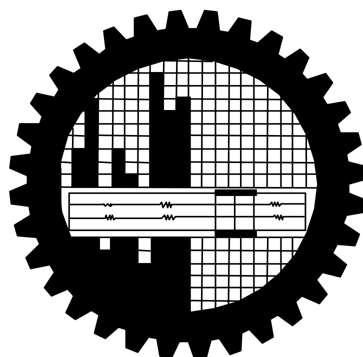
Phytochemical and Biological Investigation on the Roots of *Asparagus rosemosus*

by

Md. Ariful Hoque

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF PHILOSOPHY (M. PHIL.) IN CHEMISTRY



Department of Chemistry

BANGLADESH UNIVERSITY OF ENGINEERING AND TECHNOLOGY

Dhaka-1000, Bangladesh

2014.

Declaration

It is hereby declared that this thesis or any part of it has not been submitted elsewhere for the award of any degree or diploma.

Signature of the Candidate

Md. Ariful Hoque

Name of the Candidate

Dedicated to

My beloved parents
&
All of my inner hearted adored friends

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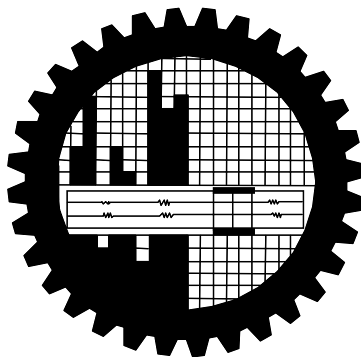

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2.0 General methods

The following sections of this chapter are a brief description of the various method followed in extraction, fractionation & purification of the compounds in the course of experimental works.

2.1 Solvents and Chemicals

Analytical or laboratory grade solvents and chemicals had used in the experiments. All solvents and reagents used in the experiments were produced from E. Merck (Germany), BDH (England). The commercial grade solvents (ethyl acetate, chloroform, methanol, ethanol, n-hexane, DMSO, dichloromethane and 1-butanol) were distilled before use.

2.2 Distillation of the solvents

The analytical grade solvents (ethyl acetate, chloroform, methanol, ethanol, n-hexane, DMSO, dichloromethane and 1-butanol) were distilled. Distilled solvents were used throughout the investigation.

Fig 2.1: Distillation process.

2.3 Evaporation

All evaporations were carried out under reduced pressure using a rotary evaporator at a bath temperature not more than 40^0C. The residual solvent in the extract and compounds were removed under high vacuum.

Fig 2.2: Rotary vacuum evaporator

2.4 Preparation of the reagents

Fig 2.3: Spray reagent/ Sprager for spray reagent.

2.4.1 Spray reagent/ Anisaldehyde-sulphuric acid (Developing reagent)

1 ml anisaldehyde

20 ml acetic acid (glacial)

10 ml sulfuric acid

170 ml methanol

2.5 Chromatographic techniques

Three types of chromatographic techniques such as Thin Layer Chromatography (TLC) and Column Chromatography (CC) & Vacuum Layer Chromatography (VLC) were used.

2.5.1 Thin layer chromatography (TLC)

Pre-coated 0.2 mm thin coatings TLC plates on aluminum sheets were used throughout the experiment.

2.5.2 Sample application

The TLC plates were spotted with a small amount of the crude extract by using a narrow glass capillary. The capillary was washed with acetone before each sample applied.

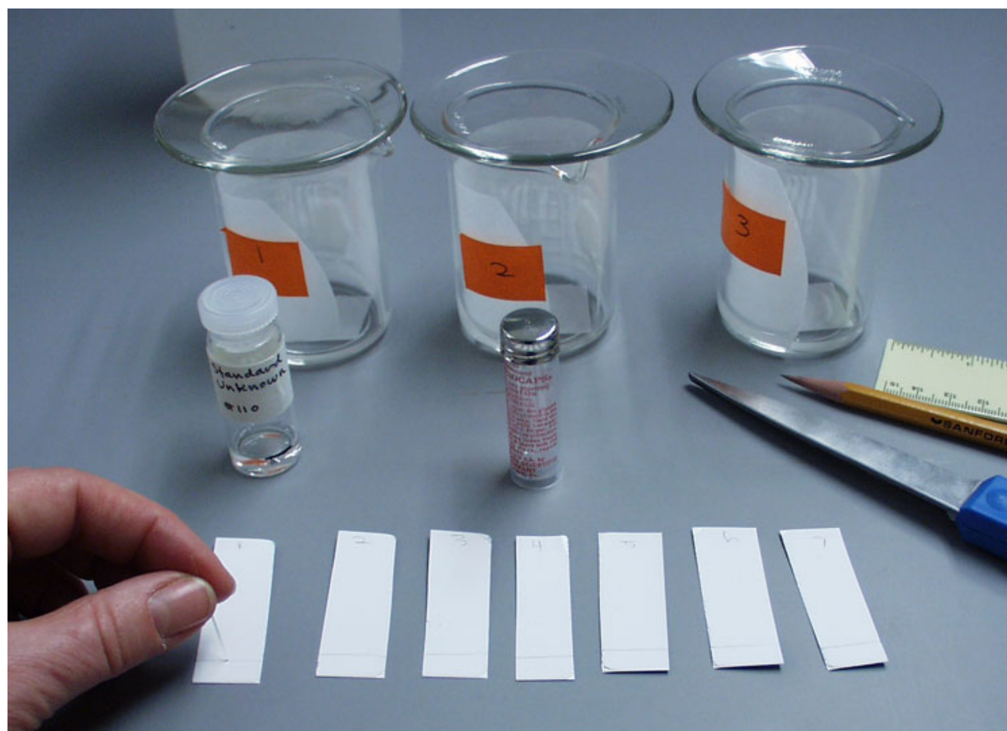

Fig 2.4: Process of spotting

2.5.3 Solvent system

The solvents of different polarity used for TLC are given below:

n-hexane : Ethyl acetate (in different ratios)

Ethyl acetate : Methanol (in different ratios)

Chloroform: Methanol (in different ratios)

n-hexane: Chloroform (in different ratios)

Ethyl acetate : Dichloromethane (in different ratios)

Dichloromethane : Methanol (in different ratios)

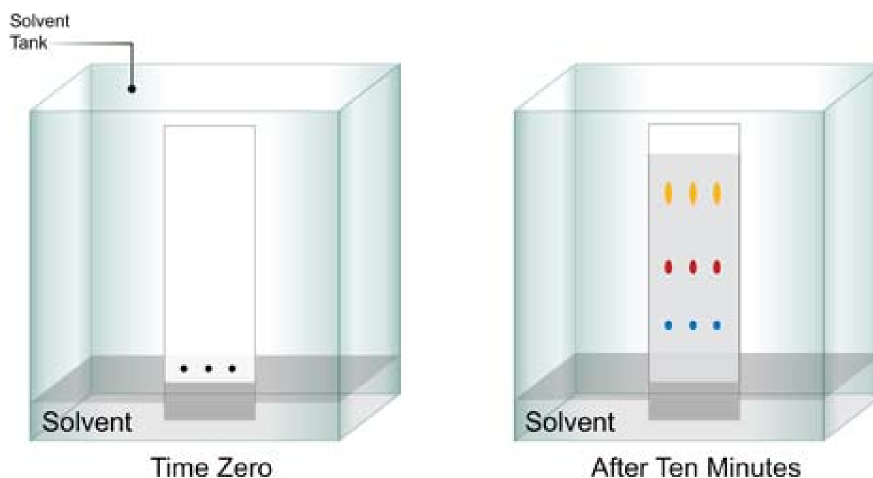

Fig 2.5: Developing of TLC plate

2.5.4 Preparation of TLC tanks

The ascending technique in glass jars & glass tanks were used to develop TLC plates. A suitable solvent system was poured into glass jar or tank in a required amount. The tank was then covered with a lid and kept for a certain period for allowing it to achieve saturation. A filter paper was usually introduced into the tank to promote the saturation process. The solvent level at the bottom of the tank must not be above the line of the spot where the sample solution was applied to the plate. As the solvent rises, the plate becomes moistened. When the solvent front moves almost near the end of the plate, the plate was then taken out and dried. The solvent front was not allowed to travel beyond the end of the silica-coated surface.

2.5.5 Detection of spots

For the location of the separated compounds, the plates were examined under UV light in different wavelengths, 254 and 361 nm.

The plates were developed by the spray reagent followed by heating in an oven at 80°C at for 5 minutes.

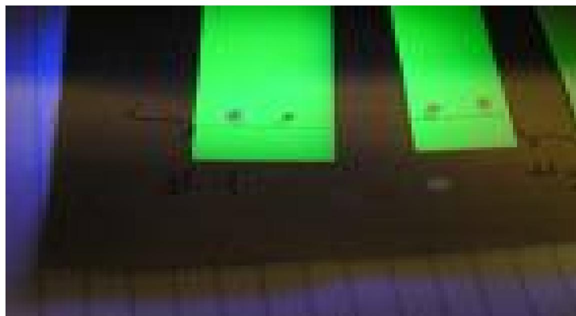

Fig 2.6: TLC plates under UV lamp

2.5.6 The R_f value

Retardation factor (R_f) is the ratio of the distance the compound travels and the distance the solvent front moves.

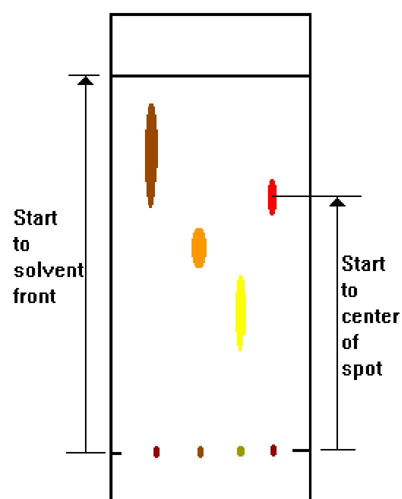

Fig 2.7: Calculation of R_f value

$$R_f = \frac{\text{Distance travelled by a substance}}{\text{Distance travelled by a solvent}}$$

Usually, the R_f value is constant for any given compound and it corresponds to a physical property of that compound.

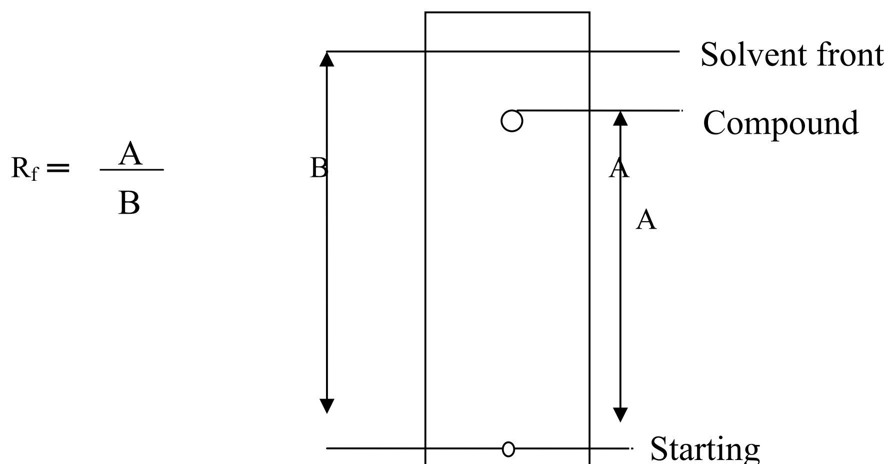

Figure-2.8: A plate for the calculation of R_f value.

2.5.7 Stationary phases of column chromatography

For normal phase column chromatography, silica gel of particle size 230-400 mesh (Merck) was used and separation was performed by gravitational flow with solvents of increasing polarity. The sample was applied into the column either as a solution or in a powdered form or by adsorbing samples by the silica gel. The eluted samples were collected in several test tubes and were monitored by TLC to make different fractions on the basis of R_f values.

For preparation of Sephadex LH-20 column, the required amount of Sephadex LH-20 gel (25-100 μm, Pharmacia, Sweden) was suspended in chloroform or DCM or methanol and the column was packed with this suspended gel.

2.5.8 Procedure for micro scale flash column chromatography

In micro scale flash chromatography, the column does not need either a pinch clamp or a stopcock at the bottom of the column to control the flow, nor does it need air-pressure connection at the top of the column. Instead, the solvent flows very slowly through the column by gravity until we apply air pressure at the top of the column with an ordinary Pasteur Pipette Bulb.

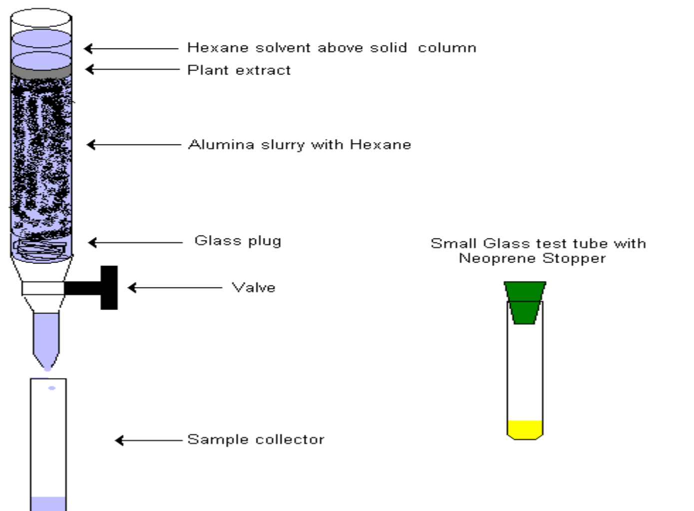

Fig 2.9: Various part of a column

2.5.9 Preparation of column (For micro scale operation)

A Pasteur pipette was plugged with a small amount of cotton to prevent the adsorbent from leaking. The pasteur pipette was filled with the slurry of column grade silica gel with a stream of solvent using a dropper. It was ensured that the "Sub Column" is free from air bubbles by recycling the solvents several times. The samples were applied at the top of the column. Elution was started with petroleum ether or n-hexane followed by increasing polarity.

2.6 Re-crystallization

Re-crystallization was employed as a final purification process. A solution of the compound in a minimum volume of the solvent in which it is soluble was prepared in hot condition. It was then left for crystallization. Sometimes, a mixture of solvents was used.

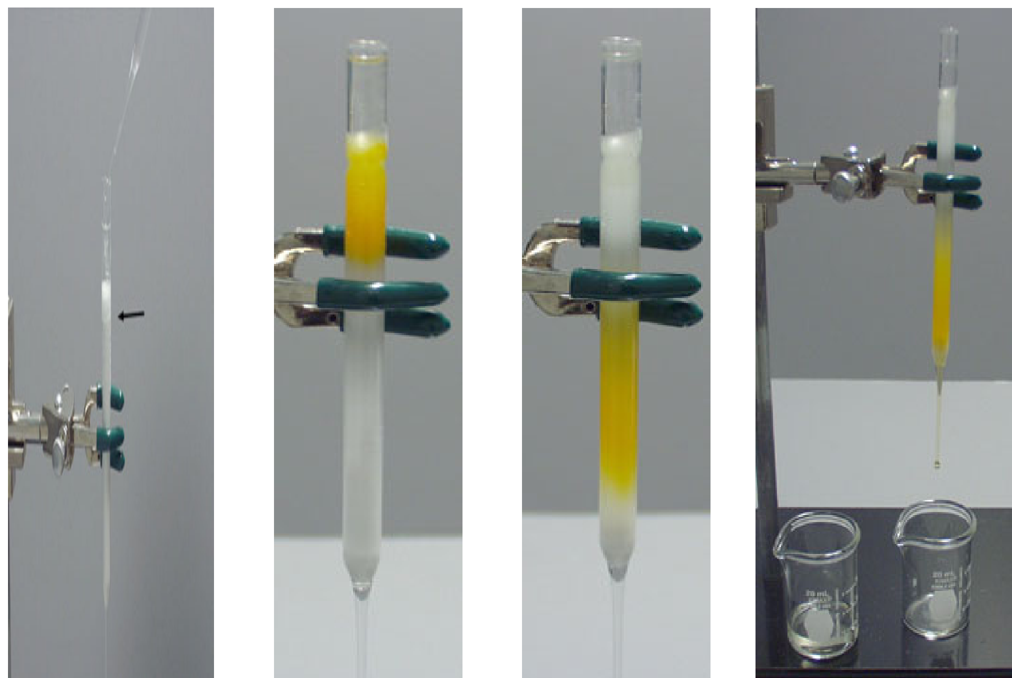

Fig 2.10: Various stages in micro scale column.

2.7 Spectroscopic Techniques

2.7.1 Infra-Red Spectroscopy (IR)

A Shimadzu IR prestige-21 (FT-IR) spectrometer was used for recording infrared spectrum. Major bands (ν_{max}) were recorded in wave number (cm^{-1}) as KBr pellets.

2.7.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a BRUKER NMR DPX-400 MHz instrument, with chemical shift data reported in ppm relative to the solvent used. The spectra were taken by using $CDCl_3$ & CD_3OD.

2.7.3 Ultra-Violet Spectroscopy

UV absorbance for obtaining the λ-max of the isolated compounds was taken by Shimadzu UV-1601 UV spectrophotometer.

2.8 Investigation of *Asparagus rosemosus*

2.8.1 Collection of the plant

The plant *Asparagus racemosus* (Locally known as Shatamuli/Shatavari) was collected from Nolchia, Nolka, Raigonj under Sirajgonj district.

2.8.2 Identification of species

The taxonomy of the plant was confirmed consulting with the National Herbarium's Botanist. A voucher specimen of this plant was deposited at Bangladesh National Herbarium.

2.8.3 Test of steroids

The roots powder (13 g) was extracted with a mixture of MeOH and $CHCl_3$ (100 ml, 1:1). This extract was concentrated and divided into two parts. One part was treated with concentrated H_2SO_4. When a reddish color is developed, indicating the presence of steroidal compound.

The other part was treated with a few drops of concentrated H_2SO_4 and 4-6 drops of acetic anhydride. When a greenish color is developed, indicating the presence of steroidal compound.

2.8.4 Test of terpenoids

A few mg of sample was dissolved in a mixture of $CHCl_3$-CH_3OH, and then few drops of conc. H_2SO_4 were added to it followed by 4-6 drops of Ac_2O. A red-violet color is developed, indicating the presence of terpenoid type compounds.

2.8.5 Test of alkaloids

Extracted 2 g of powder drug by warming for two minutes with 20 ml 1% H_2SO_4 in a 50ml conical flask on a water bath, with intermittent shaking, centrifuge; pipette off supernatant into a small conical flask. Make an initial test for alkaloids by adding to 0.1 ml in a semi-micro tube, one drop of Meyer's reagent. It gives a cream precipitate with alkaloids.

2.8.5.1 Preparation of Meyer's reagent:

It is prepared by dissolving 1.36 g of mercuric chloride in 60ml distilled water (A) & 5 g of potassium iodide in 10 ml of distilled water (B). A & B are mixed together and the volume is adjusted to 100 ml with water.

2.8.6 Extraction and isolation of the compounds from the leaves of *Asparagus rosemosus*

The roots of this plant were separated and dried under in open air. Afterwards it was powdered (~200 mesh) by a grinding machine. This powder (131 g) was used throughout this investigation. The root powder of *Asparagus rosemosus* was extracted with methanol. The extract was concentrated to dry mass (11 g) using rotary evaporator. The methanol extract was then partitioned by separatory funnel by using n-hexane, ethyl acetate and 1-butanol successively. All the crude extracts were also used for antimicrobial tests.

