CHAPTER 2

EXPERIMENTAL

2.1 Plant Materials

The rhizomes of *Amomum uliginosum* used in this study were colledted from Chiang Rai province in May 2008. The sample was identified by J.F. Maxwell of the CMU Herbarium, where a voucher specimen was deposited (08-131 J.F. Maxwell)

2.2 General Methods

2.2.1 Column Chromatography (CC)

2.2.1.1 Column Chromatography (CC)

Adsorbent: Silica gel with particle size 0.063-0.200 mm (Merck, 7734) was used in the experiments.

Packing method: Slurry packing.

Sample loading: The sample was dissolved in a small amount of suitable organic solvent, mixed with a small quantity of silica gel 60 with particle size 0.063-0.200 mm (Merck, 7734), air dried and added gently onto the top of column.

Elution: After loading of the sample, the column was eluted with suitable solvent system using gradient technique.

2.2.1.2 Flash Column Chromatography (FCC)

Adsorbent: Silica gel with particle size less than 0.063 mm (Merck, 7729) was used in the experiments.

Packing method: Slurry packing.

Sample loading: The sample was dissolved in a small amount of suitable organic solvent, mixed with a small quantity of silica gel 60 with particle size 0.063 mm (Merck, 7729), air dried and added gently onto the top of column.

Elution: After loading of the sample, the column was eluted with suitable solvent system using isocratic or gradient technique.

2.2.2Thin Layer Chromatography (TLC)

Techniques: One way, ascending.

Adsorbent: Silica gel 60 PF_{254} pre-coated on aluminum plate (Merck) size 1 × 5 cm, 2 × 5 cm and 3 × 5 cm.

2.2.3 Detection of Chromatographic plate

Ultraviolet light at 254 nm. The compound which contains unsaturated bonds especially conjugated system is visible as quenching spot under UV light at 254 nm.

Developing reagents, Anisaldehyde reagent consisted of *p*-methoxybenzaldehyde (20 ml), concentrated sulfuric acid (30 ml), water (20 ml) and ethanol (90 ml). The spot of organic compounds give specific colors with this reagent after heating at 90-110 °C for 2-4 minutes.

2.2.4 Physical Constant

Melting points were determined on an Electrothermal melting point apparatus and were uncorrected. The temperature was given in degree Celsius. Optical rotations were obtained using a JASCO-1020 polarimeter.

2.2.5 Spectroscopy

2.2.5.1 Infrared (IR) Spectra

IR spectra were recorded on a FT-IR spectrometer (Tensor 27). Spectra of solid sample were recorded as potassium bromide (KBr) pellets. Spectra of liquid sample were recorded as thin film technique.

2.2.5.2 Nuclear Magnetic Resonance (NMR) Spectra

^{1}H and ^{13}C NMR spectra were measured with a Bruker advance 400 NMR spectrometer, operating at 400 and 100 MHz, respectively.

2.2.5.3 Mass Spectra

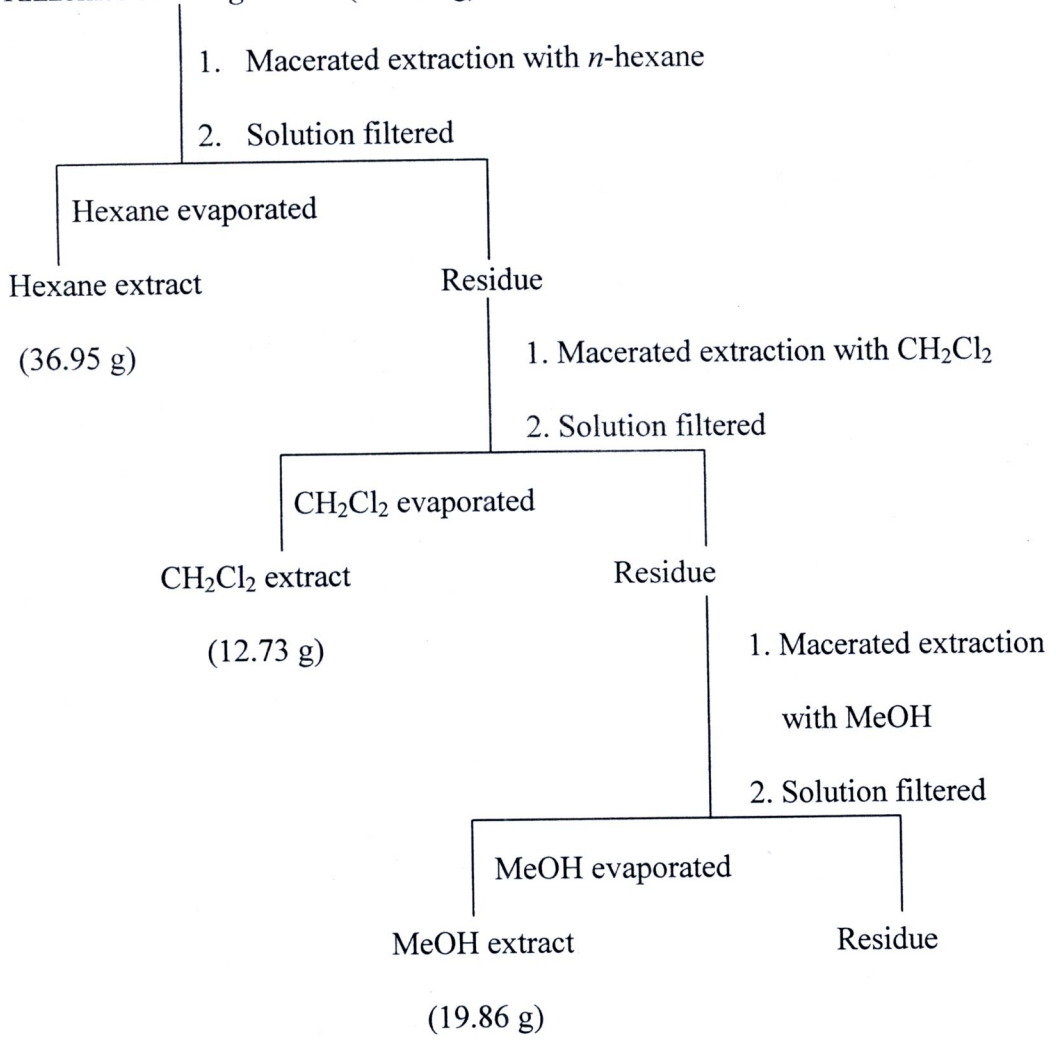
The high resolution mass spectra were performed on a Q-TOF 2TM mass spectrometer with a Z-sprayTM ES source (Micromass, Manchester, UK). Electron impact mass spectra were measured with Agilent-HP 5973 Mass Spectrometer.

2.2.5.4 Gas Chromatography-Mass Spectrometry analysis (GC/MS)

Agilent-HP 5973 Mass Spectrometer equipped with Alltech 15897 AT-1 MS capillary column (30 m x 0.25 mm, 0.25 μm film thickness). The oven temperature was programmed from 45-250 °C at the rate of 2 °C /min with final hold 12.5 min, using helium gas as a carrier gas. Individual components were identified by Wiley 275 and NIST database matching. Relative percentage of individual components were calculated based on GC peak areas without using correction factors. The injector and detector temperatures were 200 °C and 230 °C, respectively. MS were taken at 70 eV with mass range of *m/z* 29-550.

