C nmr is presented in Table 6.5. The spectral data are in close agreement with those reported in the literature (Table 6.5). The compound is therefore 3, 4', 5, 7-tetrahydroxyflavone (kaempferol) (Fig. 6.17) and this compound was isolated previously from *D. viscosa* (Wollenweber, *et al*., 1986; Getie *et a*l., 2000) and from other plant species such as *Ginkgo biloba* (Family Ginkgoaceae) (Chin *et al*., 2000).

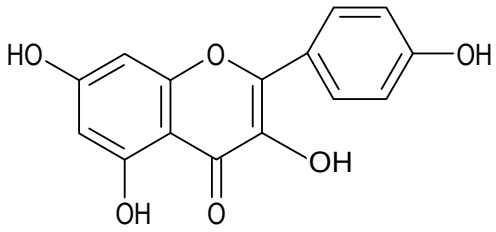
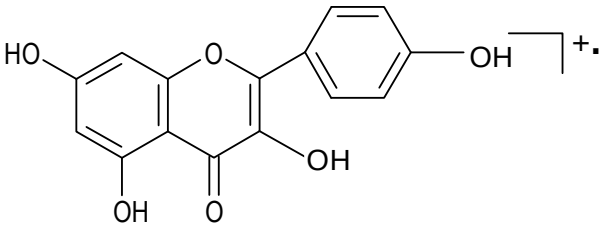


Figure 6.17: Structure of kaempferol



[M+H]$^+$ = 287.06, 30%

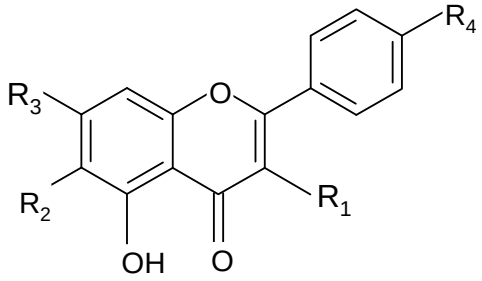
Figure 6.18: Molecular ion peak of kaempferol

Table 6.5: ^{13}C NMR data of Compound 5

^{13}C Assignment	Compound 5 (acetone-d_6)	kaempferol (DMSO-d_6) (Markham, 1982)
2	147.0	146.8
3	136.0	135.6
4	176.0	175.9
5	161.6	160.7
6	98.5	98.2
7	164.5	163.9
8	93.8	93.5
9	157.1	156.2
10	103.5	103.1
1'	122.6	121.7
2', 6'	129.8	129.5
3', 5'	115.7	115.4
4'	159.6	159.2

6.4 Conclusion

Five flavonoids (Fig. 6.19) were isolated from crude leaf extract of *D. viscosa*; 5-hydroxyl- 3, 7, 4'-trimethoxyflavone (Compound **1**); 5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone (Compound **2**); 3, 6-dimethoxy-5, 7, 4'-trihydroxyflavone (Compound **3**); 4'-O-methylkaempferol (Compound **4**) and kaempferol (Compound **5**). We isolated for the first time one compound which is 4'-O-methylkaempferol from *D. viscosa*.



Compound Name	Number	R1	R2	R3	R4
5-hydroxy -3, 7, 4'-trimethoxyflavone	**1**	OMe	H	OMe	OMe
5, 7-dihydroxy-3, 6, 4'-trimethoxyflavone	**2**	OMe	OMe	OH	OMe
5, 7, 4'-trihydroxy-3, 6-dimethoxyflavone	**3**	OMe	OMe	OH	OH
4'-O-methylkaempferol	**4**	OH	H	OH	OMe
kaempferol	**5**	OH	H	OH	OH

Figure 6.19: Five isolated compounds from *D. viscosa* leaf extracts.