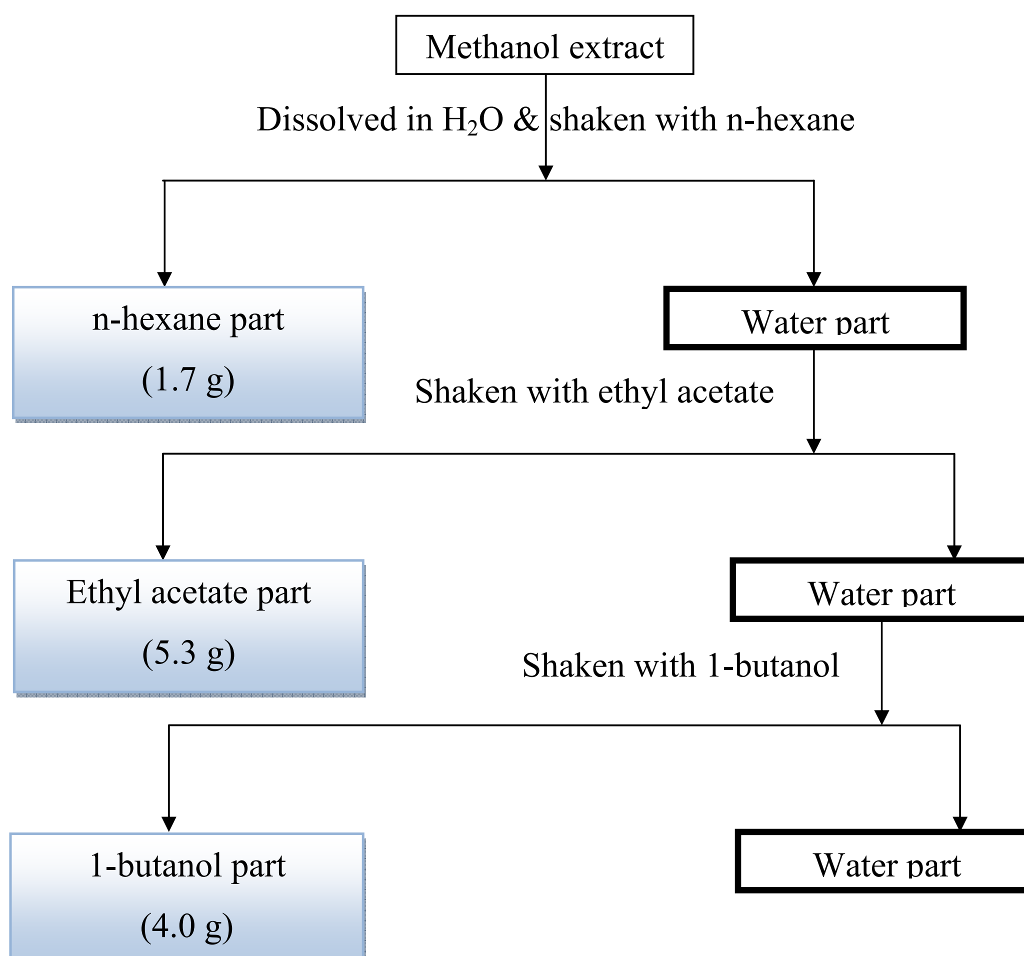

Chart 2.1: Separation by separatory funnel

2.8.7 Investigation of n-hexane extract

2.8.7.1 Thin layer chromatography (TLC)

TLC analysis of the n-hexane extract showed several spots under UV lamp following by the development by spray reagent on TLC plate.

2.8.7.2 Fractionation of the extract by vacuum liquid column chromatography (VLC)

The n-hexane extract was concentrated to dry mass (1.7 g) using rotary evaporator. The dry mass of n-hexane extract was adsorbed by the column grade silica gel. This sample was placed on the top of the bed of column packed with TLC grade silica gel. The column was first eluted with 100% n-hexane and then eluted with mixtures of n-hexane and ethyl acetate increasing the polarity of the solvents and finally with the mixtures of ethyl acetate & methanol. The eluents were collected in an amount of 200 ml in a series of conical flasks. Solvent systems used as mobile phases in the analysis of n-hexane part are listed in table-2.1.

Table-2.1: Number of fractions collected in conical flasks from vacuum liquid column chromatography (VLC) of n-hexane extract using different solvent systems

Fraction no.	Solvent system
H_1-1	n-hexane (100%)
H_1-2	n- hexane : ethyl acetate (90:10)
H_1-3	n- hexane : ethyl acetate (80:20)
H_1-4	n- hexane : ethyl acetate (70:30)
H_1-5	n- hexane : ethyl acetate (60:40)
H_1-6	n- hexane : ethyl acetate (50:50)
H_1-7	n- hexane : ethyl acetate (40:60)
H_1-8	n- hexane : ethyl acetate (30:70)
H_1-9	n- hexane : ethyl acetate (20:80)
H_1-10	n- hexane : ethyl acetate (10:90)
H_1-11	ethyl acetate (100%)
H_1-12	ethyl acetate: methanol (97.5: 2.5)
H_1-13	ethyl acetate: methanol (95:5)
H_1-14	ethyl acetate: methanol (90:10)
H_1-15	ethyl acetate: methanol (87.5:12.5)
H_1-16	ethyl acetate: methanol (85:15)
H_1-17	ethyl acetate: methanol (80:20)
H_1-18	ethyl acetate: methanol (70:30)

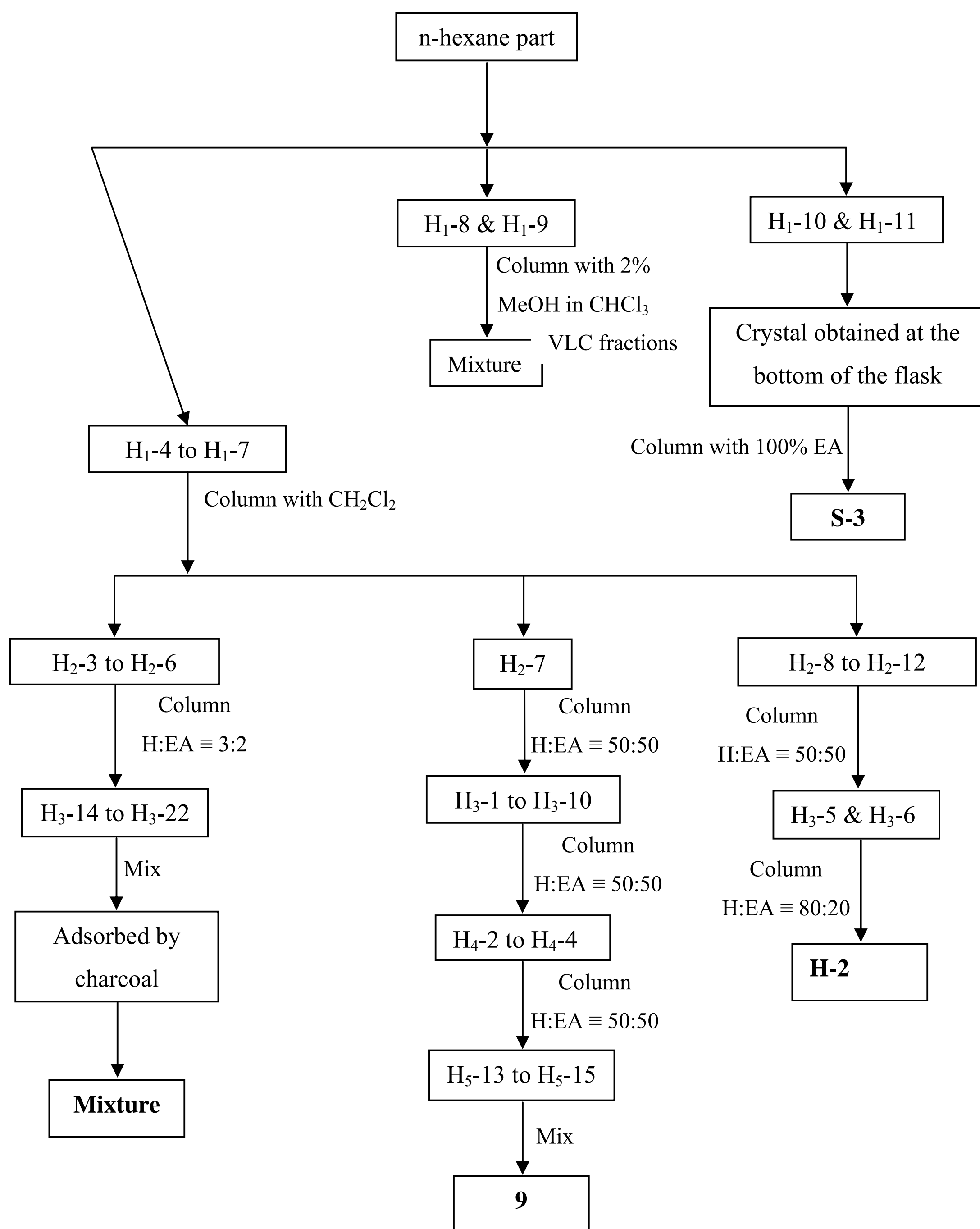

Chart 2.2: Flow chart for the isolation of compounds from n-hexane part.

2.8.7.3 Analysis of the fractions by column chromatography

Fraction H_1-1 to H_1-3 & H_1-12 to H_1-18 shows no prominent spot on TLC analysis. So, they were discarded.

Fraction H_1-4 to H_1-7 shows similar spots in TLC analysis; they were got mixed. As they contain chlorophyll, so they were done column using Sephadex LH-20 as

stationary phase & chloroform as mobile phase. Among the fractions, the TLC analyses of H2-3 to H2-6 were similar. So they got mixed & had done a column using n-hexane: EA ≡ 3:2. Then they were treated with charcoal to get the chlorophyll adsorbed by the charcoal & thus the first compound **H-2** was obtained. On the other hand, the second fraction H_2-7 shows a single compound with a little bit contamination. So, it was done a column using n-hexane : EA ≡ 50:50. But still containing some tailing in the TLC analysis, it was further treated with column with the same solvent system to get the purified compound **9.** The rest fractions of the H_1 fractions (i.e. H_1-8 to H_1-12) were mixed together for their similar R_f value. Then they were done column with the solvent system n-hexane: EA ≡ 50:50 & finally with 20% EA in n-hexane & found mixture the compound.

Since the R_f value of the fractions H_1-8 & H_1-9 were same so they were mixed together & had done a column using 2% MeOH in $CHCl_3$ to get mixture of three compound which are difficult to separated**.** In the fractions H_1-10 & H_1-11, a number of colorless crystals were formed at the bottom of the conical flasks. The TLC analysis provided the information of the little contamination of another compound which was excluded by performing a column by 100% EA as mobile phase to obtain pure compound **S-3.**

2.8.8 Investigation of the dicholoromethane extract

2.8.8.1 Thin layer chromatography (TLC)

TLC analysis of the dichloromethane extract showed several spots under UV lamp following by the development by spray reagent on TLC plate.

2.8.8.2 Fractionation of the extract by vacuum liquid column chromatography

The dry mass of dichloromethane extract (6.2 g) was mixed with column grade silica gel. This sample was placed on the top of the bed of column packed with column grade silica gel. The column was first eluted with 100% hexane and then eluted with mixtures of hexane with increasing amount of chloroform and finally with ethyl acetate. The eluents were collected in a series of test tubes. Solvent systems used as mobile phases in the analysis were listed in Table-3.2.

Table-3.2: Number of fractions collected in test tubes from column chromatography of insoluble part of methanol extracts using different solvent systems are as follows-

Hexane ml	Chloroform mL	Ethyl Acetate mL	Number of test tube
100	00	00	1-2
00	95	05	3-5
00	90	10	6-7
00	85	15	8-11
00	80	20	12-14
00	75	25	15-18
00	70	30	19-22
00	65	35	23-26
00	60	40	27-28
00	50	50	29-31
00	40	60	32-33
00	30	70	34-37
00	20	80	38-40
00	10	90	41-42
00	00	00	43-45
00	95	05	46-48
00	00	100	49-52

2.8.8.3 Screening of the fractions

Each of the fractions was monitored by TLC and the fractions of similar behaviors were combined together and these were marked as T_1, T_2, T_3, T_4& T_5 (Table-3.3).

Table-3.3: Screening of the fractions by similar TLC pattern.

No. of test tubes	Fraction no	Solvent system for TLC	No of (after spraying with vanilling)
1-10	T_1	100% Hexane	No spot was found
11-16	T_2	50:50($CHCl_3$:Hexane)	Single spot found
17-31	T_3	50:50 $CHCl_3$:Hexane)	Spot with tailing
31-43	T_4	60:40($CHCl_3$:Hexane)	Mixture
43-52	T_5	70:30($CHCl_3$:Hexane)	Mixture

2.8.8.4 Analysis of the fraction by TLC

TLC analysis of the fractions were further carried out and attempts were taken to characterize the fractions **T_2**as the TLC pattern of the fraction indicated that it would be easy to isolate pure compound from them.

2.8.8.5 Analysis of the fraction T_2

The fraction T_2 was left undisturbed at room temperature for several days. A white-solid was obtained. When the solid was obtained, a portion of the solid dissolved in dichloromethane and it was checked by TLC.

The solid obtained from the fractions $T_2$was renamed as com-3

2.9 Properties of compound S-3

2.9.1 Physical properties

The compound H-2 is a white crystalline compound. The R_f value of the compound is 0.86 in n-hexane: EA ≡ 50:50. It is soluble in chloroform, dichloromethane, ethyl acetate, methanol & ethanol. It has tested by Salkawoski method which developed a reddish color indicating that the compound may be steroid.

2.9.2 Characterization of S-3 by spectroscopic method

2.9.2.1 Infrared (IR) spectroscopy of S-3

The IR spectrum (in KBr) of the compound H-2 has important transmittance at 3446 (OH stretching), 2960 (-HC=CH- cyclic), 2937 (-C-H stretching), 1637 (C=C), 1458 (CH_2, n), 1062 (cyclic alkane) cm^{-1}.

2.9.2.2 ^{1}H-NMR spectroscopy of S-3

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of the compound H-2 has signals at δ_H (ppm) 5.347 ppm (1H, d), 5.148 ppm (1H, m), 5.010 ppm (1H, m), 3.513 ppm (1H, m; oxymethine protone), 2.262 ppm (2H, m), 1.987 ppm (2H, t), 1.835 ppm (2H, m), 1.488 ppm (6H, s) 1.001 ppm (6H, s), 0.913 ppm (3H, d), 0.807 ppm (9H, m) and 0.687 ppm (3H, d) .

2.9.2.3 ^{13}C-NMR spectroscopy of S-3

The ^{13}C-NMR spectrum in (100 MHz, $CDCl_3$)of the compound H-2 has signals at δ_C 37.30 ppm, 31.72 ppm , 71.85 ppm , 42.36 ppm, 140.81 ppm **,** 121.74 ppm , 31.96 ppm , 31.96 ppm, 50.2 ppm, 36.56 ppm , 21.13 ppm , 39.83 ppm, 40.50 ppm , 56.82 ppm , 24.34 ppm , 28.28 ppm, 56.12 ppm, 12.08 ppm , 19.43 ppm , 36.19 ppm , 18.82 ppm , 138.33 ppm, 129.31 ppm , 45.9 ppm , 29.23 ppm, 19.84 ppm , 19.08 ppm , 23.13 ppm and 12.26 ppm.

2.11 Properties of compound 9

2.11.1 Physical properties

The compound 9 is a white crystalline compound. The R_f value of the compound is 0.82 in n-hexane: EA ≡ 50:50. It is soluble in chloroform, dichloromethane, ethyl acetate, methanol & ethanol. It has tested by Salkawoski method which developed a reddish color indicating that the compound may be steroid.

2.11.2 Characterization of 9 by spectroscopic method

2.11.2.1 Infrared (IR) spectroscopy of 9

The IR spectrum (in KBr) of the compound 9 has important transmittance at 3290 (OH stretching), 1673 (C=C streching), 1565 (CH bending due to methylene group), 1445 (CH bending due to methyl group), 1275 (C-O stretching for alcoholic group) cm^{-1}.

2.11.2.2 ^{1}H-NMR spectroscopy of 9

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of the compound 9 has signals at δ_H (ppm) 5.34 ppm (1H, d), 3.51 ppm (1H, m), 2.26 ppm (2H, m), 1.98 ppm (1H, m), 1.86 ppm (3H, m), 1.56 ppm (3H, s), 0.99 ppm (4H, s) and 0.67 ppm (3H, s).

2.11.2.3 ^{13}C-NMR spectroscopy of 9

The ^{13}C-NMR spectrum in (100 MHz, $CDCl_3$) of the compound 9 has signals at δ_C 37.29 ppm , 28.26 ppm , 71.84 ppm, 42.36 ppm , 140.80 ppm**,** 121.73 ppm , 31.71 ppm, 31.95 ppm , 50.19 ppm , 36.54 ppm, 21.12 ppm , 39.82 ppm , 42.36 ppm, 56.81 ppm , 24.33 ppm, 26.16 ppm, 56.11 ppm , 11.88 ppm , 19.41 ppm , 40.50 ppm , 19.07 ppm , 34 ppm, 26.16 ppm , 45.89 ppm , 31.95 ppm , 19.83 ppm, 21.12 ppm , 24.33 ppm , and 12.01 ppm.

2.12 Properties of compound H-2

2.12.1 Physical properties

The compound H-2 is a white crystalline compound. The R_f value of the compound is 0.78 in n-hexane: EA ≡ 50:50. It is soluble in dichloromethane, ethyl acetate, methanol & ethanol. It has tested by Salkawoski method which developed a reddish color indicating that the compound may be steroid.

2.12.2 Characterization of H-2 by spectroscopic method

2.12.2.1 Infrared (IR) spectroscopy of H-2

The IR spectrum (in KBr) of the compound S-3 has important transmittance at 3490 (OH stretching), 1640 (C=C streching), 1560 (CH bending due to methylene group), 1515 (CH bending due to methyl group), 1360 (C-O stretching for alcoholic group) cm^{-1}.

2.12.2.2 ^{1}H-NMR spectroscopy of H-2

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$ + MeOH) of the compound S-3 has signals at δ_H (ppm) 5.37 ppm (1H, br s), 4.40 ppm (1H, d), 3.85 ppm (1H, dd), 3.72 ppm (1H, dd), 3.60 ppm (1H, m), 3.42 ppm (2H, t), 3.229 ppm (1H, br s), 3.22 ppm (1H, t), 2.42 ppm (1H, dd), 2.28 ppm (1H, s), 1.03 ppm (6H, s), 0.94 ppm (5H, d), 0.85 ppm (9H, m) and 0.70 ppm (3H, s).

2.12.2.3 ^{13}C-NMR spectroscopy of H-2

The ^{13}C-NMR spectrum in $CDCl_3$ + MeOH of the compound S-3 has signals at δ_C 36.8 ppm , 31.5 ppm , 78.7 ppm, 39.3 ppm , 139.9 ppm , 121.6 ppm , 31.5 ppm, 31.9 ppm, 49.8 ppm , 36.2 ppm , 20.6 ppm , 38.1 ppm , 41.9 ppm , 56.3 ppm, 23.9 ppm, 28.7 ppm , 55.6 ppm, 11.2 ppm , 19.1 ppm , 36.2 ppm, 18.4 ppm, 33.5 ppm, 27.7 ppm , 45.5 ppm, 28.7 ppm , 19.4 ppm , 18.7 ppm , 22.6 ppm , 11.2 ppm , 100.7 ppm, 73.1 ppm , 76.1 ppm , 69.6 ppm , 75.6 ppm and 61.7 ppm .