2.3 Extraction and Isolation of Bioactive Compounds

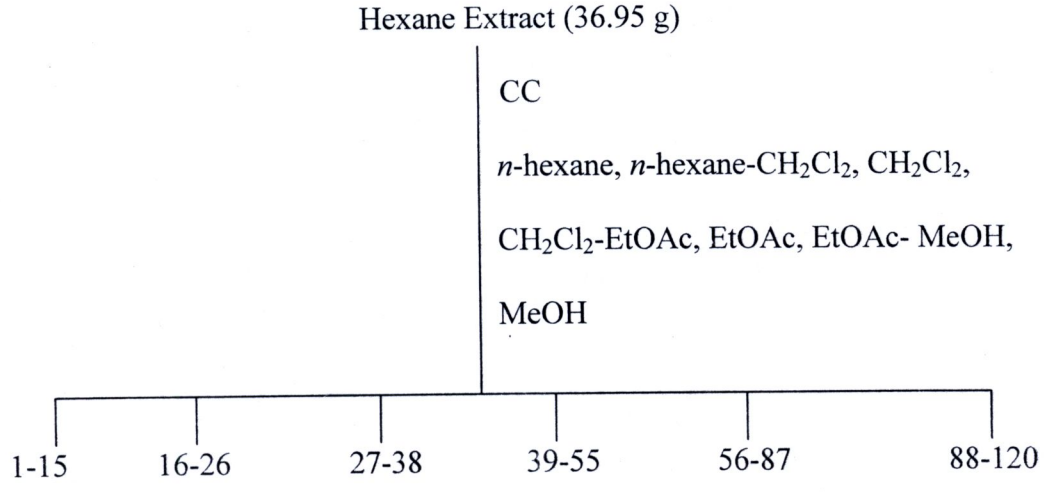
The air dried powdered rhizomes of *A.uliginosum* (1385.61 g) were succesively extracted with *n*-hexane, CH_2Cl_2 and MeOH. The extracts were evaporated to dryness under reduced pressure at temperature 40-45 °C. to obtain the hexane extract (brownish viscous oil, 36.95 g), the CH_2Cl_2 extract (brownish viscous oil, 12.73 g) and the methanol extract (dark brownish gum, 19.86 g). The extraction sequence is shown in Scheme 1.



Scheme 1 Extraction of the rhizomes of *A.uliginosum*

2.3.1 Hexane Extract

The hexane extract (36.95 g) was fractionated by flash column chromatography (FCC) eluting with *n*-hexane, *n*-hexane-CH_2Cl_2, CH_2Cl_2, CH_2Cl_2-EtOAc, EtOAc, EtOAc-MeOH and MeOH with increasing amount of the more polar solvent. The eluates were examined by TLC and 5 groups of eluting fractions were obtained (Scheme 2).



Gr. 1, Fractions 1-15 (0.1419 g)

Gr. 2, Fractions 16-26 (0.4100 g)

Gr. 3, Fractions 27-38 (0.1914 g)

Gr. 4, Fractions 39-55 (0.6600 g)

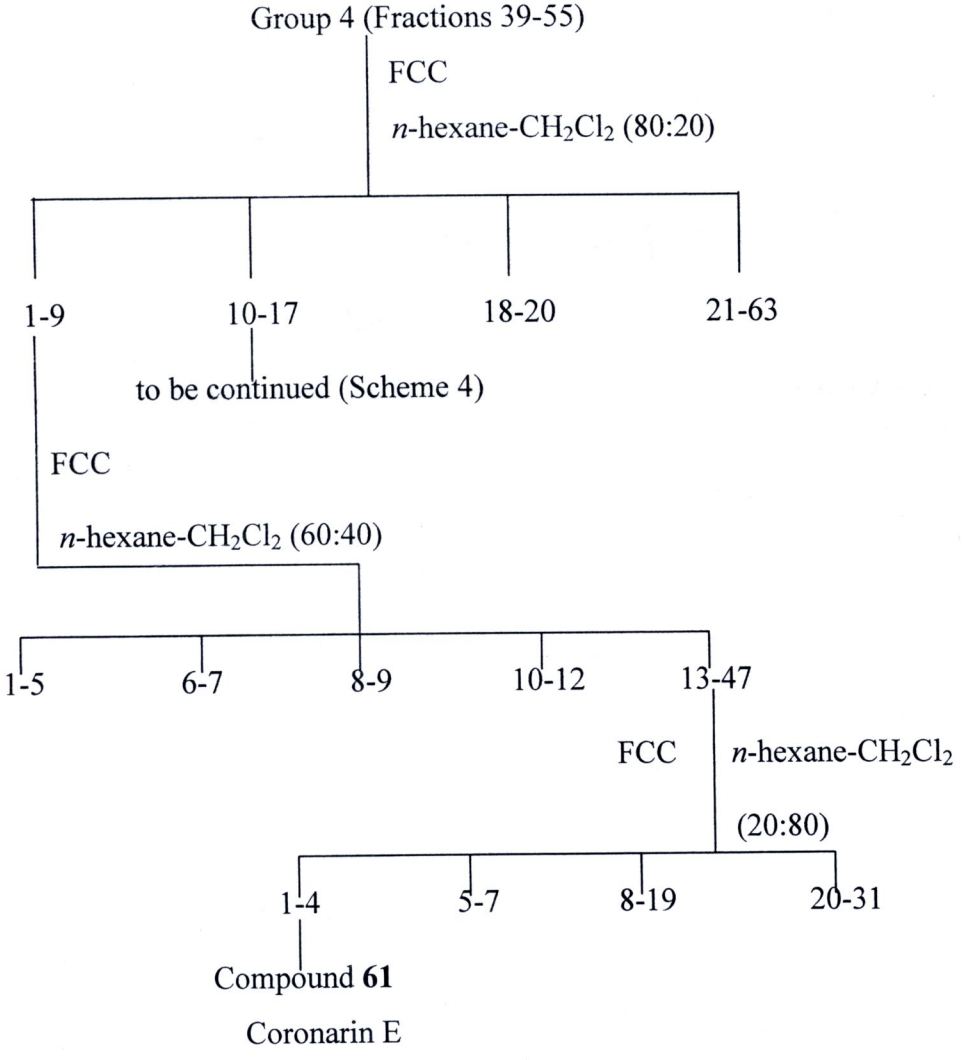
Gr. 5, Fractions 56-87 (2.5466 g)

Gr. 6, Fractions 88-120 (8.0100 g)

Scheme 2 Fractionation of the hexane extract of *A.uliginosum*

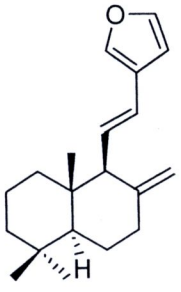
Group 4 (Fractions 39-55)

These combined fractions were rechromatographed over silica gel eluting with *n*-hexane- CH_2Cl_2 (80:20) to give 4 subfractions. Subfraction 1 (fractions 1-9) was subjected to FCC and eluted with *n*-hexane-CH_2Cl_2 (60:40) to give 5 subfractions. Subfraction 5 (fractions 13-47) was subjected to FCC and eluted with *n*-hexane-CH_2Cl_2 (20:80) to give coronarin E (**61**) as pale yellow amorphous (20.3 mg) (Scheme 3).



Scheme 3 Fractionation of group 4 (fractions 39-55) of the hexane extract

Coronarin E (**61**)



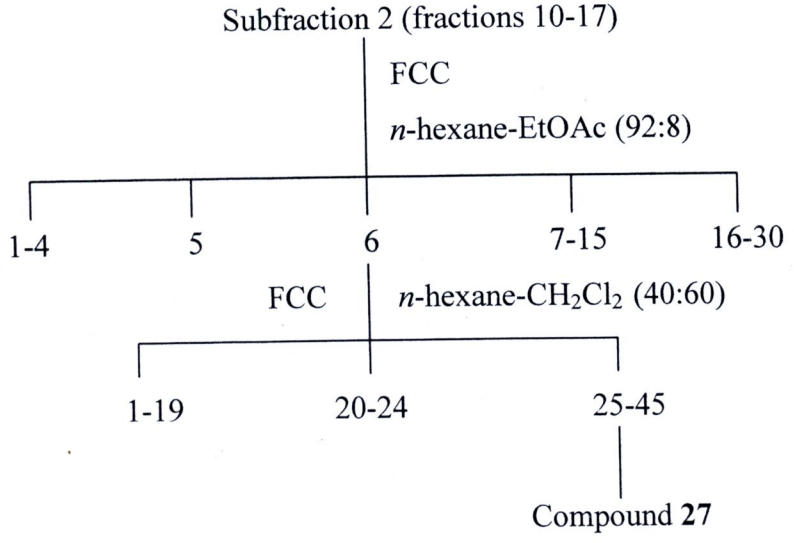
$[\alpha]_D^{29}$ + 19.70 (*c* 0.57, $CHCl_3$), Lit. $[\alpha]_D$ + 8.00 (*c* 2.12, $CHCl_3$). [51]

IR: ν_{max} 2922, 2870, 1643, 1156 cm^{-1}

^1H, ^{13}C-NMR , HMBC correlations are given in Table 8.

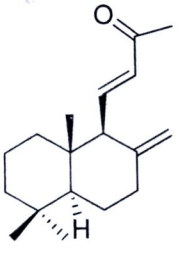
EIMS: *m/z* 284 $[M]^+$ (100); 269 (9), 147 (95), 137 (28), 69 (14), 55 (20), 41(17)

Subfraction 2 (fractions 10-17) was rechromatographed and elute with *n*-hexane-EtOAc (92:8) to give 5 subfractions. Subfraction 3 (fraction 6) was subjected to FCC and eluted with *n*-hexane-CH_2Cl_2 (40:60) to give (*E*)-15,16-bisnorlabda-8(17),11-dien-13-one (**27**) as pale yellow solid (24.7 mg) (Scheme 4).



Scheme 4 Fractionation of subfraction 2 (fractions 10-17) of Scheme 3

(*E*)-15,16-Bisnorlabda-8(17),11-dien-13-one (**27**)



$[\alpha]_D^{29}$ -8.50 (*c* 0.67, $CHCl_3$), Lit. $[\alpha]_D^{25}$ -5.50 (*c* 0.12, $CHCl_3$) [24]

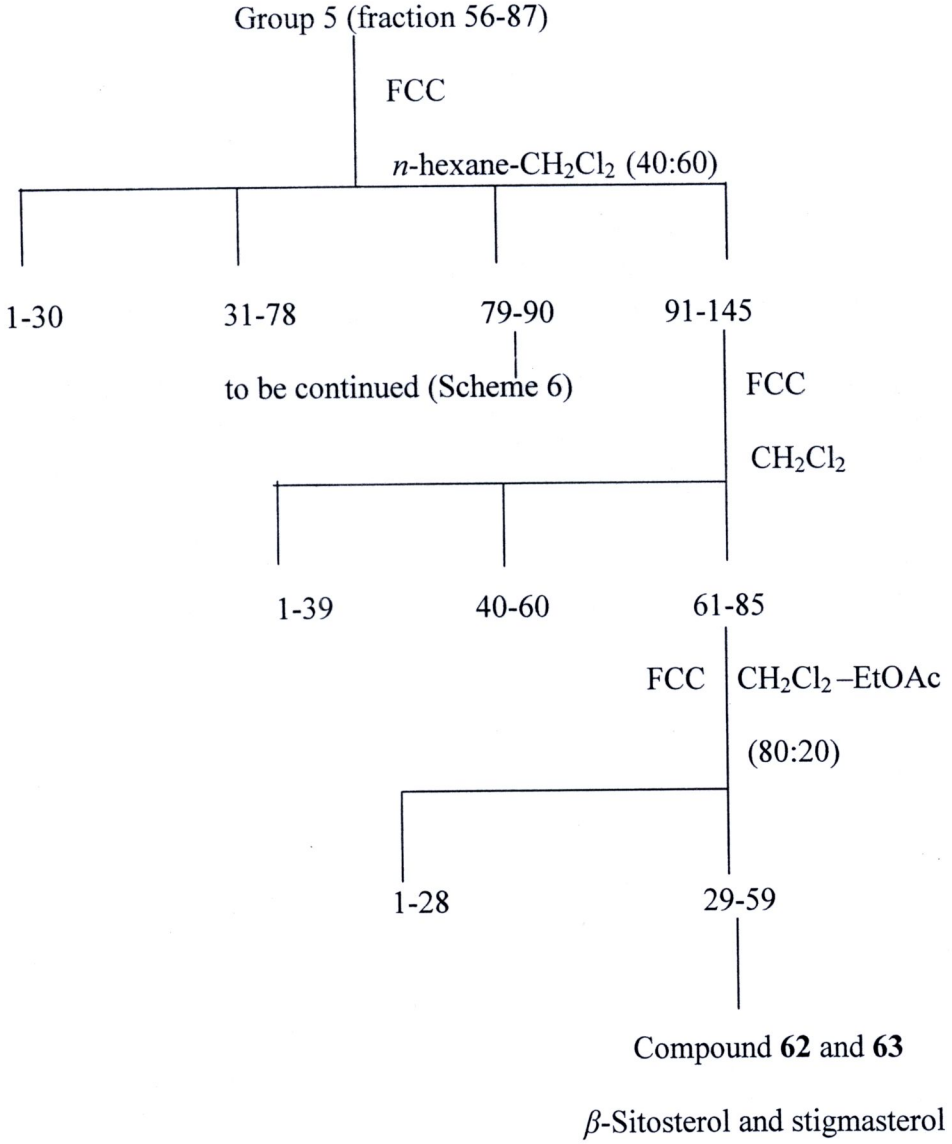
IR: ν_{max} 2936, 1664, 1645, 1258, 898 cm^{-1}

^1H, ^{13}C-NMR, HMBC correlations are given in Table 9.