2.13 Properties of compound Com-3

2.13.1 Physical properties

The compound Com-3 is a colorless crystalline compound. The R_f value of the compound is 0.89 in 2% MeOH in Ethyl acetate. It is soluble in chloroform,

dichloromethane, ethyl acetate, methanol & ethanol. It has tested for terpenoid & a red-violet color confirms that the compound Com-3 is terpenoid type compound.

2.13.2 Characterization of Com-3 by spectroscopic method

2.13.2.1 Infrared (IR) spectroscopy of Com-3

The IR spectrum (in KBr) of the compound Com-3 has important transmittance at 3302 (OH stretching), 2839 & 2924 (C-H stretching), 1749 (lactone ring), 1637 (C=C un-saturation), 900 (exocyclic methylene) cm^{-1}.

2.13.2.2 ^{1}H-NMR spectroscopy of Com-3

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of the compound R_E-1 has signals at δ_H (ppm) 7.08 (1H, s), 4.83 ppm (1H, s), 4.74 ppm (2H, s), 4.56 ppm (1H, s), 3.72 ppm (1H, d), 3.37 ppm (1H, d), 2.43 ppm (2H, m), 2.11 ppm (1H, m), 1.9 ppm (1H, m), 1.74 ppm (5H, m), 0.96 ppm (3H, s) and 0.64 ppm (3H, s) cm^{-1}.

2.13.2.3 ^{13}C-NMR spectroscopy of Com-3

The ^{13}C-NMR spectrum in $CDCl_3$ of the compound R_E-1 has signals at δ_C 38.5 ppm, 18.9 ppm, 24.5 ppm , 38.8 ppm , 56.2 ppm , 24.4 ppm , 35.3 ppm , 147.5 ppm , 56.4 ppm , 38.5 ppm, 27 ppm, 39 ppm, 134.8 ppm, 143.8 ppm , 64.9 ppm , 174.3 ppm, 106.7 ppm , 21.7 ppm , 70 ppm and 15.2 ppm.

ACKNOWLEDGEMENT

In the name of Allah, The Almighty to whom all praises me due for his infinite mercy and blessings.

I am feeling proud and honored to express my profound gratitude, indebtedness, deep appreciation and respect to my supervisor Dr. Shakila Rahman, and my Idol. She certainly is one of the most important people for me here, to transform a mechanical person into a matured, motivated, independent, extremely inquisitive and research driven person. I might go on to a different field in the future, but the initial development is the toughest part.

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AUTHOR

MD. ARIFUL HOQUE

3.1 Preliminary Investigation of the Plant Material

3.1.1 Plant material

A species of the Asparagaceae family, *Asparagus racemosus*, has been investigated in this work. The plant root was used in this investigation.

3.1.2 Extraction of the plant material

The root of *Asparagus racemosus* was collected, dried and ground to a coarse powder. The root powder was extracted with methanol, followed by hexane, dichloromethane. The methanol extract was concentrated to dry mass (11 g) using rotary evaporator.

3.1.3 Phytochemical screening

Phytochemical screening has been carried out during investigation and the presence of phytochemicals e.g. saponins, flavonoids, steroids, terpenoids, have been observed.

3.1.3 Isolation and characterization of compounds

From the various crude extracts, pure compounds were isolated applying various chromatographic techniques. The isolated pure compounds were then characterized using various spectroscopic techniques.

3.2.1 Characterization of compound-1

A) Physical properties

The compound-1was obtained as a white needle shaped crystal. The Rf value of the compound was 0.86 in n-hexane: EA = 50:50. It was soluble in chloroform, dichloromethane, ethyl acetate, methanol & ethanol. It was tested by Salkawoski and Liebermannburchard method which developed a reddish color and greenish color respectively indicating that the compound may be steroid. The melting point of this compound was found to be (138-140) °C [**48**].

B) Characterization of compound-1 by spectroscopic method

The structure of the compound-1has been established by FT-IR, ^{1}H-NMR and ^{13}C-NMR spectral evidences.

C) Infrared (IR) spectroscopy of compound-1

The IR spectrum (in KBr) of compound-1exhibit characteristic absorption at 3446.79cm^{-1} that was characteristic of O-H stretching. Absorption at 2935.55 cm^{-1} and 2855.22 cm-1 is due to aliphatic C-H stretching and bending vibrations of methyl group. Other absorption frequencies include 1653.00 cm^{-1} as a result C=C stretching however this band was weak, at 1447.18 cm-1 was a bending frequency for cyclic $(CH_2)n$ and 1375.25 cm^{-1} for C-H bending. The absorption frequency at 1055.06 cm^{-1} due to C-C vibration signified cycloalkane. The out of plane C-H vibrations of unsaturated part was observed at 883.40 cm^{-1}.These absorption frequencies resemble the absorption frequencies observed for stigmasterol [**47**].

D) ^{1}H-NMR spectroscopy of compound-1

The ^{1}H NMR spectrums showed two one proton multiplets at (δ) 3.53 and (δ) 5.36 typical for H-3 and H-6 of a steroidal nucleus. The olefinic protons H-22 and H-23 appeared as characteristic downfield signals at (δ) 5.16 ppm (1H, dd, *J*=15.0, 6.5 Hz) and 5.03 ppm (1H, dd, *J*=15.0, 9.0 Hz) respectively. Each of the signals was observed as double doublet, which indicated coupling with the neighbouring olefinic and methane protons. The ^{1}H NMR spectrums displayed two three-proton singlets at (δ) 1.00 ppm and (δ) 0.67 ppm assignable for Me-19 and Me-18 respectively. In addition, two doublets at (δ) 0.82 ppm (3H, d, 7.2 Hz) and 0.80 ppm (3H, d, 7.2 Hz) could be recognized to the two methyl groups at Me-26 and Me-27 and another three-proton doublet at (δ) 0.91 ppm (3H, d, 6.8 Hz) for Me-21. On the other hand, one three-proton triplet at (δ) 0.85 ppm (3H,t, 7.2 Hz) could be assigned to the primary methyl group attached for Me-29. **[48-51]**

E) ^{13}C-NMR spectroscopy of compound-1

The 13C NMR spectrum showed 29 carbons including an oxymethine carbon signal at δ 71·85 ppm was due to C-3*β* hydroxyl group and two signals at δ 140·81 ppm and δ 121·74 ppm, which were assigned C5 and C6 double bonds respectively as in Δ5 spirostene [**49**]. The signal at 19.41 and 12.02 ppm corresponds to angular carbon atom (C-19 and C-18 respectively). The value for C18 is lower due to γ-gauche interaction that increases the screening of the C-18 hence lower chemical shift. However, the loss of H in C-6 results in decrease in screening of the C19 leading to increase in C19 chemical shift to higher frequency. This is also tenable as in chemical shift of 19.41 ppm and 12.02 ppm (for C19 and C18 respectively). Spectra showed twenty nine carbon signal including six methyls, nine methylenes, eleven methines and three quaternary carbons which were showed in **Table-3.1** The alkene carbons appeared at δ140·81 ppm , 138.33 ppm , 129.0 ppm and 121·74 ppm. The physical and spectral data of this compound was in complete agreement to the reported data in literature [**47-55**], the compound-1 was identified as Stigmasterol. This compound-1 was first time isolated so far from the specie, *Asparagus racemosus*

Table-3.1: Compare the ^{1}H NMR and ^{13} C NMR data of the compound-1 with reference data.

C/H	^{1}H (δ ppm) Observed	^{1}H (δ ppm) Literature [48]	^{13}C (δ ppm) Observed	^{13}C (δppm) Literature [48]
1			37·29 (CH_2)	37.2
2			28·28 (CH_2)	28.2
3	3·52(1H,dd,*J*=9.6,4.8Hz)	3.53 (1H, m)	71·85 (CH)	71.82
4			42·36 (CH_2)	42.8
5			140·81 (Cq)	140.77
6	5·34 (1H, d, *J*=5·2 Hz)	5.35 (1H, m)	121·74 (CH)	121.73

C/H	^1H (δ ppm) Observed	^1H (δ ppm) Literature [48]	^{13}C (δppm) Observed	^{13}C (δppm) Literature [48]
7	1.99 (2H, m)	2.00 (2H, m)	31·72 (CH_2)	31.9
8			34.01 (CH)	33.96
9			50·20 (CH)	50.14
10			36·54 (Cq)	36.5
11			26·17 (CH_2)	26.08
12			39·83 (CH_2)	39.7
13			42·37 (Cq)	42.2
14			56·82 (CH)	56.88
15			24·33 (CH_2)	24.31
16	1.84 (2H, m)	1.86 (2H, m)	29.23 (CH_2)	29.16
17			56·12 (CH)	56.06
18	0·67 (3H, s, Me)	0.68 (3H, s, Me)	12.02 (CH_3)	12.05
19	1.00 (3H, s, Me)	1.02 (3H, s, Me)	19·41 (CH_3)	19.0

C/H	**^{1}H (δppm)Obtained**	**^{1}H (δppm) Literature [48]**	**^{13}C (δppm) Obtained**	**^{13}C (δppm) Literature [48]**
20	2.28 (1H, m)	2.30 (1H, m)	40·50 (CH)	40.5
21	0·92 (3H,d,J=6.0Hz,Me)	0.93(3H,d,J=8.0Hz,Me)	21.13 (CH_3)	21.22
22	5.16(1H,dd,*J*=15.0,8.4Hz)	5.18 (1H, m)	138.33(CH_2)	138.33
23	5.03(1H,dd,*J*=15.0,8.4Hz)	5.00(1H, m)	129.0 (CH_2)	129.28
24			51.28 (CH)	51.25
25			45·91 (CH)	45.84
26	0·81 (3H,d,J=7.2Hz,Me)	0.82(3H,d,J=6.7Hz,Me)	19·42 (CH_3)	19.4
27	0·79 (3H,d,J=7·2Hz,Me)	0.79(3H,d,J=6.7Hz,Me)	19.84 (CH_3)	20.21
28			24·34 (CH_2)	24.4
29	0·85 (3H,d,J=8.0Hz,Me)	0.85(3H,t,J=7.4Hz,Me)	12·26 (CH_3)	12.26

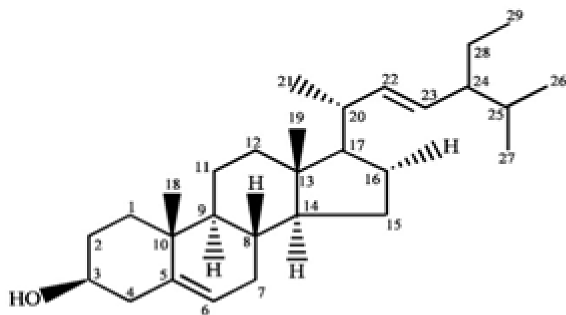

Fig 3.1: The probable structure of the compound-1 as stigmasterol.

3.3 Characterization of compound-2

A) Physical properties

The compound-2 was obtained as white powdered crystal. The R_f value of the compound was 0.33 in toluene: ethyl acetate = 95:5. It was soluble in chloroform, dichloro- methane, ethyl acetate, methanol and ethanol. It was tested by Salkawoski and Liebermann burchard method which developed a reddish color and greenish color respectively indicating that the compound may be steroid. The melting point of this compound was found to be 130°- 132° C [**52**].

B) Characterization of compound-2 by spectroscopic method

The structure of the compound-2has been established by FT-IR, ^{1}H-NMR, ^{13}C-NMR, DEPT-90 and DEPT-135 spectral evidences.

C) Infrared (IR) spectroscopy of compound-2

The IR spectrum (in KBr) of compound-2 exhibit characteristic absorption band at 3421.72 cm^{-1} that was characteristic of O-H stretching. Absorption at 2935.55 cm-1 and 2855.22 cm-1 is due to aliphatic C-H stretching and bending vibrations of methyl group. Other absorption frequencies include 1653.00 cm^{-1} as a result C=C stretching however this band was weak at 1447.18 cm^{-1} was a bending frequency for cyclic (CH_2) and 1375.25 cm^{-1} for C-H bending. The absorption

frequency at 1051.78 cm^{-1} due to due to C-C vibration signified cycloalkane. These absorption frequencies resemble the absorption frequencies observed for *β*-sitosterol [**52**].

D) ^{1}H-NMR spectroscopy of compound-2

The ^{1}H NMR spectrum of the compound-2showed two one-proton multiplet at δ 3.51 ppm and δ 5.34 ppm typical for H-3 and H-6 of a steroidal nucleus. The spectrum further revealed two singlets at δ 0.67 and δ 1.00 ppm each integrating for three protons assignable to two tertiary methyl groups at Me-18 and Me-19 respectively. The spectrum also showed two doublets at δ 0.79 ppm (J =7.2 Hz) and δ 0.83 ppm (J =7.2 Hz) which could be attributed to two methyl groups at Me-27 and Me-26 respectively. On the other hand, two doublet at δ 0.92 ppm (J = 6.0 Hz) andδ 0.85 (J = 8.0 Hz) could be assigned to the primary methyl groups attached at Me-21 and Me-29, respectively [**52-55].**

E) ^{13}**C-NMR spectroscopy of compound-2:** The ^{13}C NMR spectrum showed 29 carbons including an oxymethine carbon signal at δ 71.84 and two olefinic carbons at δ 140.80 and δ 121.73 ppm.The double bonded unsaturation at δ 140·80 ppm and δ 121·73 ppm which were assigned C5 and C6 double bonds respectively as in Δ5 spirostene [**49**]. If we compare DEPT 135 & DEPT 90 experiments for compound-2then we confirmed that this compound was having six methyl (CH_3) groups, eleven methylene (CH_2) groups, nine methine (CH) groups and three quaternary carbons (C) groups which are shown in **Table-3.2**. The physical and spectral data of the compound was in complete agreement to the reported data in literature [**47**, **50, 52-55**] the compound-2 was identified as *β*-sitosterol. The compound-2 was first time isolated so far from the specie, *Asparagus racemosus.*

Figure 3.2: Compare the ^{1}HNMR and ^{13}CNMR sperta data fo the compound-2 with reference data.

C/H	**^{1}H (δ ppm) Observed**	**^{1}H (δ ppm) Literature [52]**	**^{13}C (δ ppm) Observed**	**^{13}C (δ ppm) Literature [52]**
1			37·29 (CH_2)	37.28
2			31.95 (CH_2)	31.93
3	3·51 (1H,m)	3.07(1H,dd, *J*= 11.0,5.0 Hz)	71·84 (CH)	71.82
4			42·36 (CH_2)	42.33
5			140·80 (Cq)	140.70
6	5·34(1H,d,*J*=5·2Hz)	5.11 (1H, t, *J*= 3.3 Hz)	121·73 (CH)	121.72
7			31·71 (CH_2)	31.69

C/H	^{1}H (δ ppm) Observed	^{1}H (δ ppm) Literature [52]	^{13}C (δ ppm) Observed	^{13}C (δ ppm) Literature [52]
8			31·95 (CH)	31.93
9			50·19 (CH)	50.17
10			36·18 (Cq)	36.12
11			21·12 (CH_2)	21.10
12			39·82 (CH_2)	39.80
13			42·36 (Cq)	42.33
14			56·81 (CH)	56.79
15			24·33 (CH_2)	24.37
16			28.26 (CH_2)	28.25
17			56·11 (CH)	56.09
18	0·67 (3H, s, Me)	0.68 (3H, s, Me	11·88 (CH_3)	11.86
19	1.00 (3H, s, Me)	1.02 (3H, s, Me)	19·41 (CH_3)	19.40
20			36.54 (CH)	36.52
21	0·92(3H,d,J=6.0Hz,Me)	0.94 (3H,d,J=6.5Hz,Me)	19·07 (CH_3)	18.79

C/H	^{1}H (δ ppm) Observed	^{1}H (δ ppm) Literature [52]	^{13}C (δ ppm) Observed	^{13}C (δ ppm) Literature [52]
22			34·00 (CH_2)	33.98
23			26·16 (CH_2)	26.14
24			45·89 (CH)	45.88
25			29.23 (CH)	28.91
26	0·83(3H,d,J=7.2Hz,Me)	0.85(3H,d,J=6.7Hz,Me	19·83 (CH_3)	19.80
27	0·79(3H,d,J=7·2Hz,Me)	0.83(3H,d,J=6.7Hz,Me)	18.81 (CH_3)	18.79
28			23.12 (CH_2)	23.10
29	0·85(3H,d,J=8.0Hz,Me)	0.88(3H,t,J=7.4Hz,Me)	12·01 (CH_3)	11.99

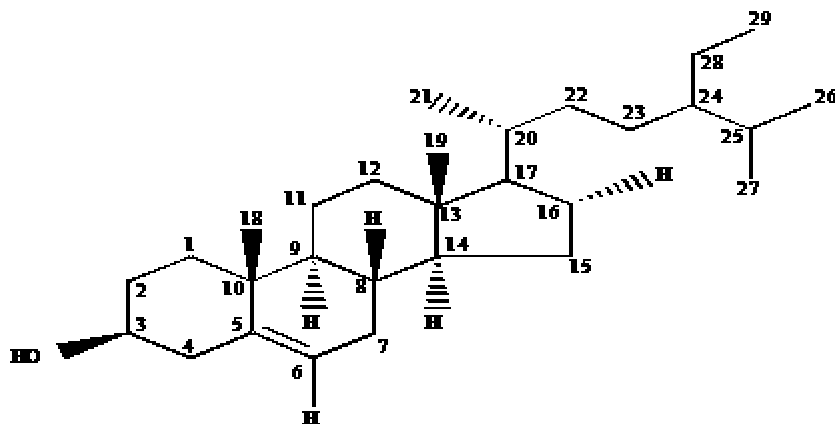

Fig 3.2: the probable structure of compound -2 as β-sitosterol .

3.3 Characterization of compound-3

From Dichloromethane part chloroform and ethyl acetate 85:15 to 75:25 was name as the fraction $T_{2.}$ After the sub-column a white-solid was obtained. When the solid was obtained, a portion of the solid dissolved in dichloromethane. The white solid compound was isolated with slight impurities and it was obtained as a white crystalline solid. During screening it was observed that compound-3 (Rf = 0.57, n- Hexane: $CHCl_3$-50:50).

A) Physical properties

Compound -3 was soluble in a mixture of chloroform and dichloromethane. Melting point of the sample was found to be 277-279°C. It gave violet color with vanilling-sulphuric acid reagent indicating that compound -3 might be a triterpene.

B) Characterization of compound-3 by spectroscopic method

The structure of the compound-3 has been established by FT-IR, ^{1}HNMR, ^{13}NMR, DEPT-90 and DEPT-135 spectral evidences.

C) IR Spectroscopy:

The IR (KBr pellet) spectrum of the compound-3 had major absorbance at 2947.28 cm^{-1} for CH_3 stretching, 1735.8 cm^{-1} for ester C=O stretching, 1366.59 cm^{-1} and 1453.39.11 cm^{-1} for C-H stretching, 1244.11 cm^{-1} for C-O bond and weak 874.66 cm^{-1} for C-H bending.

D) ^{1} H NMR Spectroscopy

The ^{1}H NMR spectrum of the compound-3 revealed the signals at C δ1.672, 0.787, 0.823, 0.841, 0.863, and 0.926 and 1.118 ppm for seven methyl groups. Olifinic protons of H-29 showed two broad singlets atδ 4.672 and δ 4.556. A singlet at δ2.036 was observed for methyl group of acelate H-32. A multiplete at δ 4.479 was observed for H-3.

Table 3.3: ^{1}H-NMR data of compound-3 compare with reference value

No of Proton ^{1}H NMR	Experiment value of ^{1}H NMR Compound-3	Reported Value ^{1}H NMR[54]
H-3	4.479 (1H, m)	4.47 (1H, m,)
H-19	2.371 (1H, m)	2.37 (dt, 1H)
H-23	0.856 (3H, s)	0.85 (3H, s)
H-24	0.841 (3H, s)	0.84 (3H, s)
H-25	0.863 (3H, s)	0.86 (3H, s)
H-26	1.018 (3H, s)	1.03(3H, s)
H-27	0.926(3H, s)	0.94(3H, s)
H-28	0.787 (3H, s)	0.79 (3H, s)
H-29a	4.556 (1H, s)	4.57 (1H, s)
H-29b	4.672 (1H, s)	4.68 (1H, s)
H-30	1.672 (3H, s)	1.68 (3H, s)
$CH_3-C(=O)-$	2.036 (3H. S)	2.04 (3H,s)

Here, ^1H NMR spectra shown in tentative structure of the compound fig.: 3.3

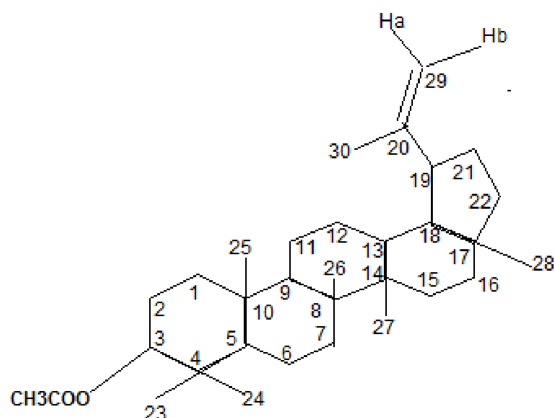

Fig. 3.3: ^1H NMR spectra of compound-3

E) ^{13}C NMR Spectroscopy

The ^{13}C NMR spectrums of compound -3 gave 32 singals indicating that the compound contained 32 carbons. By DEPT-135 and their expansions all the carbons were distinguished. DEPT technic indicated the presence of 8methyl, 11 methylene, 6 methine, 6 quaternary carbons in the compound-3.

In the ^{13}C NMR spectrum a signal at δ 171.03 ppm indicate the presence of C=O ester group in the compound. The deshielded peak at δ 150.99 ppm was assigned to the quarternary carbon attached with an olefinic carbon, which was supported by the presence of a signal at δ 109.38 ppm due to the olefinic carbon CH_2. The signal at δ 81.00 ppm was assigned a methane carbon attached with a C-O group.

Table 3.3: 13 C spectra data of Compound -3 compare with reported value**.**

No. Carbon	Types of Carbon	Chemical shift (δ) value in ppm	
		Compound-3 (δ)	**Reported Value (δ) [54]**
1	CH_2	38.41	38.42
2	CH_2	23.74	23.7
3	CHOH	81.0	81.00
4	C	37.82	37.82
5	CH	55.41	55.41
6	CH_2	18.23	18.23
7	CH_2	34.24	34.25
8	C	40.88	40.88
9	CH	50.37	50.38
10	C	37.11	37.11
11	CH_2	20.97	20.97
12	CH_2	25.12	25.14
13	CH	38.07	38.08
14	C	42.85	42.85
15	CH_2	27.46	27.46
16	CH_2	35.60	35.60
17	C	43.02	43.02
18	CH	48.32	48.32
19	CH	48.03	48.03
20	C	150.99	150.97
21	CH_2	29.86	29.87
22	CH_2	39.68	40.02
23	CH_3	27.97	27.09
24	CH_3	16.20	16.19
25	CH_3	16.52	16.51
26	CH_3	16.00	16.00

No. Carbon	Types of Carbon	Chemical shift (δ) value in ppm	
		Compound-3 (δ)	Reported Value (δ) [54]
27	CH_3	14.53	14.52
28	CH_3	18.02	18.02
29	CH_2	109.38	109.36
30	CH3	19.31	19.31
$-C(=O)-O-$ 3	$-C(=O)-O-$	171.03	171.01
$CH_3-C(=O)-O-$ 3	$CH_3-C(=O)-O-$	21.34	21.32

Here, 13C NMR spectra of compound-3 shown in fig.: 3.4

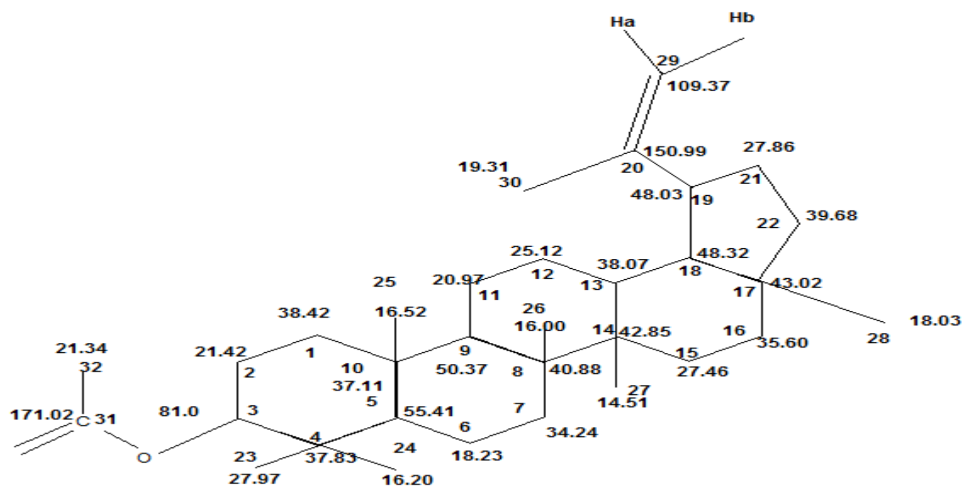

Fig. 3.4: ^{13}C NMR spectra of compound-3

By comparing the various types of compound-3 spectroscopic (IR, ^{1}H NMR, ^{13}C NMR, and DEPT) data of compound-3 with the reference of the compound, it has beenconfirmed that the structure of the compound-3 is Lupeol acetate is **[(3β)-lup-20(29)-en-3-ol acetate].**

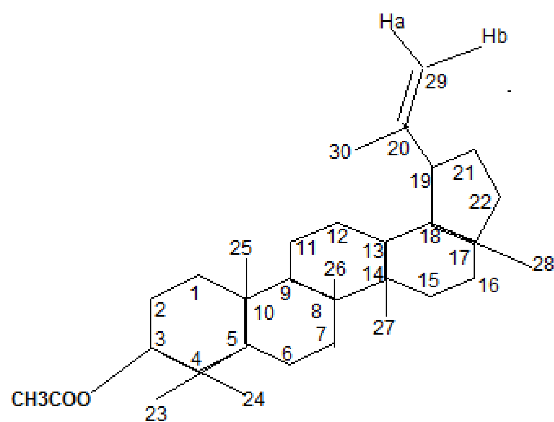

Fig. 3.5: Lupeol acetate [(3β)-lup-20(29)-en-3-ol acetate].

3.4 Characterization of compound-4

3.2.4 Characterization of H-2 as daucosterol

A) Physical properties

The compound H-2 is a white crystalline compound. The R_f value of the compound is 0.78 in n-hexane : EA ≡ 50:50. It is soluble in chloroform, dichloromethane, ethyl acetate, methanol & ethanol. It has tested by Salkawoski method which developed a reddish color indicating that the compound may be steroid.

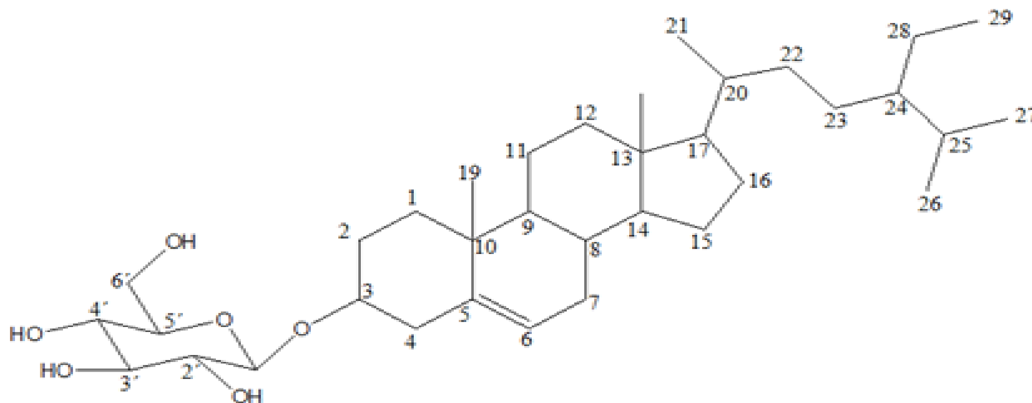

Fig. 3.6: Structure of daucosterol [β-sitosterol glucoside]

B) Characterization of H-2 by spectroscopic methods

The structure of the compound H-2 has established by IR, ^{1}H-NMR, ^{13}C-NMR, DEPT-90 & DEPT-135 spectral evidences.

C) IR Spectroscopy:

On subjection to I.R Spectroscopic analysis (in KBr), the observed absorption bands are 3490 cm^{-1} that is characteristic of O-H stretching. Absorption at 2960 cm^{-1} is due to cyclic olefinic –C=CH- stretching. Other absorption frequencies include 1640 cm^{-1} as a result of C=C absorption, however, this band is weak. 1458 cm^{-1} is a bending frequency for cyclic (CH_2) n and 1360 cm^{-1} for $–CH_2$ (CH_3) 2γ.

D) ^{1} H NMR Spectroscopy

The ^{1}H-NMR spectra gave a pattern very similar to that of β-sitosterol with the exception than an additional signal for a glucose moiety. The ^{1}H-NMR spectrum of the compound S-3 revealed the peaks at δ 0.71, .093 and 1.03 ppm due to methyl groups of a steroid. A number of multiplates between 1.09-2.05 ppm and a multiplate δ at 0.084 ppm were due to methylene and methyl protons present in the compound. The broad multiplate at δ 3.60 ppm indicates the presence of oxymethine proton flanked with two different methylene groups ($-CH_2$-CHOH-CH_2-). Two low intense multiplates at δ 5.16 and δ 5.05 ppm in the spectrum indicated the presence of two

olefinic protons attached with two methylene groups (>CH-CH=CH-CH<) in side chain of the compound.

A broad singlet at δ 5.38 ppm indicated the presence of a double bond in between a quarternary carbon and a methylene carbon i.e. presence of olefinic proton. The peaks between δ 4.40 - 3.61 ppm and δ 3.30 – 3.20 ppm are confirms the glucose moiety in the compound.

E) ^{13}C NMR Spectroscopy

The ^{13}C-NMR spectrum of the compound H-2 showed 35 carbons including an oxymethine carbon signal at δ 78.7 and two olefinic carbon at δ 139.9 & δ 121·6. The double bonded unsaturation at δ 139.9 & δ 121·6 was characteristics of spirostene. It gave signals for six methyl groups at δ 11·2 (C-18), 19·1 (C-19), 18.4 (C-21), 19·4 (C-26), 18.7 (C-27) and 11.2 (C-29), twelve methylene, fourteen methine and three quaternary carbons. Distinction among methyle, methylene, methane & quaternary carbons was approved by ^{13}C-NMR spectrum recorded by DEPT 135 & DEPT 90 experiments.

Table 3.4: ^{13}C-NMR data of compound H-2 compared with published data of daucosterol:

Carbon no.	Type of carbon	H-2 (Chemical shift in ppm)	Daucosterol (Chemical shift in ppm)
1	CH_2	36.8	37.0
2	CH_2	31.5	31.6
3	CH	78.7	78.9
4	CH_2	39.3	39.5
5	C	139.9	141.6
6	CH	121.6	121.9
7	CH_2	31.5	31.6
8	CH	31.9	31.7
9	CH	49.8	50.0
10	C	36.2	36.5
11	CH_2	20.6	20.8
12	CH_2	38.1	38.5

Carbon no.	Type of carbon	H-2 (Chemical shift in ppm)	Daucosterol (Chemical shift in ppm)
13	C	41.9	42.1
14	CH	56.3	56.5
15	CH_2	23.9	24.5
16	CH_2	28.7	28.7
17	CH	55.6	55.8
18	CH_3	11.2	12.0
19	CH_3	19.1	19.0
20	CH	36.2	36.0
21	CH_3	18.4	18.5
22	CH_2	33.5	34.0
23	CH_2	27.7	28.0
24	CH	45.5	45.7
25	CH	28.7	28.9
26	CH_3	19.4	19.5
27	CH_3	18.7	18.7
28	CH_2	22.6	22.8
29	CH_3	11.2	12.0
1′	CH	100.7	100.9
2′	CH	73.1	73.3
3′	CH	76.1	76.2
4′	CH	69.6	70.0
5′	CH	75.6	75.4
6′	CH_2	61.7	61.7

By comparing the various types of compound H-2 spectroscopic (IR, ^{1}H NMR, ^{13}C NMR, and DEPT) data of compound H-2 with the reference of the compound, it has been confirmed that the structure of the compound H-2 is Daucosterol. The structure is

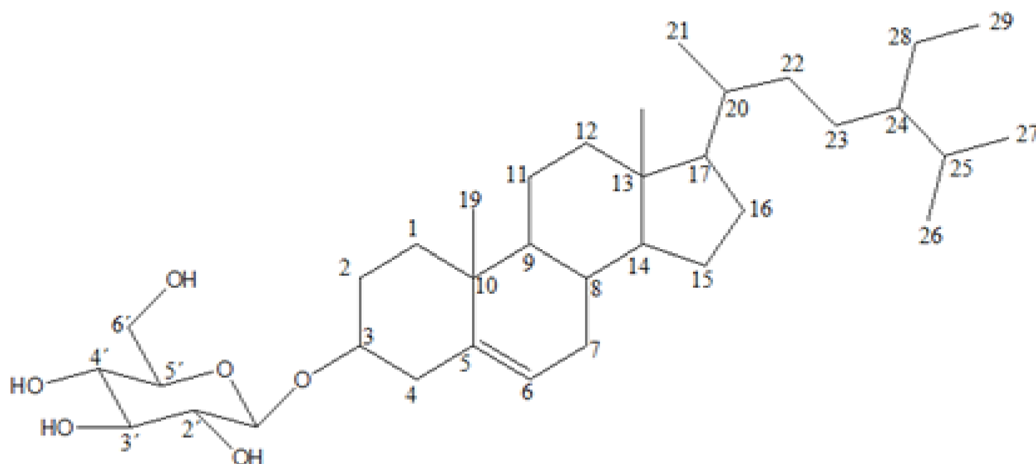

Fig. 3.7: Structure of daucosterol [β-sitosterol glucoside]

Abbreviations and Symbols

CC	Column Chromatography
VLC	Vacuum Liquid Chromatography
TLC	Thin-Layer Chromatography
TMS	Tetra methyl silane
UV/VIS	Ultraviolet/visible Spectroscopy
IR	Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance(Spectroscopy)
^{1}H NMR	Proton Nuclear Magnetic Resonance
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
DEPT	Distortionless Enhancement by Polarization Transfer
NOE	Nuclear-Overhauser-Enhancement
HSQC	Heteronuclear Single Quantum Correlation
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
ROESY	Rotating Frame Overhauser Enhancement Spectroscopy
MS	Mass Spectrometry
m/z	Mass/charge
δ	Chemical shift
δH	Hydrogen NMR Chemical Shift
δC	Carbon NMR Chemical Shift
s	Single
d	Doublet
dd	Double doublet
br t	Broad triplet
m	Multiplet
J J-	Coupling (also called indirect dipole dipole coupling)
ppm	Parts per million
Hz	Hertz
MHz	Mega hertz

Abbreviations and Symbols

λmax	Maximal absorbance wavelength
Rf	Retardation factor/ Retention Factor
Rt	Retention Time
RP	Reversed Phase
Tab.	Table
IC_{50}	The half maximal inhibitory concentration
LC_{50}	The half maximal lethal concentration
CD_3OD	Methanol-d_4
CHC_{l3}	Chloroform
CDC_{l3}	Chloroform- d_4
MeOH/ CH_3OH	Methanol
HCl	Hydrochloric acid
H_2O	Water
ACN	Acetonitrile
CH_2Cl_2 / DCM	Dichloromethane
EA	Ethyl acetate
DMSO	Dimethyl sulphoxide
DCM	Dichloromethane
H_2SO_4	Sulphuric acid
NaCl	Sodium Chloride
KBr	Potassium bromide
VS	Vincristine Sulphate
µL	Micro Liter
µg	Micro gram
mL	Mili-Liter
mm	Mili-meter

4.1 Introduction

Bacteria and fungi are responsible for many infectious diseases. The increasing clinical implications of drug resistant fungal and bacterial pathogens have lent additional urgency to antimicrobial drug research. The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the *in vitro* fungal and bacterial growth. This ability may be estimated by any of the following three methods.

i) Disc diffusion method

ii) Serial dilution method

iii) Bioautographic method

But there is no standardized method for expressing the results of antimicrobial screening [67]. Some investigators use the diameter of zone of inhibition and/or the minimum weight of extract to inhibit the growth of microorganisms. However, a great number of factors viz., the extraction methods [68, 69] in columns volume, culture medium composition [70, 71] and incubation temperature [72] can influence the results.

Among the above mentioned techniques the disc diffusion [70] is a widely accepted *in vitro* investigation for preliminary screening of test agents which may possess antimicrobial activity. It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials. However, no distinction between bacteriostatic and bacteriocidal activity can be made by this method [73].

4.2 Principle of Disc Diffusion Method

Solutions of known concentration (µg/mL) of the test samples are made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) are then impregnated with known amounts of the test substances using micropipette. Discs containing the test material are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs and blank discs (impregnated with solvents) are used as positive and negative control. These plates are then kept at low temperature (40C) for 24 hours to allow maximum diffusion. During this time dried discs absorb water from the surrounding media and then the test materials are dissolved and diffused out of the sample disc. The diffusion occurs according to the physical law that controls the diffusion of molecules through agar gel [**75**]. As a result there is a gradual change of test materials concentration in the media surrounding the discs.

The plates are then incubated at 37ºC for 24 hours to allow maximum growth of the organisms. If the test materials have any antimicrobial activity, it will inhibit the growth of the microorganisms and a clear, distinct zone of inhibition will be visualized surrounding the medium. The antimicrobial activity of the test agent is determined by measuring the diameter of zone of inhibition expressed in millimeter. The experiment is carried out more than once and the mean of the readings is required [**70**].

In the present study the crude extract, some column fractions as well as five purified compounds were tested for antimicrobial activity by disc diffusion method.

4.3 Experimental

4.3.1 Apparatus and Reagents

Filter paper	discs Petridishes	Inoculating loop
Sterile cotton	sterile forceps	Spirit burner
Micropipette	Screw cap test tubes	Nosemask and Hand gloves
Laminar air flow hood	Autoclave	Incubator
Refrigerator	Nutrient Agar Medium	Ethanol
Chloroform		

4.3.2 Test materials

4.3.2.1 Test materials of *Asparagus rosemosus*

1. Four crude extracts (**crude methanol, n-Hexane, dichloromethane**and**butanol**).

2. Four column fractions (15**, T_4, F_2, and F_{10}**) of various extract.

3. Four pure compounds (compound-1, compound-2**,** compound-3and compound-4**).**

4.3.3 Test Organisms

The bacterial strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka. Both Gram positive and Gram-negative organisms were taken for the test and they are listed in the Table 5.1.