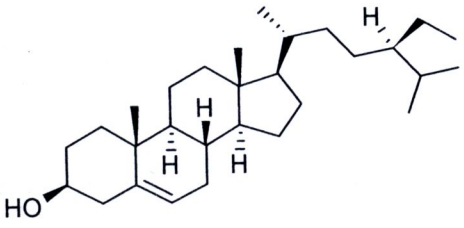
EIMS: *m/z* 260 $[M]^+$ (47) ; 245 (16), 217 (35), 137 (60), 81 (92), 69 (30), 43(58)

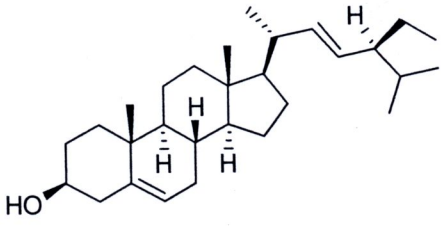
Group 5 (fractions 56-87)

Group 5 (fractions 56-87) were rechromatographed over silica gel and eluted with *n*-hexane-CH_2Cl_2 (40:60) to give 4 subfractions. Subfraction 4 (fractions 91-145) was subjected to FCC and eluted with CH_2Cl_2 to give 3 subfractions. Subfraction 3 (61-85) was subjected to FCC and eluted with CH_2Cl_2 –EtOAc (80:20) to give a mixture of *β*-sitosterol (**62**) and sigmasterol (**63**) as white crystal (0.2 g) (Scheme 5)



Scheme 5 Fractionation of group 5 (fractions 56-87) of the hexane extract





β-Sitosterol **(62)**

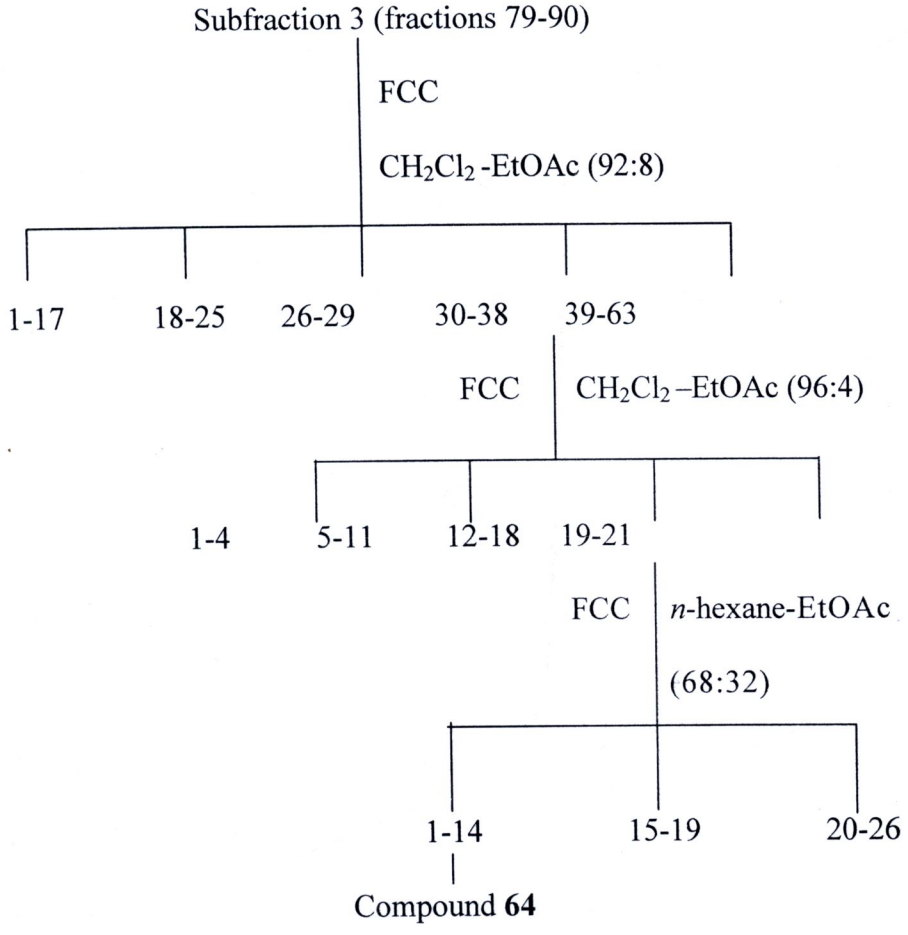
Sigmasterol (**63**)

IR: ν_{max} 3384, 3218, 3025, 2868, 1665, 1462, 1383 , 1046 cm^{-1}

^{1}H, ^{13}C-NMR, HMBC correlations are given in Table 10.

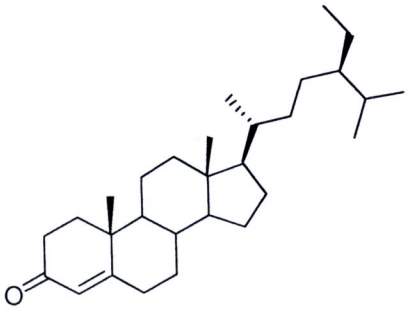
EIMS: *m/z* 414 $[M]^{+}$ (92) ; 396(100), 81(78)

Subfraction 3 (fractions 79-90) was rechromatographed and elute with CH_2Cl_2 -EtOAc (92:8) to give 5 subfractions. Subfraction 4 (fractions 30-38) was subjected to FCC and eluted with CH_2Cl_2 –EtOAc (96:4) to give 4 subfractions. Subfraction 3 (12-18) was subjected to FCC and eluted with *n*-hexane-EtOAc (68:32) to give stigmast-4-en-3-one (**64**) as white crystal (1.5 mg) (Scheme 6).



Scheme 6 Fractionation of subfraction 3 (fractions 79-90) of Scheme 5

Stigmast-4-en-3-one (**64**)



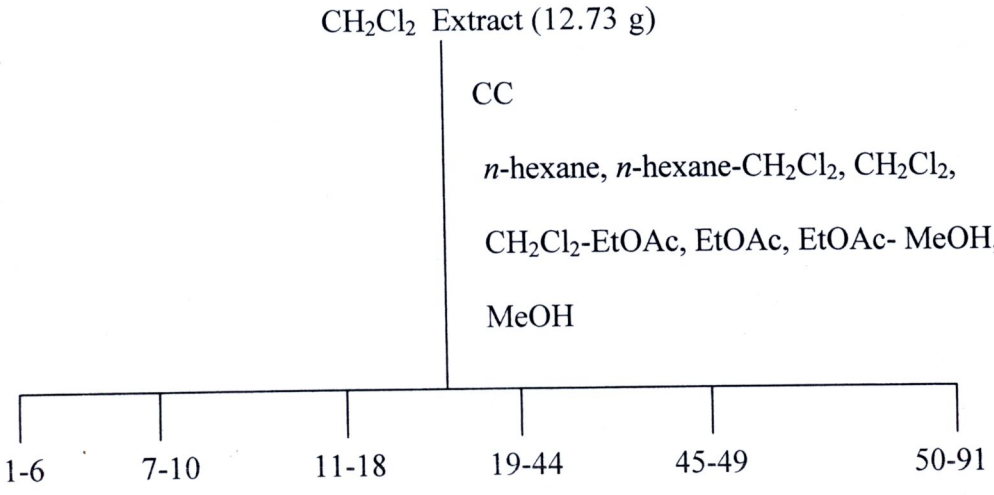
IR: ν_{max} 2940, 1676, 1462, 1382 cm^{-1}

^{1}H, ^{13}C-NMR, HMBC correlations are given in Table 11.

EIMS: *m/z* 412 $[M]^{+}$ (95); 397, 370, 289, 229, 124(100)

2.3.2 CH_2Cl_2 Extract

The CH_2Cl_2 extract (12.73 g) was fractionated by flash column chromatography (FCC) eluting with *n*-hexane, *n*-hexane-CH_2Cl_2, CH_2Cl_2, CH_2Cl_2-EtOAc, EtOAc, EtOAc-MeOH and MeOH with increasing amount of the more polar solvent. The eluates were examined by TLC and 6 groups of eluting fractions were obtained (Scheme 7).