Table 4.1: List of Test Bacteria

Gram-positive Bacteria	Gram-negative Bacteria
Bacillus cereus	*Escherichia coli*
Bacillus megaterium	*Klebsiella sp.*
Bacillus subtilis	*Protes sp.*
Staphylococcus aureus	*Salmonella typhi*
Bacillus polymyxa	*Shigellasonnei*
Streptococcus pneumonia	*Pseudomonas Aureus*
Mycobacterium tuberculosis	*Vibrio cholera*

4.3.4 Culture medium and their composition

The following media is used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms.

a. Nutrient agar medium

Ingredients	**Amounts**
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extracts	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s. to	100 mL
P^H	7.2 ± 0.1 at 25ºC

b. Nutrient broth medium

Ingredients	**Amounts**
Bacto beef extract	0.3 gm
Bacto peptone	0.5 gm
Distilled water q.s.to	100 mL
pH	7.2 ± 0.1 at 25ºC

c. Muller – Hunton medium

Ingredients	**Amounts**
Beef infusion	30 gm
Casamino acid	1.75 gm
Starch	0.15 gm
Bacto agar	1.70 gm
Distilled water q.s. to	100 mL
pH	7.3 ± 0.2 at 25ºC

***d. Tryptic soya broth medium* (TSB)**

Ingredients	**Amounts**
Bactotryptone	1.7 gm
Bactosoytone	0.3 gm
Bacto dextrose	0.25 gm
Sodium chloride	0.5 gm
Di potassium hydrogen Phosphate	0.25 gm
Distilled water q.s. to	100 mL
pH	7.3 ± 0.2 at 25ºC

Nutrient agar medium (DIFCO) used most frequently for testing the sensitivity of the organisms to the test materials and to prepare fresh cultures.

4.3.4.1 Preparation of medium

To prepare required volume of this medium, calculated amount of each of the constituents was taken in a conical flask and distilled water was added to it to make the required volume. The contents were heated in a water bath to make a clear solution. The P^H(at 25ºC) was adjusted at 7.2 –7.6 using NaOH or HCl. 10 mL and 5 mL of the medium was then transferred in screw cap test tubes to prepare plates and slants respectively. The test tubes were then capped and sterilized by autoclaving at 15-lbs. pressure/sq. inch at 121ºC for 20 minutes. The slants were used for making fresh culture of bacteria and fungi that were in turn used for sensitivity study.

Figure 4.1: Autoclave

4.3.5 Sterilization procedures

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petri-dishes and other glass-wares were sterilized by autoclaving at a temperature of 121ºC and a

pressure of 15-lbs. /sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

4.3.6 Preparation of subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37ºC for their optimum growth. These fresh cultures were used for the sensitivity test.

4.3.7 Preparation of the test plates

The test organisms were transferred from the subculture to the test tubes containing about 10 mL of melted and sterilized agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The bacterial and fungal suspension was immediately transferred to the sterilized petridishes. The petridishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media.

4.3.8 Preparation of discs

Three types of discs were used for antimicrobial screening.

4.3.8.1 Standard discs

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, kanamycin (30µg/disc) disc was used as the reference.

4.3.8.2 Blank discs

These were used as negative controls which ensure that the residual solvent (left over the discs even after air-drying) and the filter paper were not active themselves.

4.3.8.3 Preparation of sample discs with test samples

Measured amount of each test sample was dissolved in specific volume of solvent to obtain the *desired* concentrations in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

4.3.8.3.1 Preparation of sample discs with test samples of *Asperagusrosemosus.*

Crude **methanol, n-hexane, dichloromethane and butanol** extracts and four column fractions (**15, T_4, F_2 and F_{10}** of various extract and four pure compounds (**compound-1, compound-2, compound-3 and compound-4)** were tested for antimicrobial activity against a number of both Gram-positive and Gram-negative bacteria.

a) Test sample for crude extracts

Crude extracts were tested for antimicrobial activity using sample concentration of 500 µg per disc.

b) Test samples for column fractions of the various crude extract

The antimicrobial sensitivity of the column fractions were tested using sample concentration of 400 µg per disc.

c) Test samples for pure compounds

Pure compound was tested for antimicrobial activity using sample concentration of 300 µg per disc.

4.3.9 Preparation and application of the test samples

The test samples were weighed accurately and calculated amounts of the solvents were added accordingly using micropipette to the dried samples to get desired concentrations. The test samples were applied to previously sterilized discs using adjustable micropipette under aseptic conditions.

4.3.9.1 Diffusion and Incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at 4ºC for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37ºC for 24 hours.

Figure 4.4: Incubator before and after inverted petridishes

4.3.10 Determination of antimicrobial activity by the zone of inhibition

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the Antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

Figure 4.5: Counting of zone of inhibition

4.4 Results and discussion of *in vitro* Antimicrobial screening of *Asparagus rosemosus*.

Crude **methanol, n-hexane, dichloromethane and butanol** extracts and four column fractions (**15, T_4, F_2 and F_{10}** of various extract and four pure compounds (**compound-1 (Stigma sterol), compound-2 (β sitosterol), compound-3 (Lupeol acetate) and compound-4 (Daucosterol)** were tested for antimicrobial activity against a number of both Gram-positive and Gram-negative bacteria. Standard disc of Kanamycin (30 μg/disc) was used for comparison purpose.

The crude ethanol extract exhibited good antimicrobial activity against most of the test organisms **(Table: 4.2)** and n-Hexane, dichloromethane, butanol extracts exhibited moderate antimicrobial activity against the test organisms **(Table: 4.2)**.The four column fractions of exhibited low to mild **(Table: 4.3)** and the four pure compounds exhibited moderate antimicrobial activity against most of the test organisms **(Table: 4.4)**.

The zone of inhibition produced by crude methanol extract was found to be 8-14 mm and n-Hexane, dichloromethane, butanolextracts were found to be 7-15 at a concentration of 500μg/disc. and Similarly, and four column fractions (**15, T_4, F_2 and F_{10}**) of various extract yielded zones of inhibition 7 – 13 mm at a concentration of 400 μg/disc and four pure compounds Compound-1, Compound-2, Compound-3 and Compound-4 yielded zones of inhibition 7 – 12 mm at a concentration of 300 μg/disc

The crude methanol extract was screened against 14 test bacteria. This extract showed good activity against all the test Gram-positive and Gram-negative bacteria. n-Hexane, dichloromethane, butanol extracts were screened against 14 test bacteria. These extract showed moderate activity against most of the test Gram-positive and Gram-negative bacteria but these extract were found to be resistant to the *Bacillus megaterium, Salmonella typhi, Vibrio cholera* and *Bacillus polymyxa*and*Bacillus megaterium, Streptococcus pneumonia, Vibrio cholera* respectively.

The column fraction 15 of the crude extract of n-Hexane was screened against 8 test bacteria. This fraction showed poor to mild activity against Gram-positive bacterium *Bacillus cereus,Bacillus subtilis, Staphylococcus aureus, Bacillus polymyxa, Mycobacterium tuberculosis* and Gram-negative bacteria *Escherichia coli, Klebsiella sp., Shigellasonnei.*

The column fractions $\mathbf{T_4}$of the crude extract of chloroform were screened against 8 test bacteria. This fraction showed poor to mild activity against Gram-positive bacterium *Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus*, *Mycobacterium tuberculosis* and Gram-negative bacteria *Escherichia coli, Shigellasonnei, Vibrio cholera.*

The column fractions $\mathbf{F_2}$and $\mathbf{F_{10}}$ of the crude extract of butanol were screened against 8 test bacteria. This fraction showed poor to mild activity against Gram-positive bacterium *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus*, *Mycobacterium tuberculosis* and Gram-negative bacteria *Escherichia coli, Shigellasonnei.*

Fourteen (14) bacterial strains were taken to study the antimicrobial activity of the pure compounds Compound-3, Compound-2, Compound-4and Compound-1at a concentration of 300µg/disc.These compounds showed moderate activity against all the Gram-positive bacteria *Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus, Streptococcus pneumonia, B. polymyxa, Mycobacterium tuberculosis* and Gram-negative bacteria *E.coli, Klebsiella sp., Proteus sp., Salmonella typhi, Shigellasonnei, Pseudomonas Aureus, Vibrio cholera* but Compound-1 resistant to *Streptococcus pneumonia, Mycobacterium tuberculosi;*Compound-3resistant to *Proteus sp., Shigellasonnei;* Compound-4resistant to *Salmonella typhi, Pseudomonas Aureus* and Compound-2 resistant to *Salmonella typhi*.

Table-4.2: Anti-microbial activity of the four crude extracts (Methanol, dichloromethane, n-hexane and butanol) of *Asparagus rosemosus* .

Name of the Bacteria	Methanol extract	n-hexane extract	Dichloromethane extract	Butanol extract	Kanamycin
	500µg/disc				30µg/disc
Gram-positive bacteria					
Bacillus cereus	12	8	13	8	34
Bacillus megaterium	9	10	..	..	34
Bacillus subtilis	12	7	12	11	32
Staphylococcus aureus	14	11	..	8	35
Bacillus polymyxa	..	..	..	8	33
Streptococcus pneumonia	12	9	9	..	34
Mycobacterium tuberculosis	…	12	15	9	31
Gram- negative bacteria					
Escherichia coli	10	..	13	12	35
Klebsiella sp.	..	..	..	7	32
Protes sp.	11	..	..	..	30
Salmonella typhi	..	8	..	..	33
Shigellasonnei	6	..	12	7	34
Pseudomonas Aureus	12	..	8	..	34
Vibrio cholera	8	9	9	nd	31

N.B.: "--" Indicates „No activity   and "nd" indicates „Not done   .

Table-4.3: Anti-microbial activity of the four column fractions (15, T_4, F_2, F_{10}) of various extract of *Asparagus rosemosus.*

Name of the Bacteria	15	T_4	F_2	F_{10}	Kanamycin
	400µg/disc				30µg/disc
Gram-positive bacteria					
Bacillus cereus	8	9	7	12	34
Bacillus megaterium	--	7	--	--	34
Bacillus subtilis	9	9	8	12	32
Staphylococcus aureus	8	8	12	10	35
Bacillus polymyxa	10	--	--	--	33
Streptococcus pneumonia	--	--	--	--	34
Mycobacterium tuberculosis	10	10	7	12	31
Gram- negative bacteria					
Escherichia coli	12	10	7	13	35
Klebsiella sp.	10	--	--	--	32
Protes sp.	--	--	--	--	30
Salmonella typhi	--	--	---	--	33
Shigellasonnei	10	10	7	12	34
Pseudomonas Aureus	--	7	12	10	34
Vibrio cholera	--	9	--	--	31

N.B.: "--" Indicates „No activity and "nd" indicates „Not done .

Table-4.4: Anti-microbial activity of the four compounds (Com-1, Com-2, Com-3 and Com-4) of *Asparagus rosemosus*.

Name of the Bacteria	Com-1 Stigmasterol	Com-2 β-sitosterol	Com-3 Lupeol acetate	Com-4 Daucosterol	Kanamycin
	300µg/disc				30µg/disc
Gram-positive bacteria					
Bacillus cereus	8	9	7	12	34
Bacillus megaterium	--	7	--	--	34
Bacillus subtilis	9	9	8	19	32
Staphylococcus aureus	8	..	..	8	35
Bacillus polymyxa	7	--	--	--	33
Streptococcus pneumonia	--	--	--	--	34
Mycobacterium tuberculosis	11	8	7	12	31
Escherichia coli	11	10	7	8	35
Klebsiella sp.	8	--	--	--	32
Protes sp.	--	--	--	--	30
Salmonella typhi	--	--	---	--	33
Shigella sonnei	7	10	7	9	34
Pseudomonas Aureus	--	7	12	10	34
Vibrio cholera	--	9	--	--	31

N.B.: "--" Indicates „No activity and "nd" indicates „Not done .

ABSTRACT

The plant *Asparagus racemosus* of Aspargaceae family is used various diseases upset stomach (dyspepsia), constipation, stomach spasm and stomach ulcers. It is also used for fluid retention, pain, anxiety, diarrhea, bronchitis, dementia and diabetes. The plant root was enriched with important biologically active compounds such as sarsasapogenin, adscendin (A, B), asparanin (A, B, C). So the plant is selected for further investigation. The plant's dried root was extracted with various solvent such as methanol, dichloromethane and various solvent extract was obtained. During this investigation four compounds were isolated from various plant extracts. All the compounds were identified preliminary by chemical methods and then the structure elucidations of the compounds were performed by various spectral methods (IR, ^{1}H NMR, ^{13}C NMR and DEPT) and were confirmed in comparison with the result that is available in the published literatures. In this project, three steroidal type compounds stigmasterol [(3S,8S,9S, 10R,13R,14S,17R)-17-[(E,2R,5S)-5-ethyl-6methylhept-3-en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol] , β-sitosterol [17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol], Daucosterol [β-Sitosterol glucoside] and one triterpinoid compound Lueol acetated [(3β)-lup-20(29)-en-3ol acetate] find out from the roots of the plant.

Stigmasterol and β-sitosterolshowed low anti- microbial activity against gram positive bacteria (Bacillus cereus, Bacillus polymyxa) and gram negative bacteria (Klebsiella sp., Shigella sonnei,)

Lupeol acetate also showed low anti microbial activity against gram positive (Bacillus cereus, Bacillus subtilis, Mycobacterium tuberculosis) and gram negative Shigella sonnei.

The steroid Daucosterol showed moderate activity against Bacillus subtillis and gram negative bacteria Pseudomonas.

Thus the isolation, characterization and investigation of anti microbial activity of the compounds from the root of plant *Asparagus resemosus* might generate much synthetic and biological interest in this field of the natural products.

5.0 Conclusion

A species of the Asparagaceae family, *Asparagus racemosus*, has been investigated in this work. During this investigation six compounds were isolated from this plant extract. Among them structural elucidation of the four compounds were performed. The structural elucidation of the remaining compound was not carried out as it ^{1}H NMR gives evidence for being a fatty material. All the compounds were identified preliminiary by chemical methods and then the structure elucidations of the compounds were performed by various spectra methods (IR, ^{1}H NMR, ^{13}C NMR and DEPT) and were confermed comparison with the result that are available in the published literatures. Three steroidal type compounds stigmasterol, β-sitosterol, Daucosterol and one triterpinoid compound Lueol acetated extracted from the roots of the plant.

The various crude extract (methanol, n-hexane, dichloroform, butanol) column fractions 15, T4, F2 and F10, and four compounds (stigmasterol, β-sitosterol , Lupeol acetated and Daucosterol) were investigated for anti microbial test.

Methanol, n-hexane, dichloroform and butanol crude extract show moderate to low activity against gram positive bacteria (*Bacillus cereus, Bacillus megaterium, Bacillus subtillis, Staphylococcus aureus, Bacillus polymyxa, Mycobacterium tuberculosis*) and gram negative Bacteria (*Escherichia coli,Klebsiellasp, Protessp, shigella sonnei, Pseudomonas Aureus, Vibro cholera*) . Thecoloum fractions were also show moderate to low anti microbial activity some of the gram positive bacteria and gram negative bacteria.

Stigmasteroland β-sitosterolshowed low anti- microbial activity against gram positive bacteria (Bacillus cereus, Bacillus polymyxa) and low in gram negative bacteria (Klebsiella sp., Shigellasonnei,)

Lupeol acetate showed low anti microbial activity against gram positive (Bacillus cereus, Bacillus subtilis, Mycobacterium tuberculosis) and gram negative Shigellasonnei.

Daucosterol showed moderate activity against Bacillus subtillis and gram negative bacteria Pseudomonas Aureus.

Since the plant Asparagus rosemosus contains various bioactive compounds, so it may be concluded that this plant might play a vital role as a medicinal plant.

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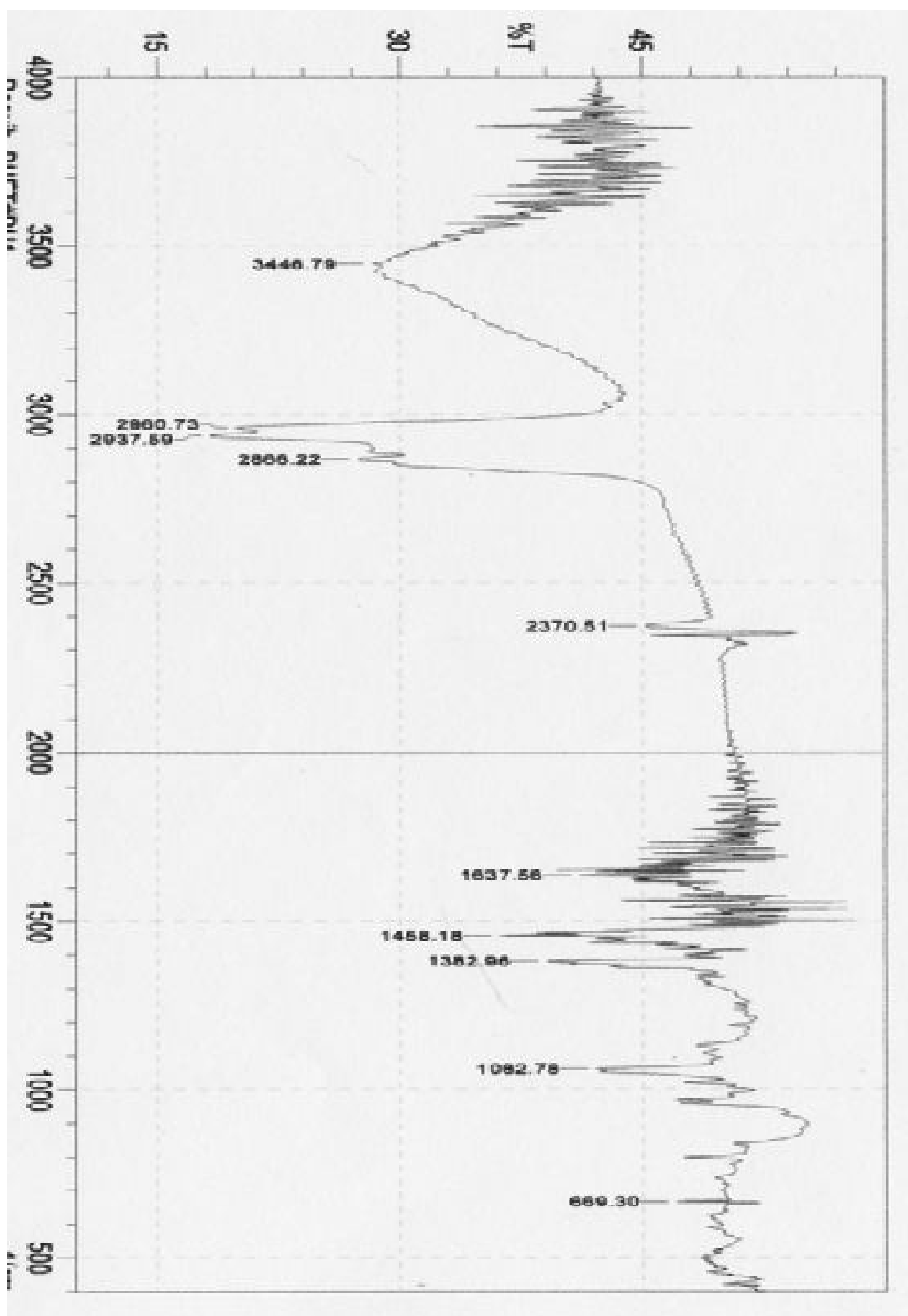


Fig1.1: IR Spectra of the compound-1 or S-3

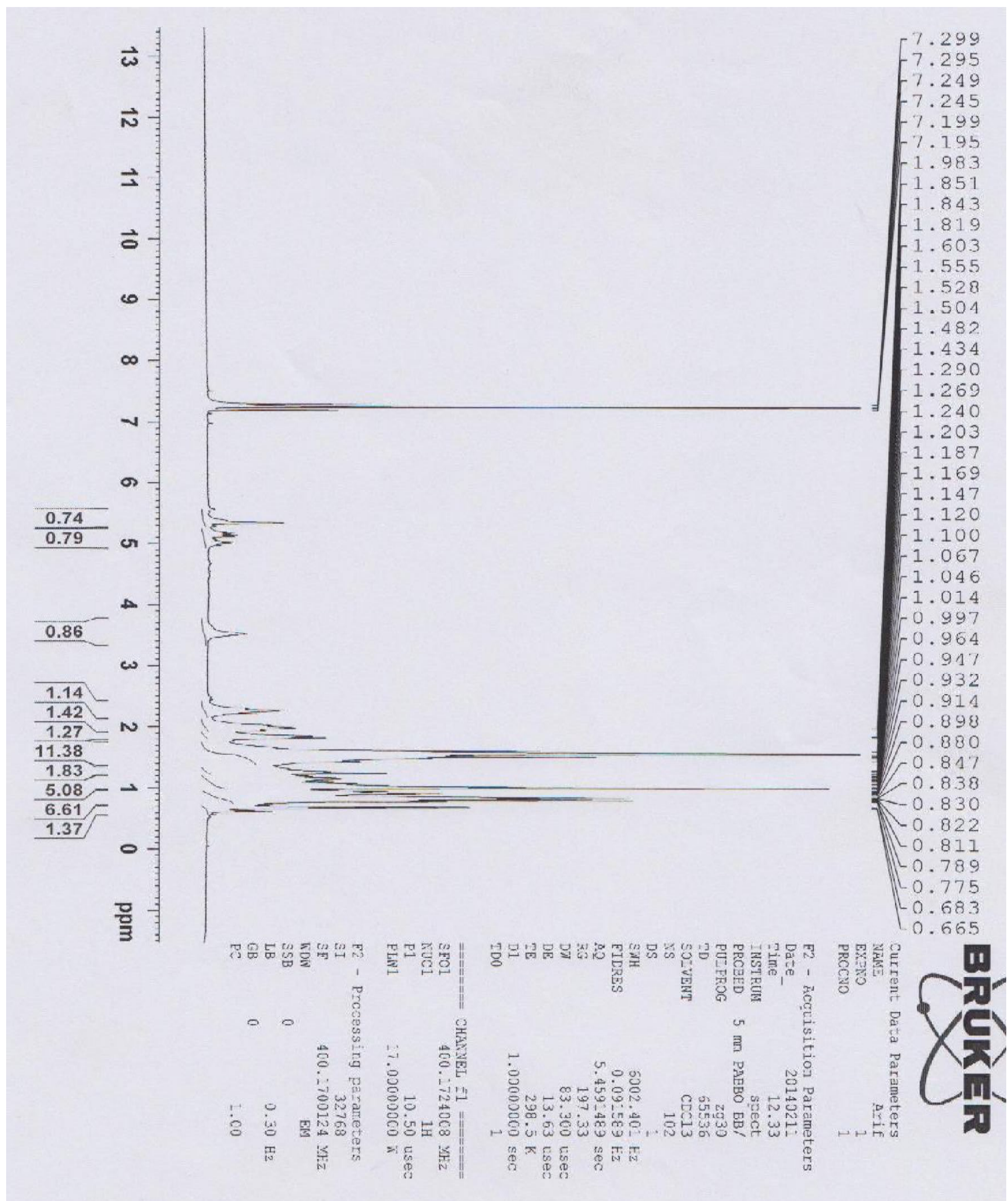


Fig 1.2: 1H NMR spectra of Compound-1 or S-3

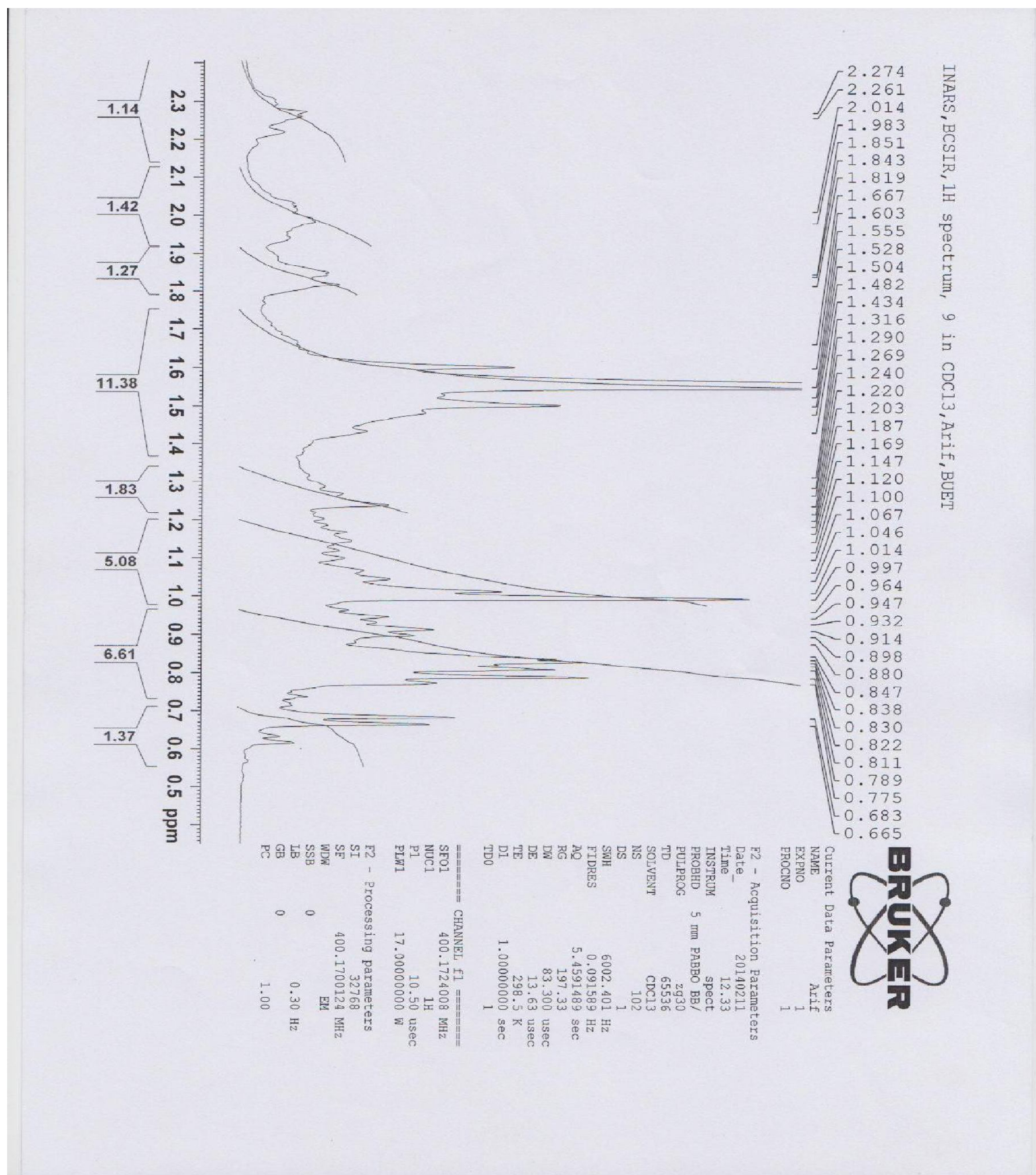


Fig 1.3 1H NMR (Expantion) Spectra of the compound-1 or S-3

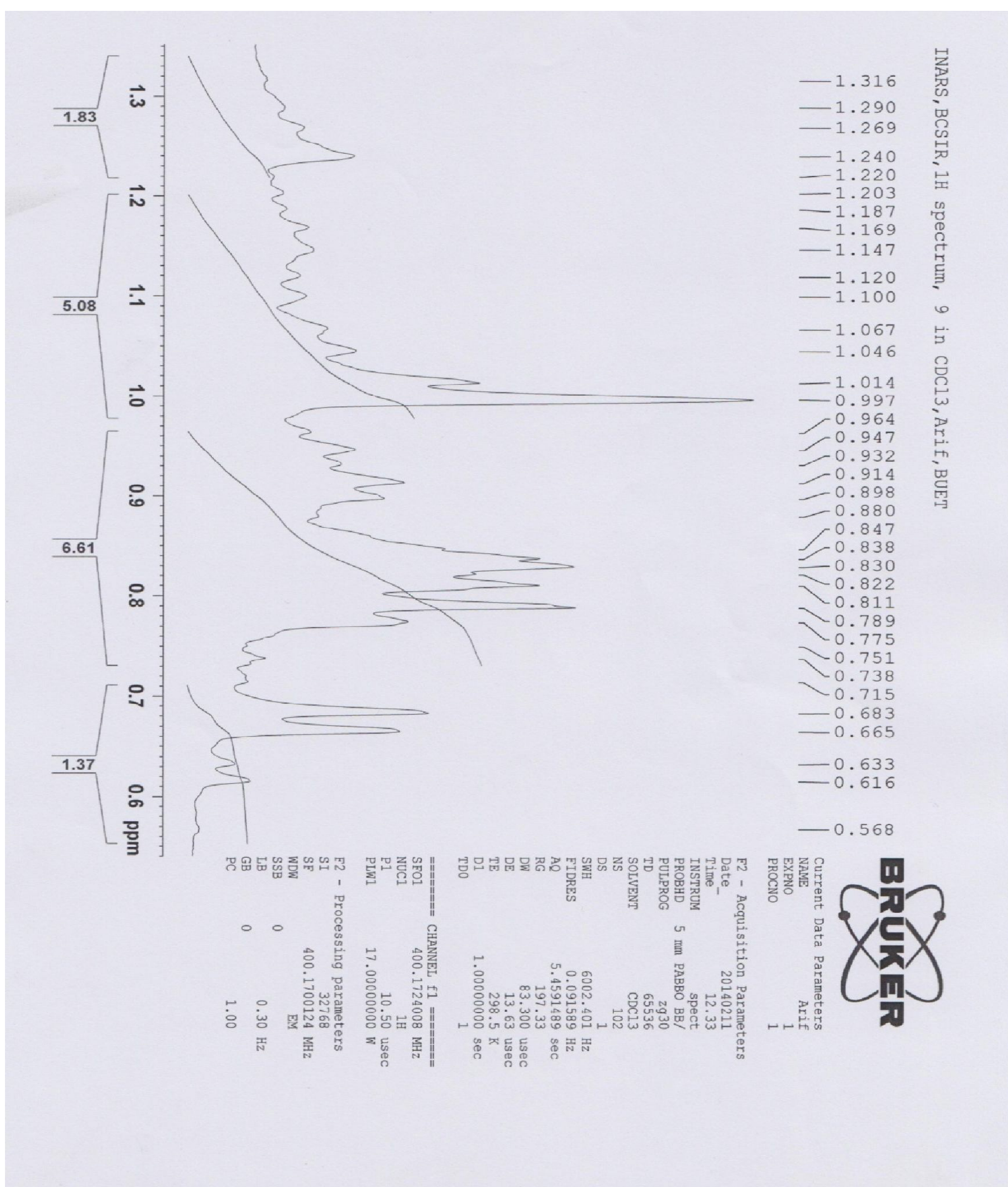


Fig 1.4: [1] H NMR (Expantion-1) of S-3 or Compound-1

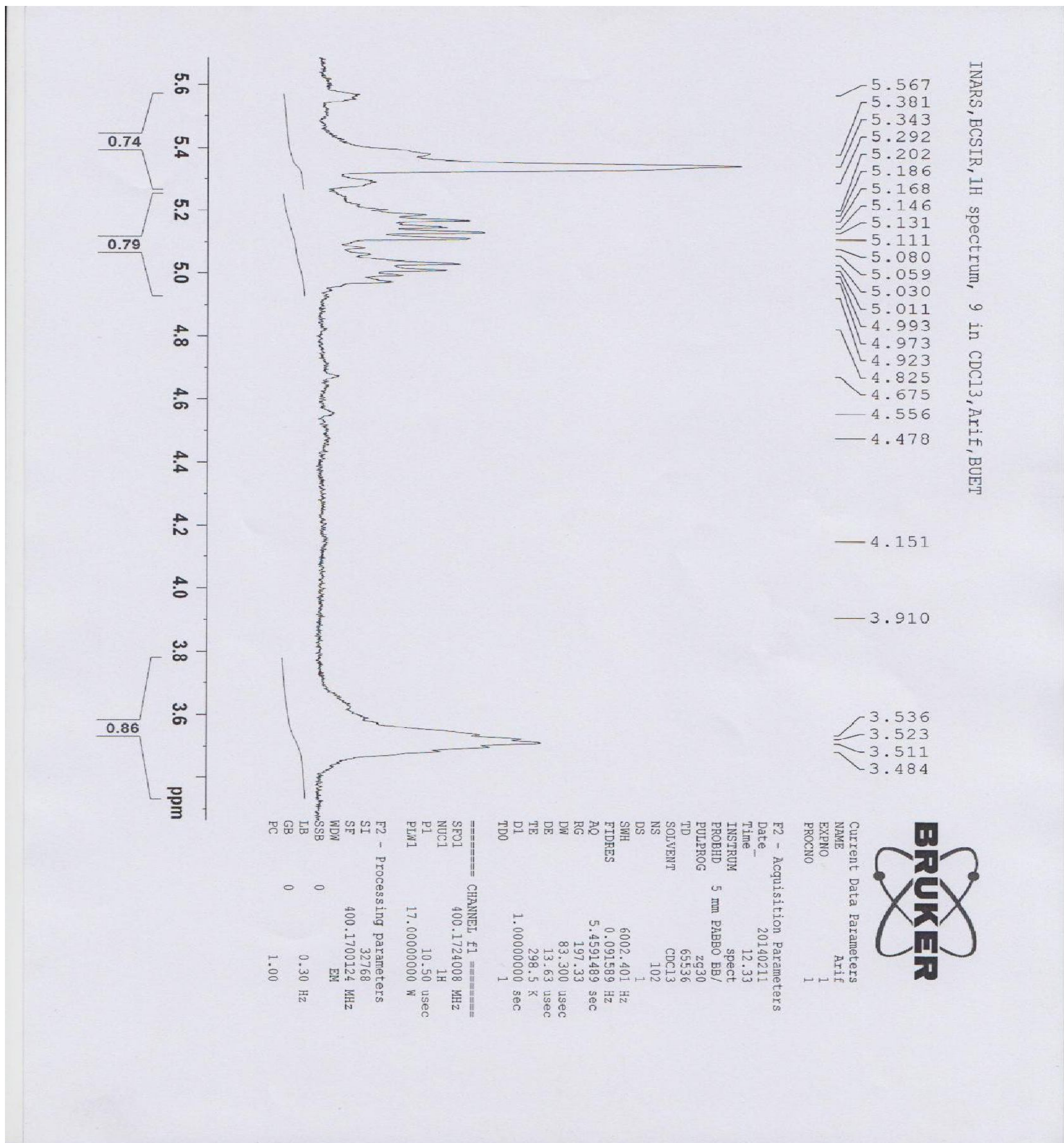


Fig 1.5: [1] H NMR (Expantion-2) of S-3 or Compound-1

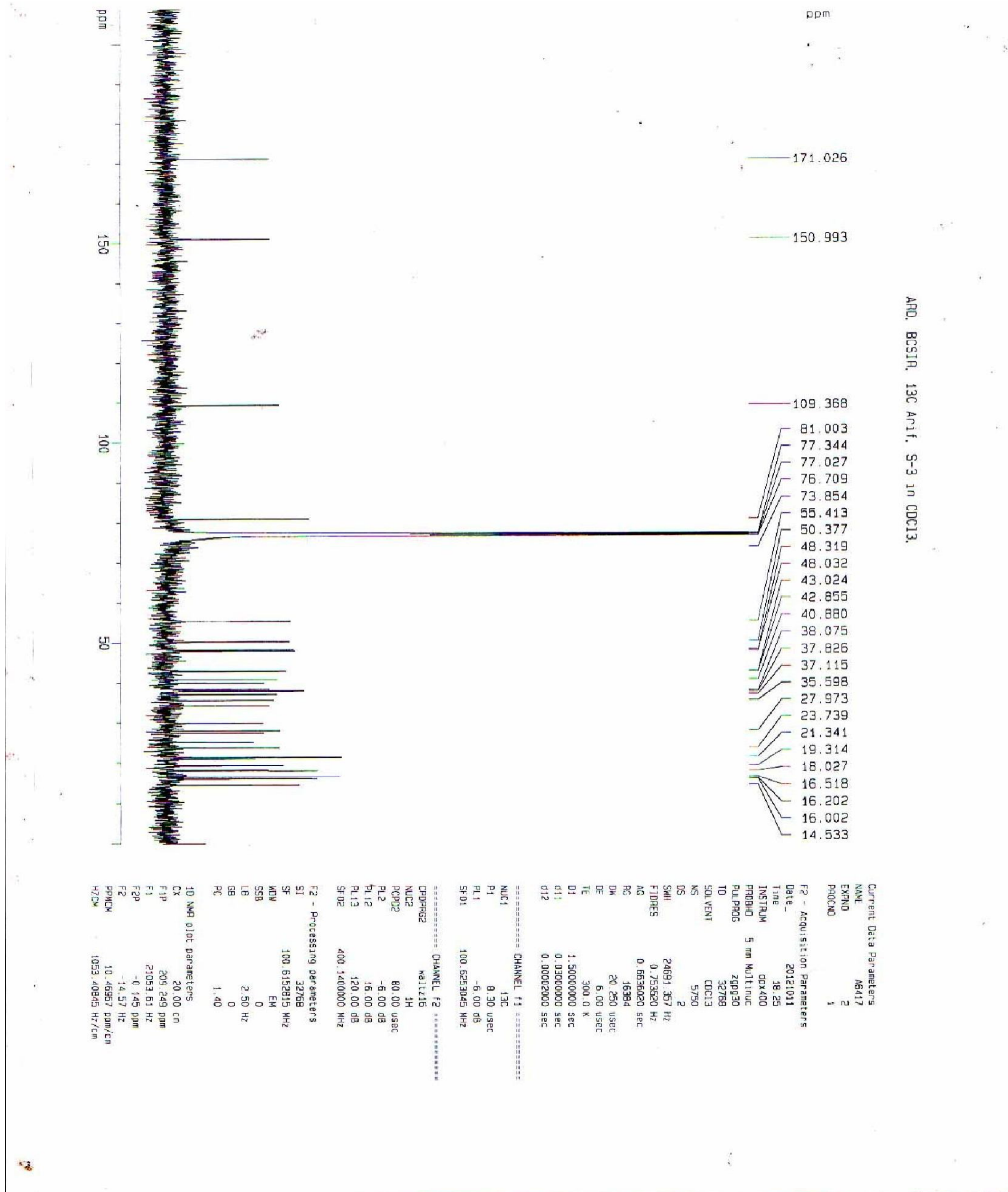


Fig 1.6: 13 C NMR Spectra of S-3 Compound-1

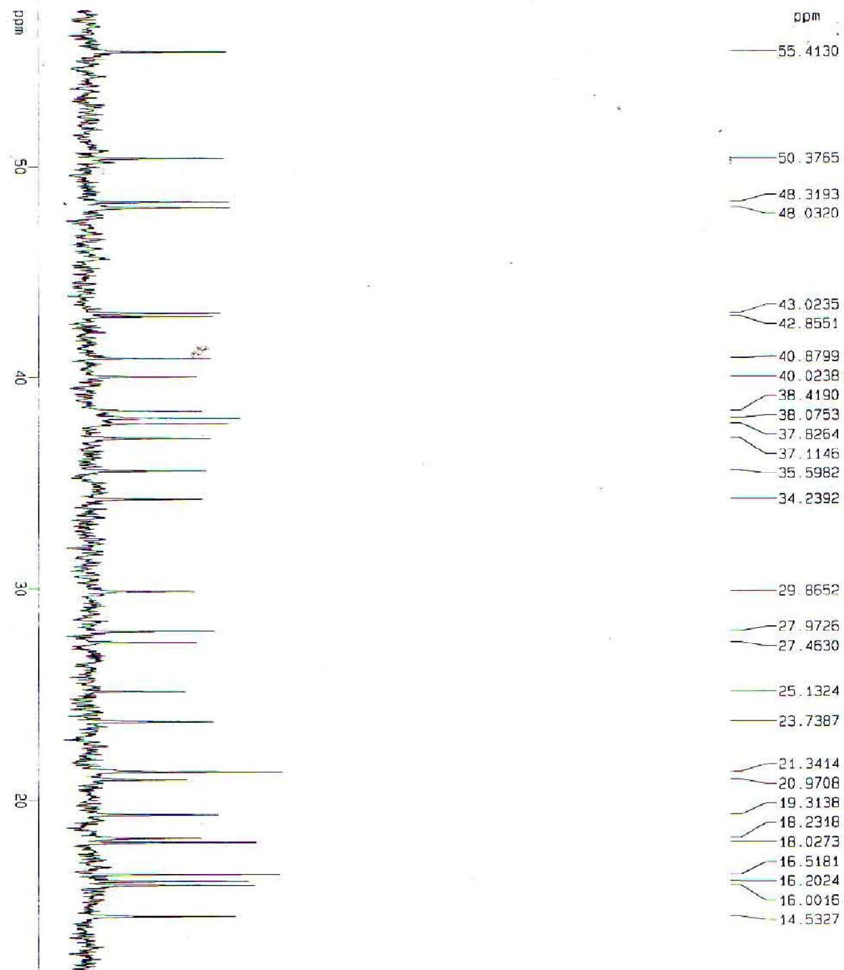
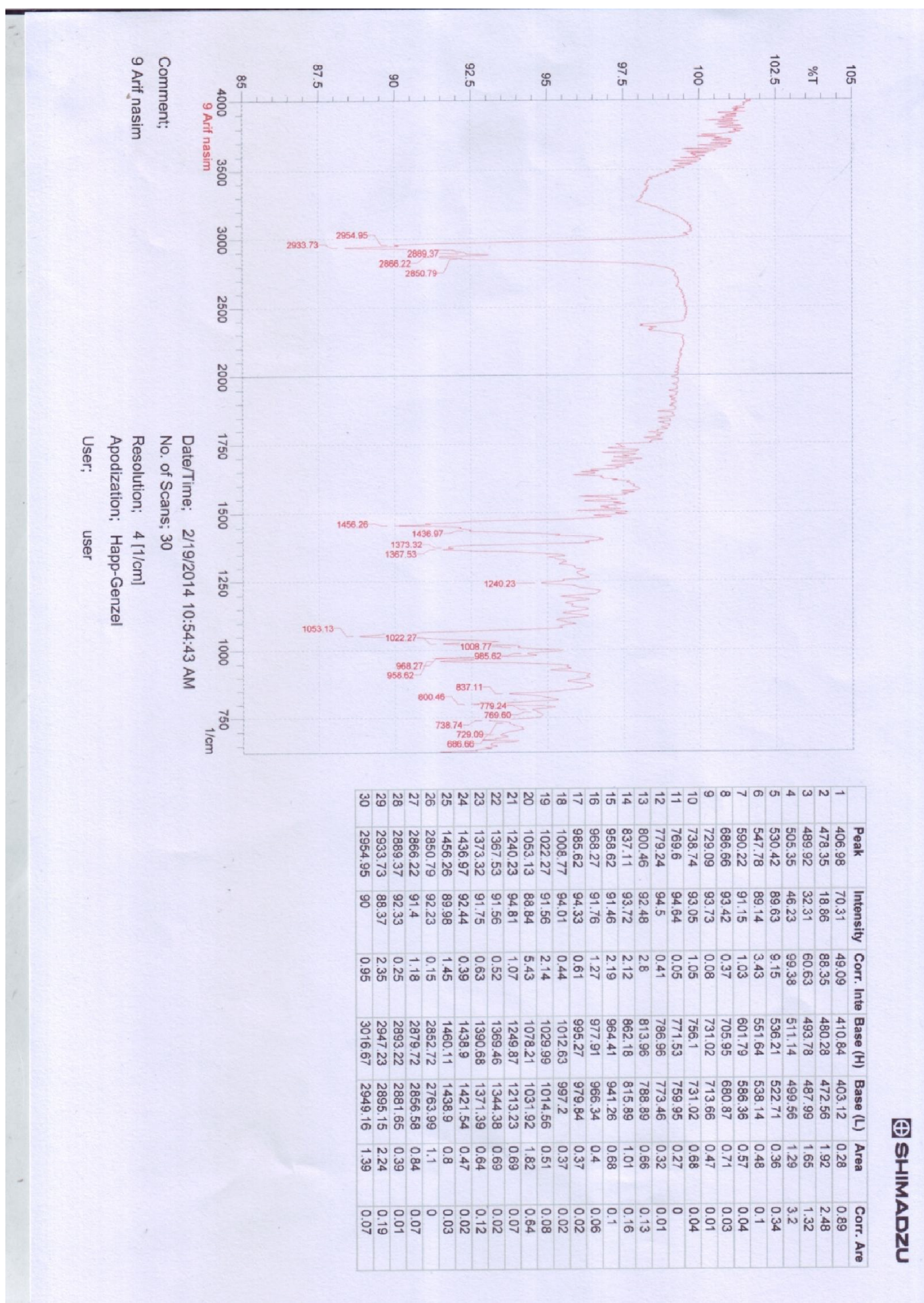


Fig 1.7: 13 C NMR (Expantion-1) Spectra of S-3 Compound-1



	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	406.98	70.31	49.09	410.84	403.12	0.28	0.89
2	478.35	18.86	88.35	480.28	472.56	1.92	2.48
3	489.92	32.31	60.63	493.78	487.99	1.65	1.32
4	505.35	46.23	99.38	511.14	499.56	1.29	3.2
5	530.42	89.63	9.15	536.21	522.71	0.36	0.34
6	547.78	89.14	3.43	551.64	538.14	0.48	0.1
7	590.22	91.15	1.03	601.79	586.36	0.57	0.04
8	686.66	93.42	0.37	705.95	680.87	0.71	0.03
9	729.09	93.73	0.08	731.02	713.66	0.47	0.01
10	738.74	93.05	1.05	756.1	731.02	0.68	0.04
11	769.6	94.64	0.05	771.53	759.95	0.27	0
12	779.24	94.5	0.41	786.96	773.46	0.32	0.01
13	800.46	92.48	2.8	813.96	788.89	0.66	0.13
14	837.11	93.72	2.12	862.18	815.89	1.01	0.16
15	958.62	91.46	2.19	964.41	941.26	0.68	0.1
16	968.27	91.76	1.27	977.91	966.34	0.4	0.06
17	985.62	94.33	0.61	995.27	979.84	0.37	0.02
18	1008.77	94.01	0.44	1012.63	997.2	0.37	0.02
19	1022.27	91.56	2.14	1029.99	1014.56	0.51	0.08
20	1053.13	88.84	5.43	1078.21	1031.92	1.82	0.64
21	1240.23	94.81	1.07	1249.87	1213.23	0.69	0.07
22	1367.53	91.56	0.52	1369.46	1344.38	0.69	0.02
23	1373.32	91.75	0.63	1390.68	1371.39	0.64	0.12
24	1436.97	92.44	0.39	1438.9	1421.54	0.47	0.02
25	1456.26	89.98	1.45	1460.11	1438.9	0.8	0.03
26	2850.79	92.23	0.15	2852.72	2763.99	1.1	0
27	2866.22	91.4	1.18	2879.72	2856.58	0.84	0.07
28	2889.37	92.33	0.25	2893.22	2881.65	0.39	0.01
29	2933.73	88.37	2.35	2947.23	2895.15	2.24	0.19
30	2954.95	90	0.95	3016.67	2949.16	1.39	0.07