Gr. 1, Fractions 1-6 (0.2515 g)

Gr. 2, Fractions 7-10 (0.1161 g)

Gr. 3, Fractions 11-18 (0.6535 g)

Gr. 4, Fractions 19-44 (3.0132 g)

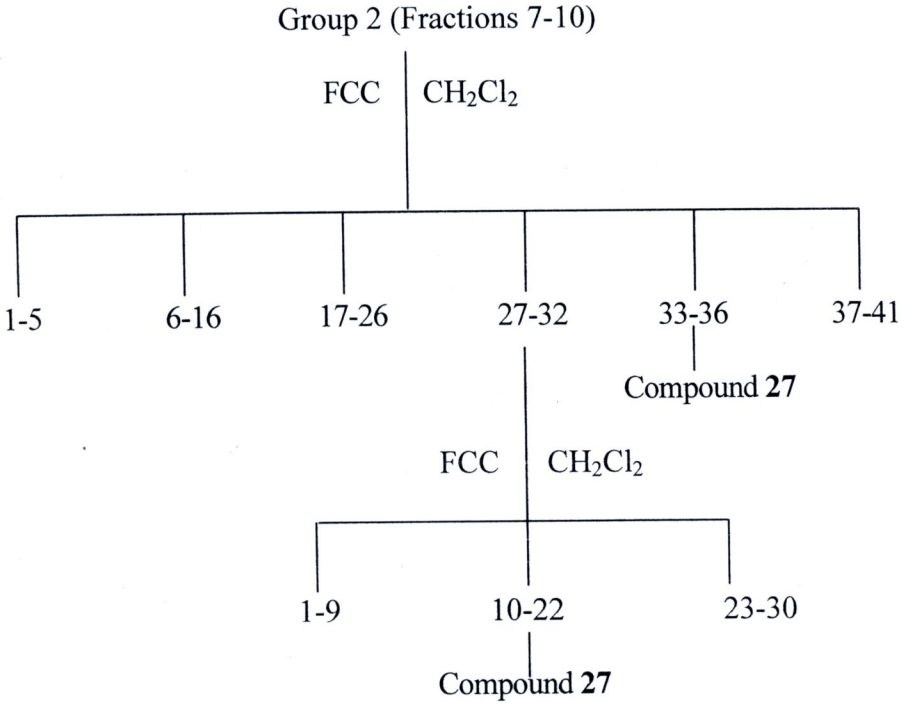
Gr. 5, Fractions 45-49 (0.6643 g)

Gr. 6, Fractions 50-91 (7.9752 g)

Scheme 7 Fractionation of the CH_2Cl_2 extract of *A.uliginosum*

Group 2 (Fractions 7-10)

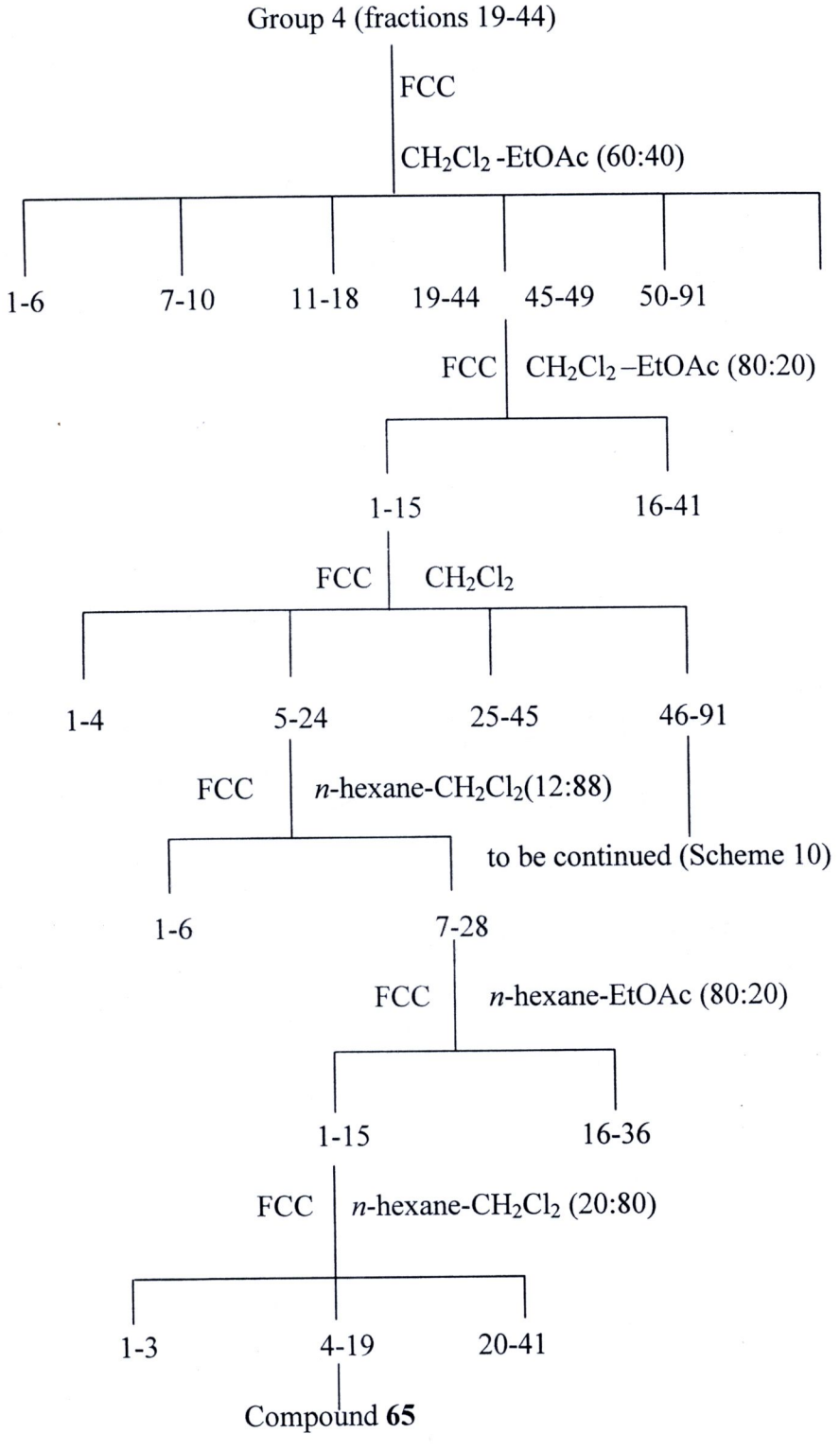
These combined fractions were rechromatographed over silica gel eluting with CH_2Cl_2 to afford (*E*)-15, 16-bisnorlabda-8(17),11-dien-13-one (**27**) as pale yellow solid (22.5 mg) (Scheme 8).



Scheme 8 Fractionation of group 2 (fractions 7-10) of the CH_2Cl_2 extract

Group 4 (fraction 19-44)

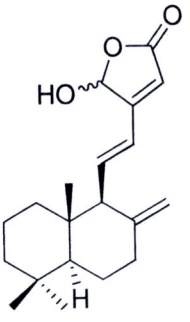
Group 4 (fractions 19-44) were rechromatographed over silica gel and eluted with CH_2Cl_2 -EtOAc (80:20) to give 6 subfractions. Subfraction 4 (fractions 19-44) was subjected to FCC and eluted with CH_2Cl_2 –EtOAc (80:20) to give 2 subfractions. Subfraction 1 (fractions 1-15) was subjected to FCC and eluted with CH_2Cl_2 to give 4 subfractions. Subfraction 2 (fractions 5-24) was subjected to FCC and eluted with *n*-hexane-CH_2Cl_2(12:88) to give 2 subfractions. Subfraction 2 (fractions 7-28) was subjected to FCC and eluted with *n*-hexane-EtOAc (80:20) to give 2 subfractions. Subfraction 1 (fractions 1-15) was subjected to FCC and eluted with *n*-hexane-CH_2Cl_2 (20:80) to give 16-hydroxylabda-8(17), 11, 13-trien-15, 16-olide (**65**) as pale yellow solid (9.3 mg) (Scheme 9**).**



16-Hydroxylabda-8(17), 11, 13-trien-15, 16-olide

Scheme 9 Fractionation of group 4 (fractions 19-44) of the CH_2Cl_2 extract

16-Hydroxylabda-8(17),11,13-trien-15,16-olide (**65**)



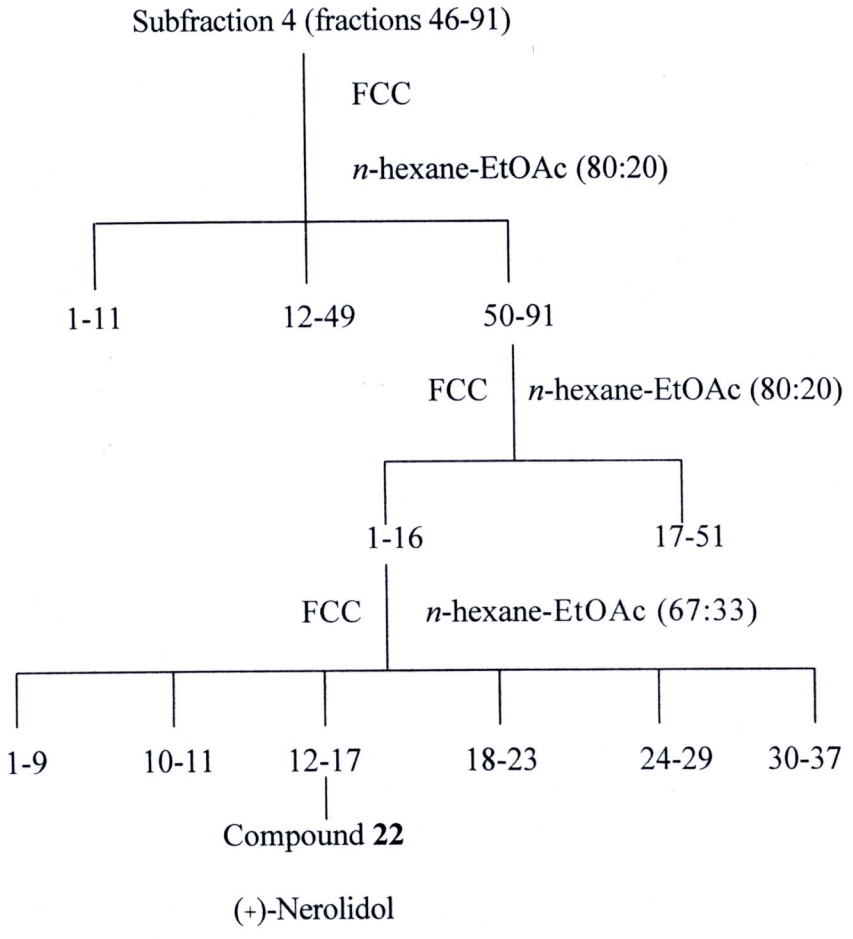
$[\alpha]_D^{29}$ +24.09 (*c* 0.63, $CHCl_3$).

IR: ν_{max} 3373, 2927, 1747, 1643, 1129, 891 cm^{-1}

^1H, ^{13}C-NMR, HMBC correlations are given in Table 12.

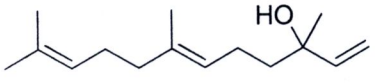
HREIMS: $[M+Na]^+$ 339.1938 (calcd for $C_{20}H_{28}O_3Na$, 339.1936)

Subfraction 4 (fractions 46-91) was rechromatographed and elute with *n*-hexane-EtOAc (80:20) to give 3 subfractions. Subfraction 3 (fractions 50-91) was subjected to FCC and eluted with *n*-hexane-EtOAc (80:20) to give 2 subfractions. Subfraction 1 (fractions 1-16) was subjected to FCC and eluted with *n*-hexane-EtOAc (67:33) to give (+)-nerolidol (**22**) as colorless oil (24 mg) (Scheme 10).



Scheme 10 Fractionation of subfraction 4 (fractions 46-91) of Scheme 9

(+)-Nerolidol



$[\alpha]_D^{29}$ +10.43 (*c* 0.41, $CHCl_3$), Lit. $[\alpha]_D^{29}$ +11.20 (*c* 1.7, $CHCl_3$)[4]

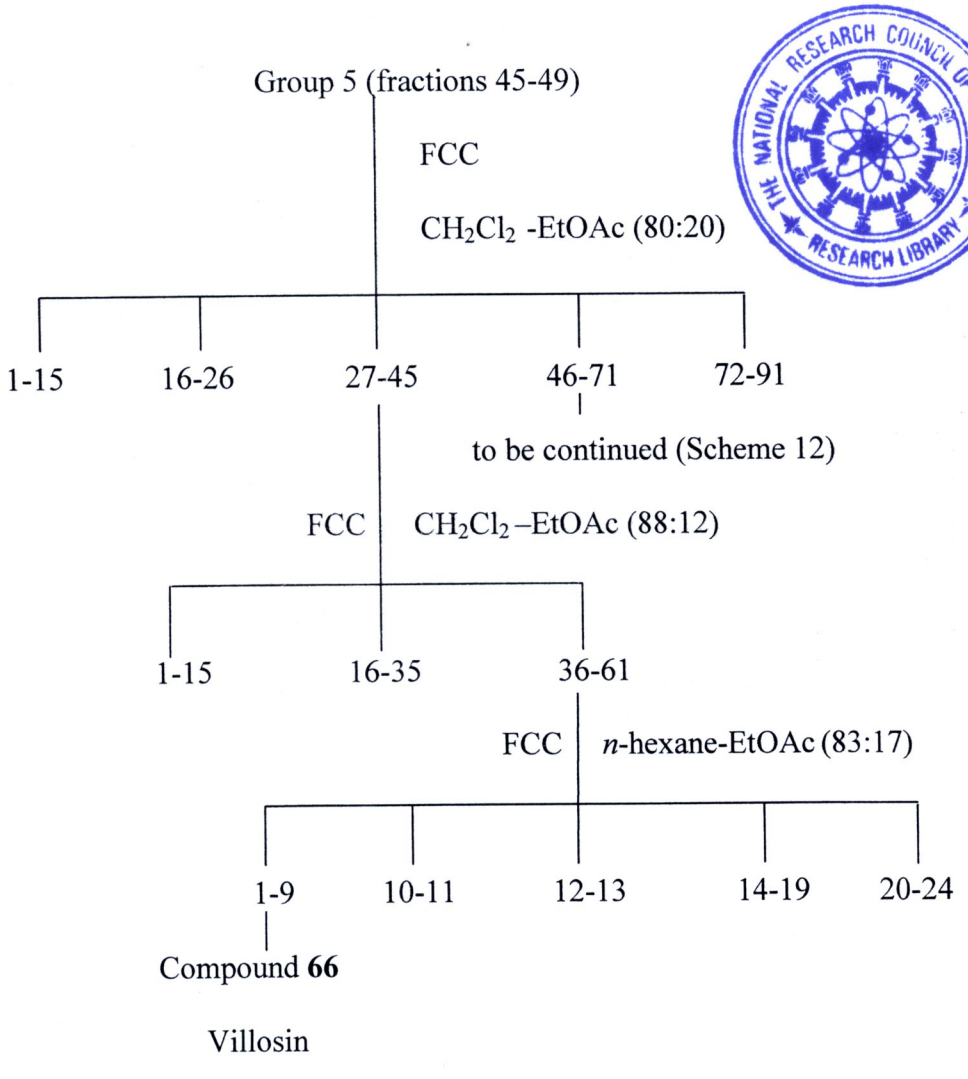
IR: ν_{max} 3422, 2969, 2925, 1640, 1450, 1376, 994, 919 cm^{-1}

^1H, ^{13}C-NMR, HMBC correlations are given in Table 13.