Fig 2.1: IR of the 9 or compound- 2

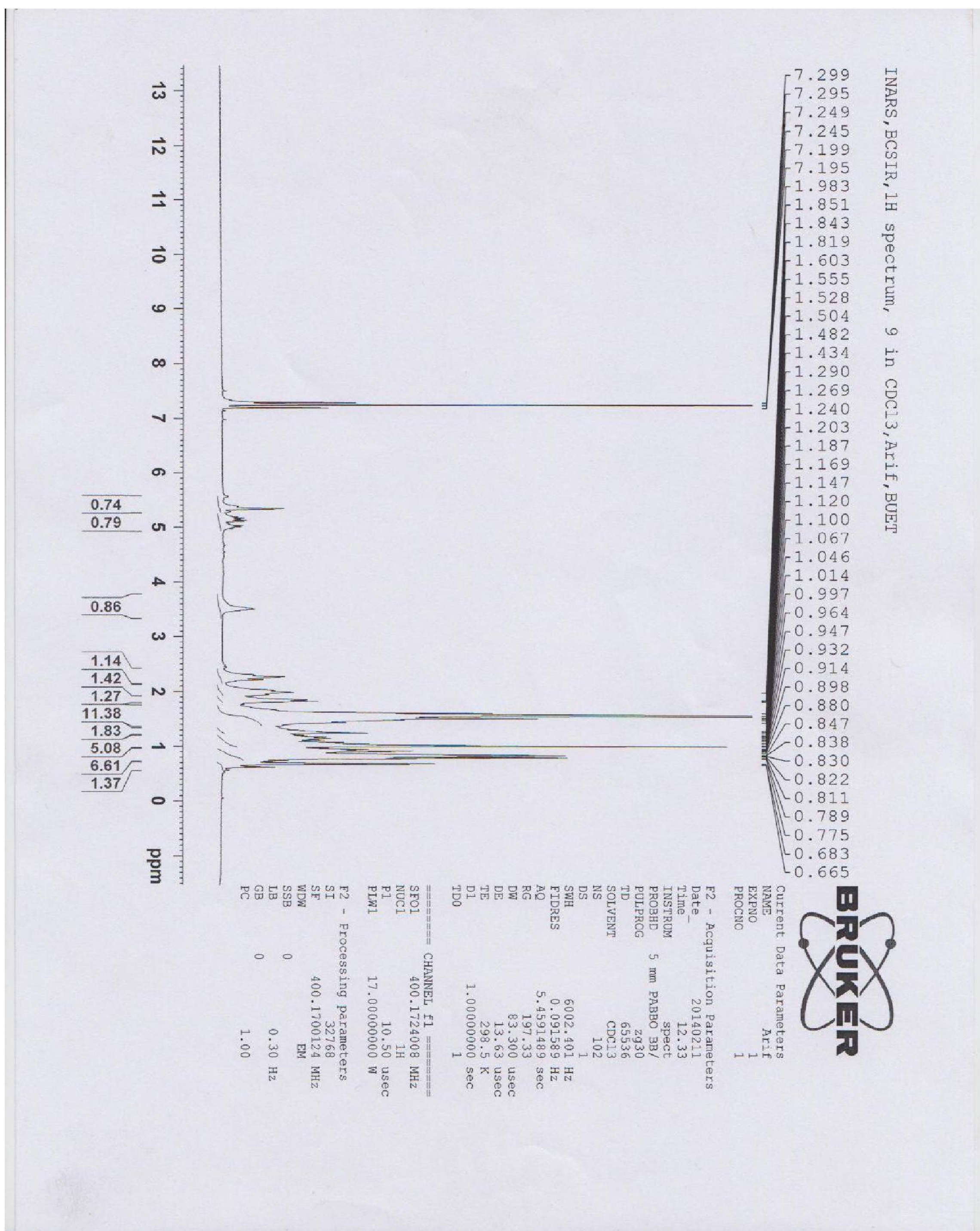


Fig 2.2: ^{1}H NMR Spectra of 9 or Compound-2

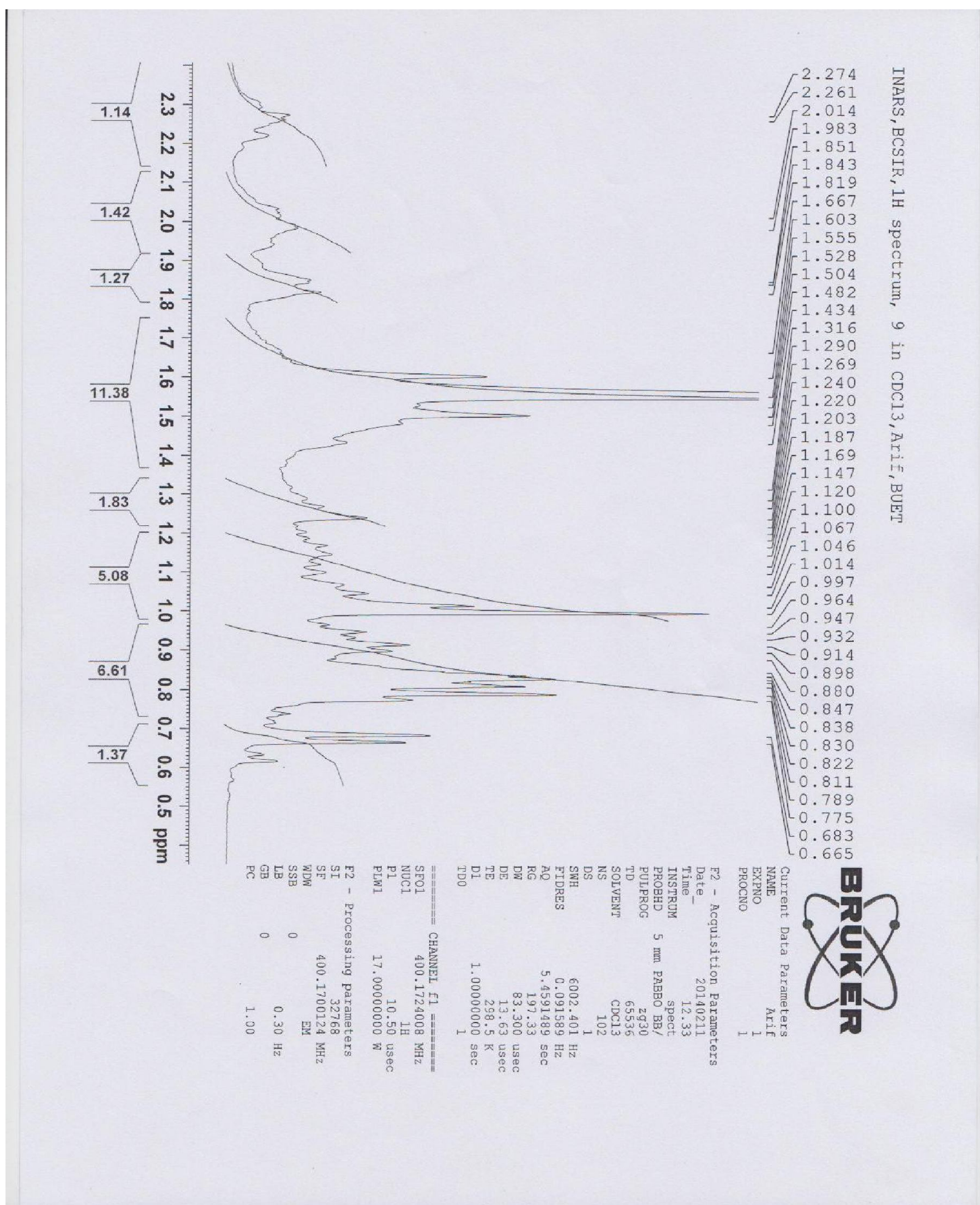


Fig 2.2: ^{1}H NMR (Expansion-1) Spectra of 9 or Compound-2

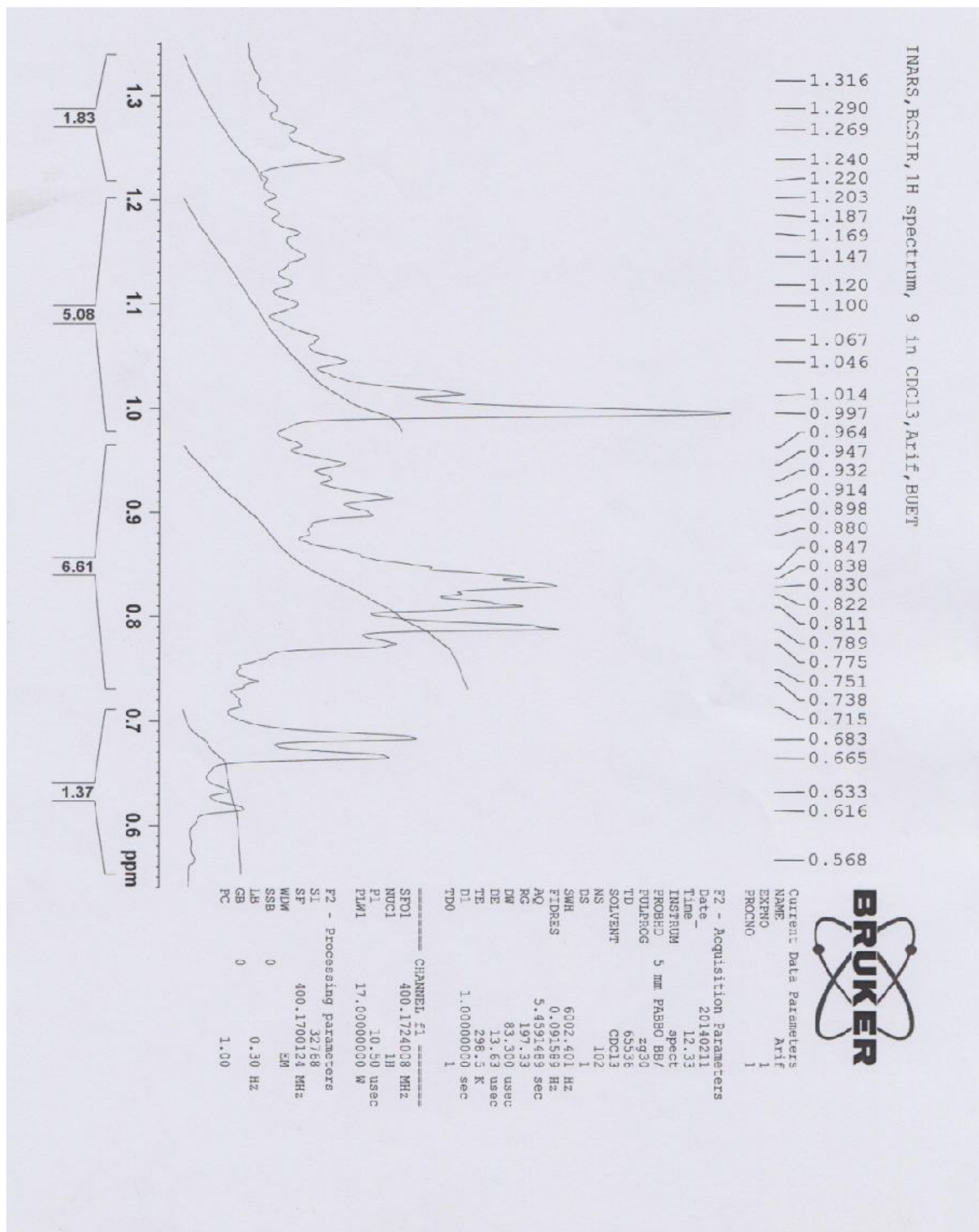


Fig 2.2: ^{1}H NMR (Expansion-2) Spectra of 9 or Compound-2

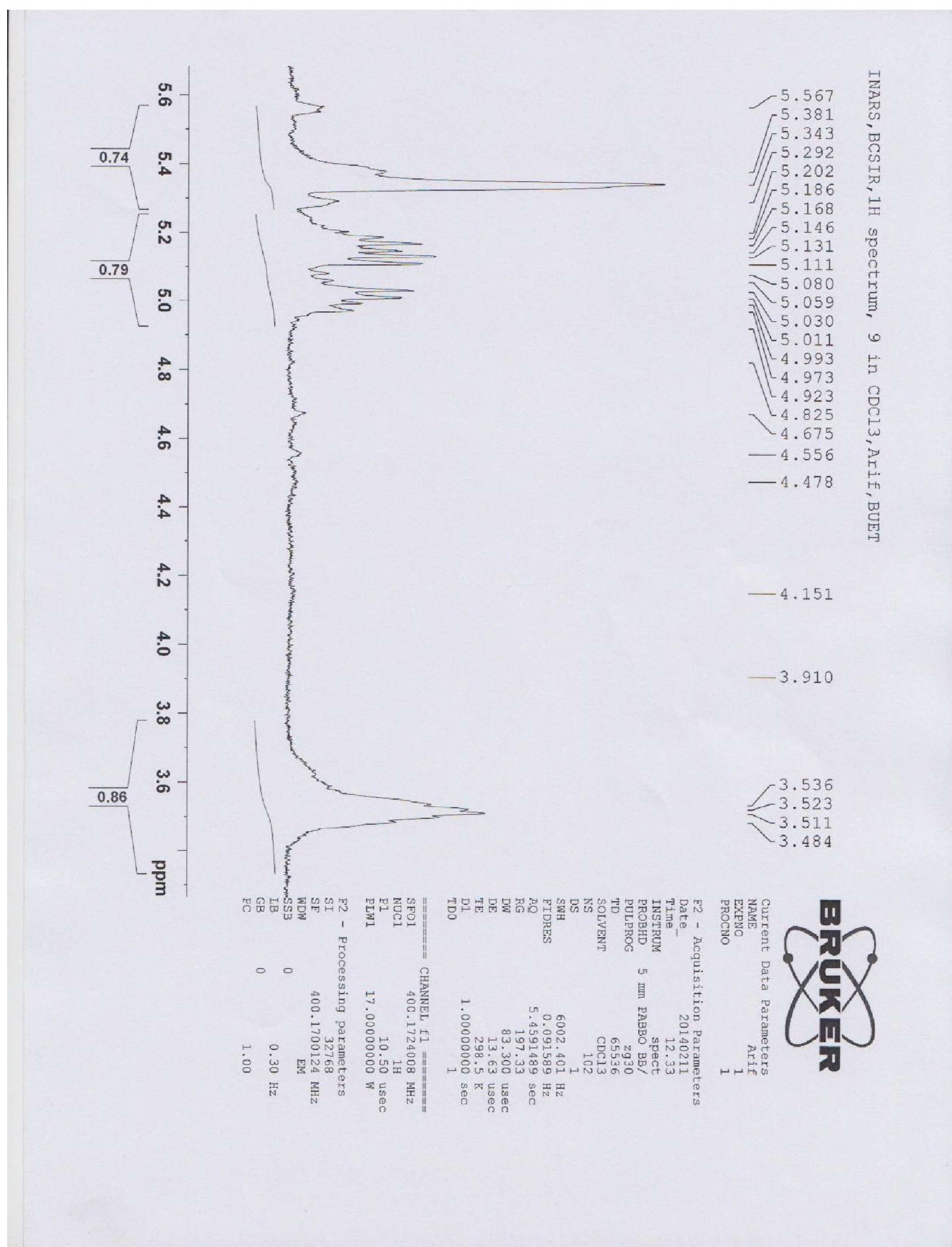


Fig 2.3: ^{1}H NMR (Expansion-3) Spectra of 9 or Compound-2

Fig 2.4: ^{1}H NMR (Expansion-4) Spectra of 9 or Compound-2