EIMS: *m/z* 222: $[M]^+$; 204(2), 189(7), 161(28), 136(32), 121(20), 107(47), 93(79)

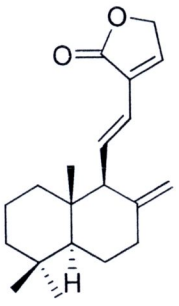
Group 5 (fractions 45-49)

Group 5 (fractions 45-49) were rechromatographed over silica gel and eluted with CH_2Cl_2 -EtOAc (80:20) to give 5 subfractions. Subfraction 3 (fractions 27-45) was subjected to FCC and eluted with CH_2Cl_2 –EtOAc (88:12) to give 3 subfractions. Subfraction 3 (fractions 36-61) was subjected to FCC and eluted with *n*-hexane-EtOAc (83:17) to give villosin **(66)** as white crystal (20.1 mg) (Scheme 11).



Scheme 11 Fractionation of group 5 (fractions 45-49) of the CH_2Cl_2 extract

Villosin (**66**)



$[\alpha]_D^{29}$ +3.94 (*c* 0.17, $CHCl_3$).

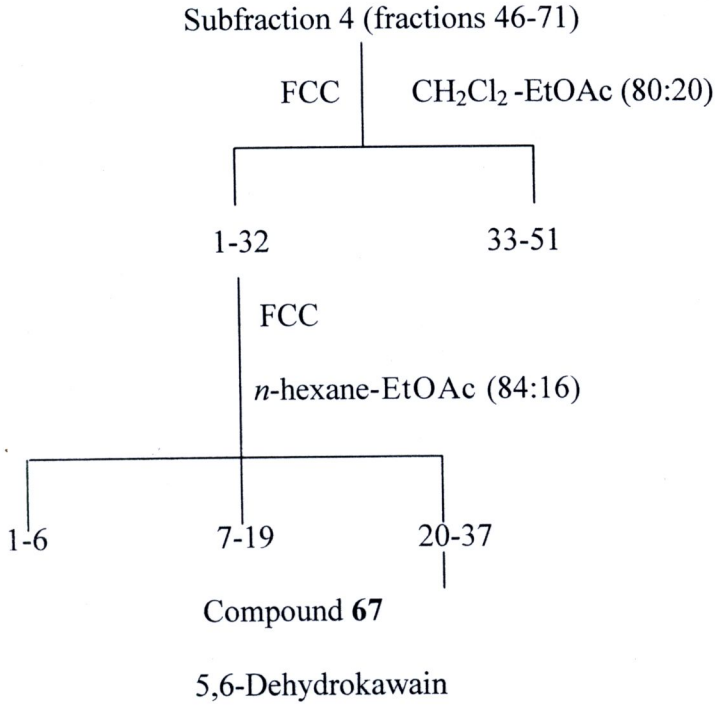
M.p. 124.5-125.0 °C.

IR: ν_{max} 2925, 1754, 1640, 1086, 1052, 947, 902, 833 cm^{-1}.

^1H, ^{13}C-NMR, HMBC correlations are given in Table 14.

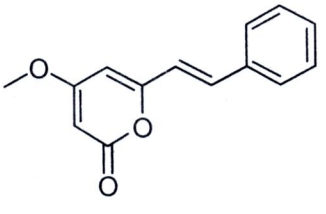
EIMS: *m/z* 300 $[M]^+$ (19) ; 285 (10), 257 (4), 189 (6), 137 (100), 123 (22), 55 (15), 41 (20)

Subfraction 4 (fractions 46-71) was rechromatographed and eluted with CH_2Cl_2 -EtOAc (80:20) to give 2 subfractions. Subfraction 1 (fractions 1-32) was subjected to FCC and eluted with *n*-hexane-EtOAc (84:16) to give 5,6-dehydrokawain (**67**) as pale yellow crystals (4.3 mg) (Scheme 12).



Scheme 12 Fractionation of Subfraction 4 (fractions 46-71) of the CH_2Cl_2 extract

5,6-Dehydrokawain (**67**)



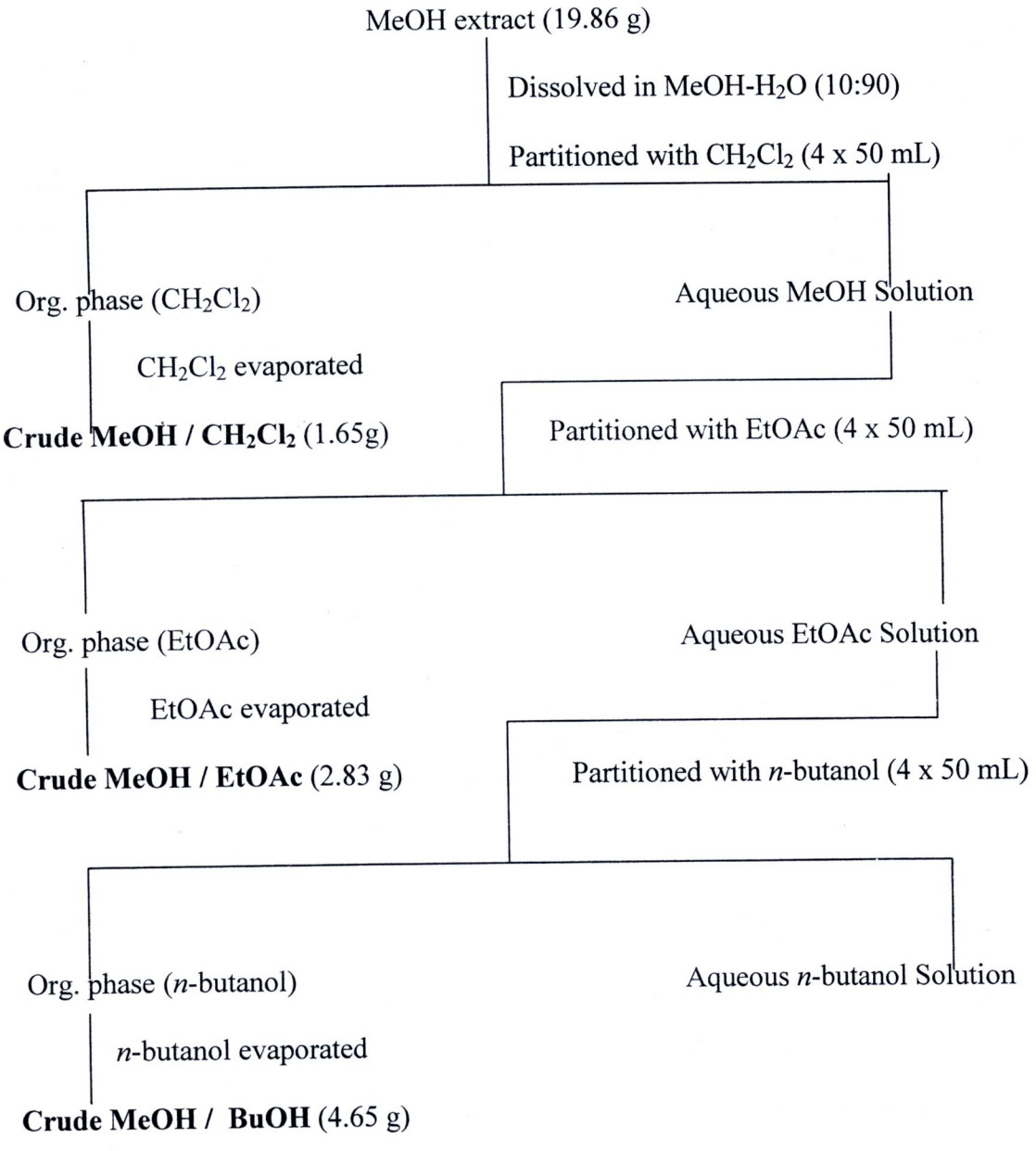
IR: ν_{max} 3040, 1713,1610, 1452 cm^{-1}.

^1H, ^{13}C-NMR, HMBC correlations are given in Table 15.

EIMS: *m/z* 288 $[M]^+$ (100) , 200 (52), 157 (55), 77 (50), 69 (42)

2.3.3. MeOH Extract

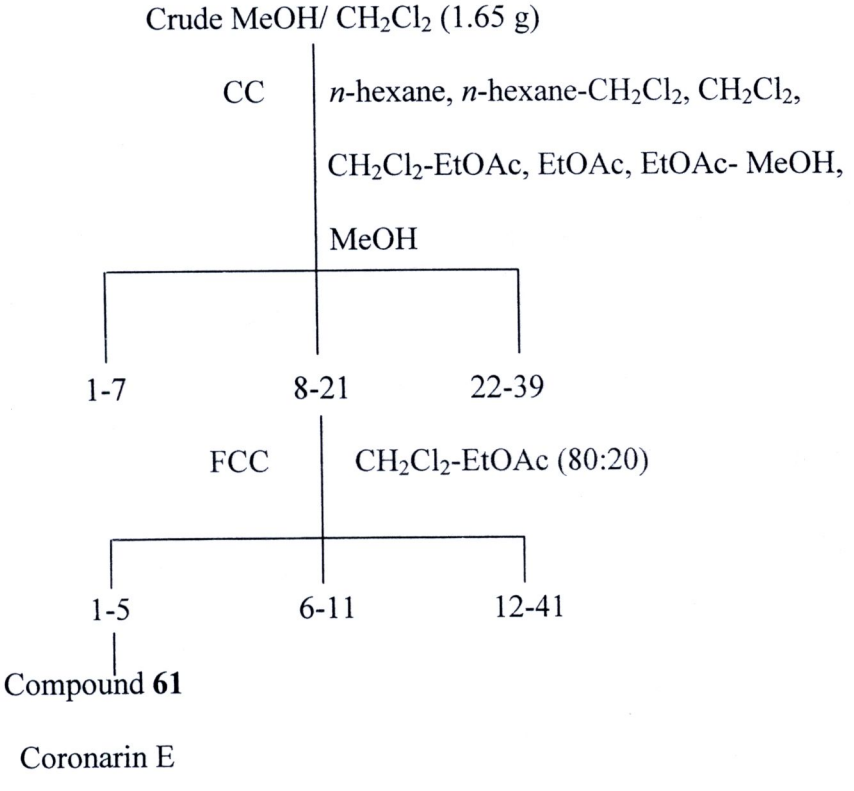
The MeOH extract (19.86 g) was dissolved in MeOH-H_2O (10:90) and then partitioned 3-4 times with CH_2Cl_2 in a separatory funnel. The organic layer were combined and evaporated to dryness to give a dark yellow-brown viscous liquid as the crude extract (1.65 g). The aqueous phase was partitioned 3-4 times with EtOAc in separatory funnel. The organic layer were combined and evaporated to dryness to give a dark yellow-brown viscous liquid as the crude extract (2.83 g). The aqueous phase was partitioned 3-4 times with *n*-butanol in separatory funnel. The organic layer were combined and evaporated to dryness to give a dark yellow-brown viscous liquid as the crude extract (4.65 g) (Scheme 13).



Scheme 13 Partition of the MeOH extract of *A. uliginosum*

Crude MeOH/ CH_2Cl_2 (1.65 g)

The MeOH/CH_2Cl_2 extract (1.65 g) was rechromatographed over silica gel using *n*-hexane, *n*-hexane-CH_2Cl_2, CH_2Cl_2-EtOAc, EtOAc and MeOH as eluent with increasing amount of the more polar solvent to afford 3 subfractions. Subfraction 2 (fractions 8-21) was subjected to FCC and eluted with CH_2Cl_2- EtOAc (80:20) to give coronarin E (**61**) as pale yellow amorphous (12.3 mg) (Scheme 14).



Scheme 14 Fractionation of the crude MeOH/ CH_2Cl_2 extract of *A.uliginosum*

2.4 Biological Activities

2.4.1 Antimycobacterial Assay:

The antimycobacterial activity was assessed against *Mycobacterial tuberculosis* H37Ra using the green fluorescent protein microplate assay (GFPMA).[48] The lowest drug concentration effecting and inhibition of ≥ 90 % was considered the MIC. The standard drugs, rifampicine, streptomycin, isoniazid and oflaxacin showed MIC values of 0.003-0.012, 0.156-0.313, 0.023-0.046 and 0.391-0.781 μg/mL respectively. The antimycobacterial assay of compounds **27, 61, 65** and **66** is shown in **Table 8**.

2.4.2 Cytotoxicity Assays

The cytotoxicity assays against human oral epidermoid carcinoma (KB), human breast cancer (BC) and human small cell lung cancer (NCI-H187) were performed employing colorimetric method.[49] The standard drugs doxorubicin showed IC_{50} values against these cell lines at 0.117, 0.663 and 0.053 μg/mL respectively, and ellipticine exhibited IC_{50} values against KB, NCI-H187 and noncancerous Vero cells at 0.302, 0.440 and 1.345 μg/mL. The cytotoxicity evaluation of compounds **27, 61, 65** and **66** is shown in **Table 9**.

Table 8 Anti- *Mycobacterial tuberculosis* (Anti-TB) H37Ra of compounds **27, 61, 65** and **66**

Compound	Anti-TB (MIC, μg/mL)
27	Inactive
61	12.50
65	6.25
66	Inactive
Rifampicin	0.003-0.012
Streptomycin	0.156-0.313
Isoniazid	0.023-0.046
Oflaxacin	0.391-0.781

Table 9 Cytotoxic Activities of compounds **27, 61, 65** and **66**

Compound	Cytotoxicity (IC_{50}, μg/mL)			
	KB[a]	BC[b]	NCI-H187[c]	Vero cell
(*E*)-15,16-bisnorlabda-8(17), 11-dien-13-one (**27**)	36.99	49.22	21.67	Non cytotoxicity
Coronarin E (**61**)	9.67	Inactive	18.06	25.37
16-hydroxylabda-8(17),11,13-trien-15,16-olide (**65**)	0.91	2.89	0.72	5.37
villosin (**66**)	4.10	8.50	0.12	Non cytotoxicity
Doxorubicin	0.117	0.663	0.053	-
Ellipticine	0.302	-	0.440	1.345

[a] human oral epidermoid carcinoma

[b] human breast cancer

[c] human small cell lung cancer