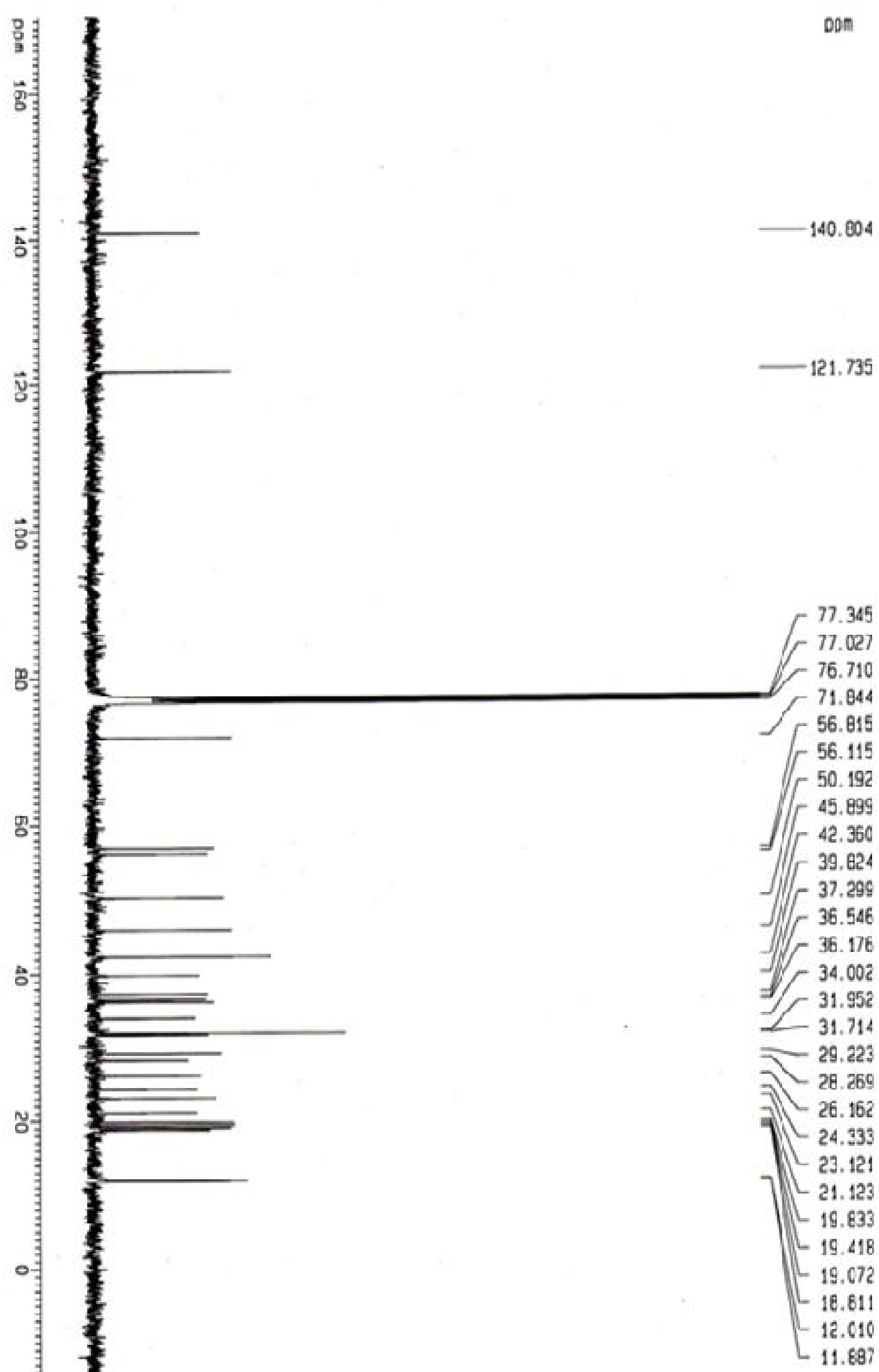
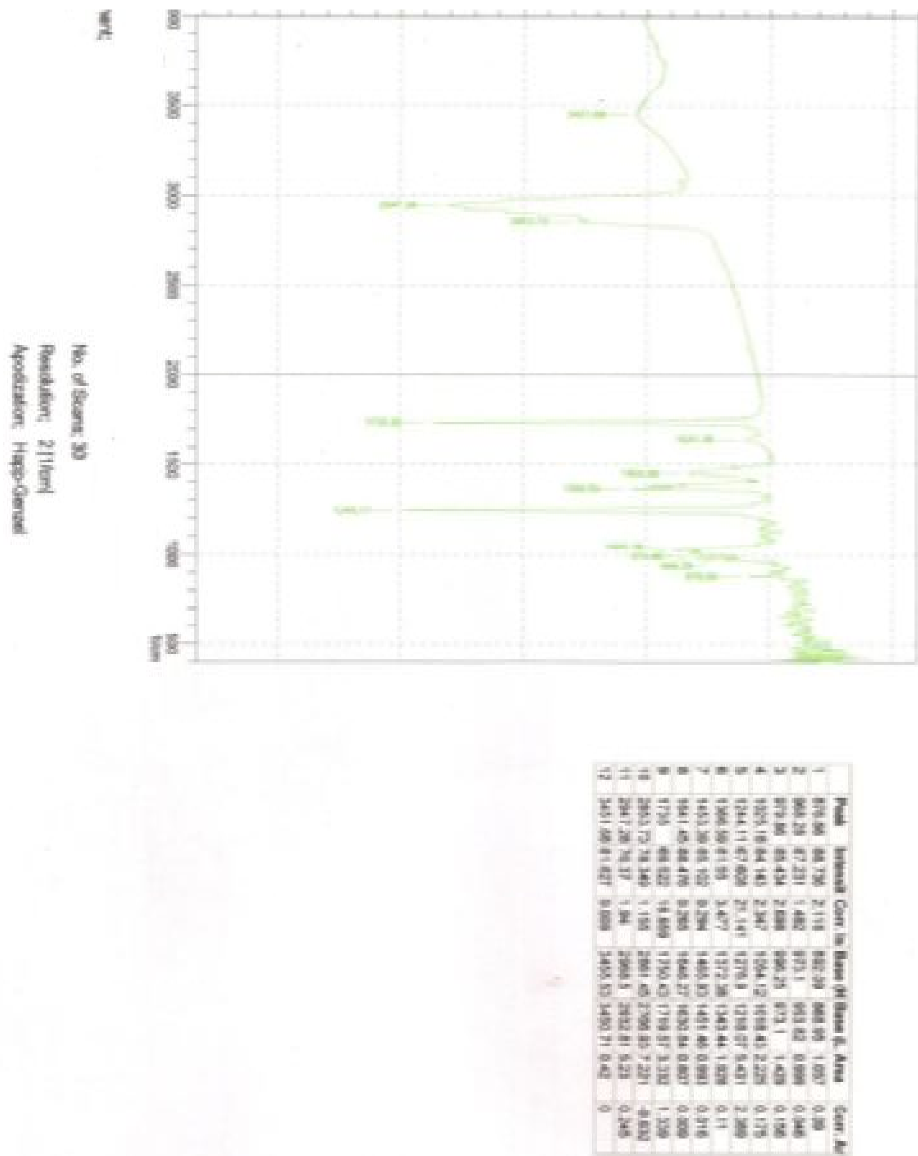


Fig 2.4: ^{13}C NMR Spectra of 9 or Compound-2



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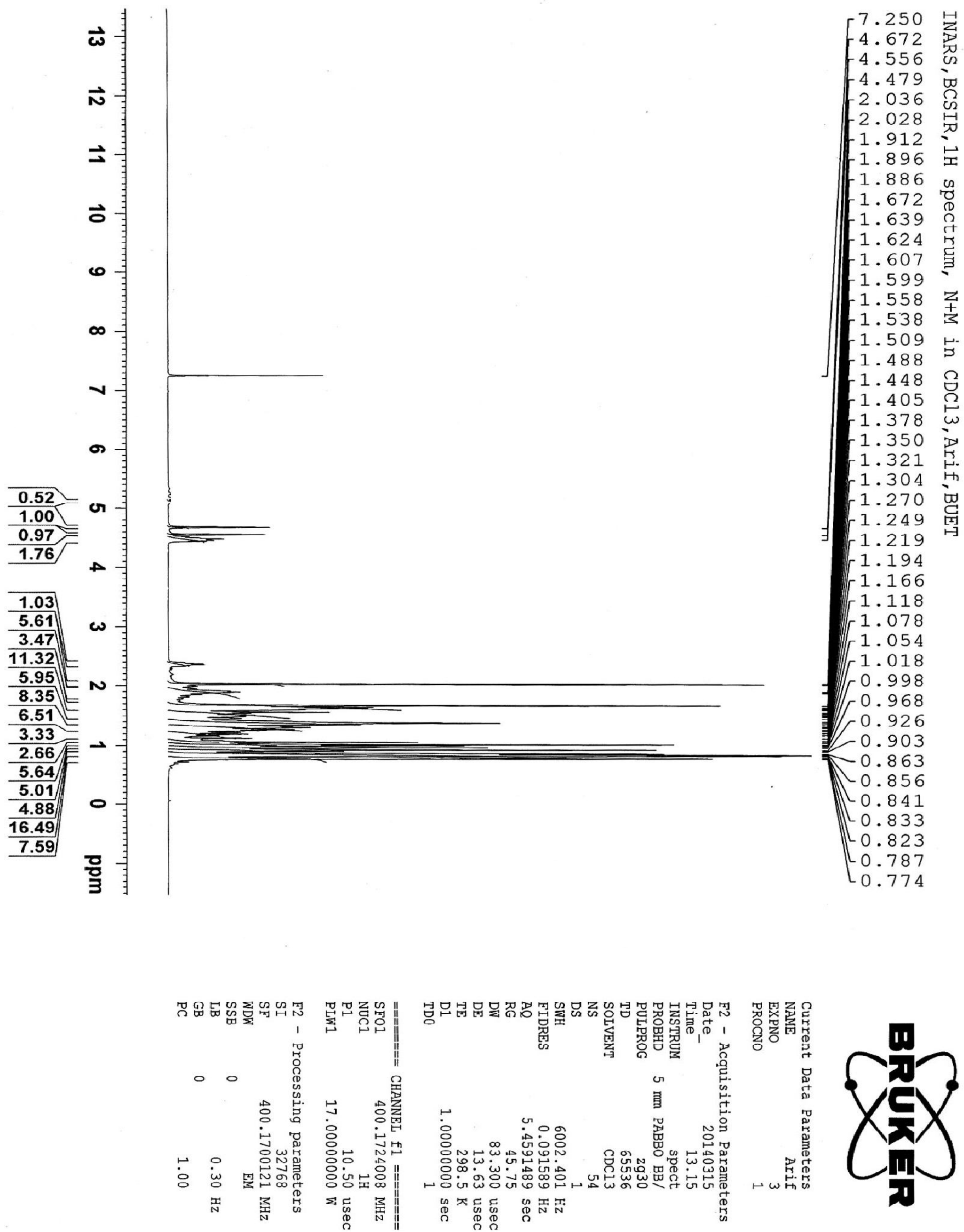
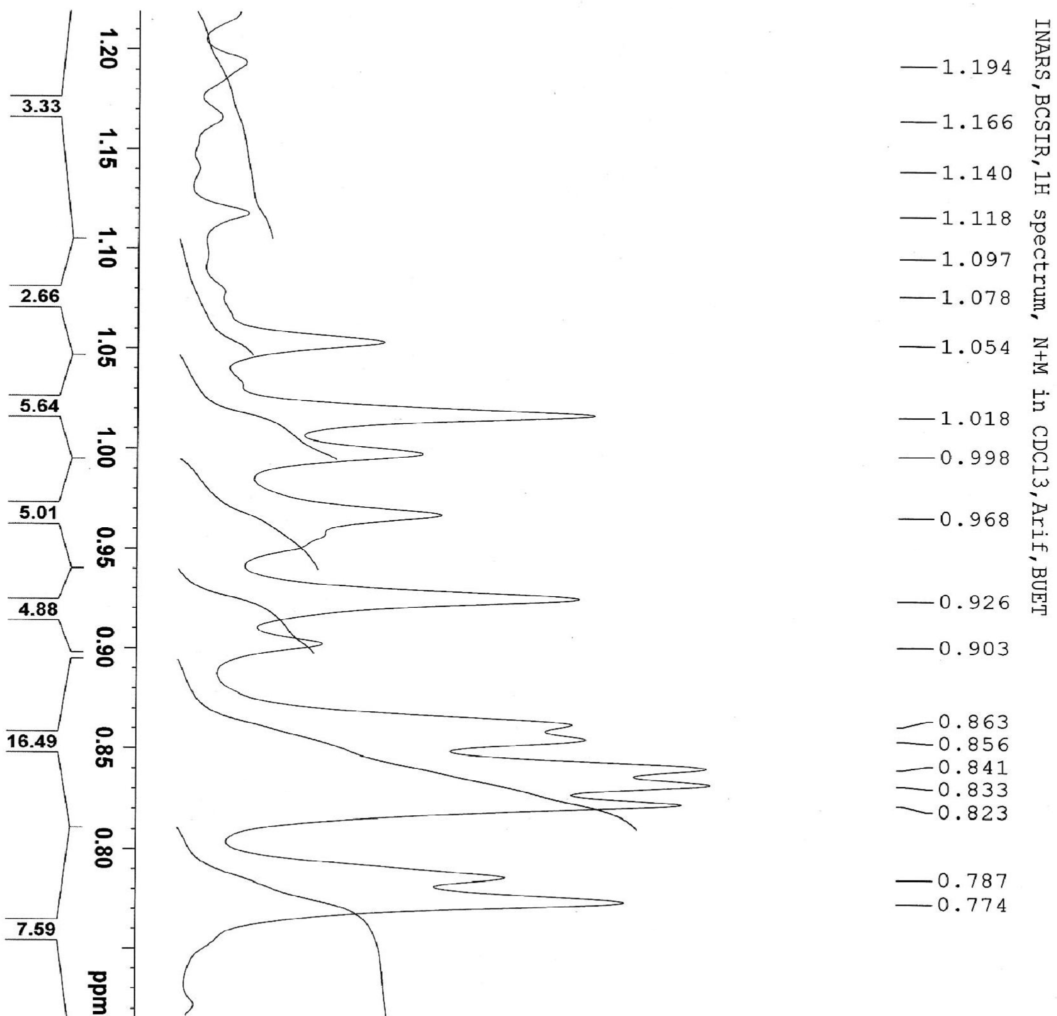


Fig 3.1: ^{1}H NMR spectra of the compound-3



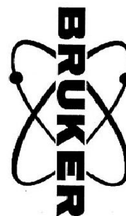



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Fig 3.2: ^{1}H NMR spectra (Expansion-1) of compound -3

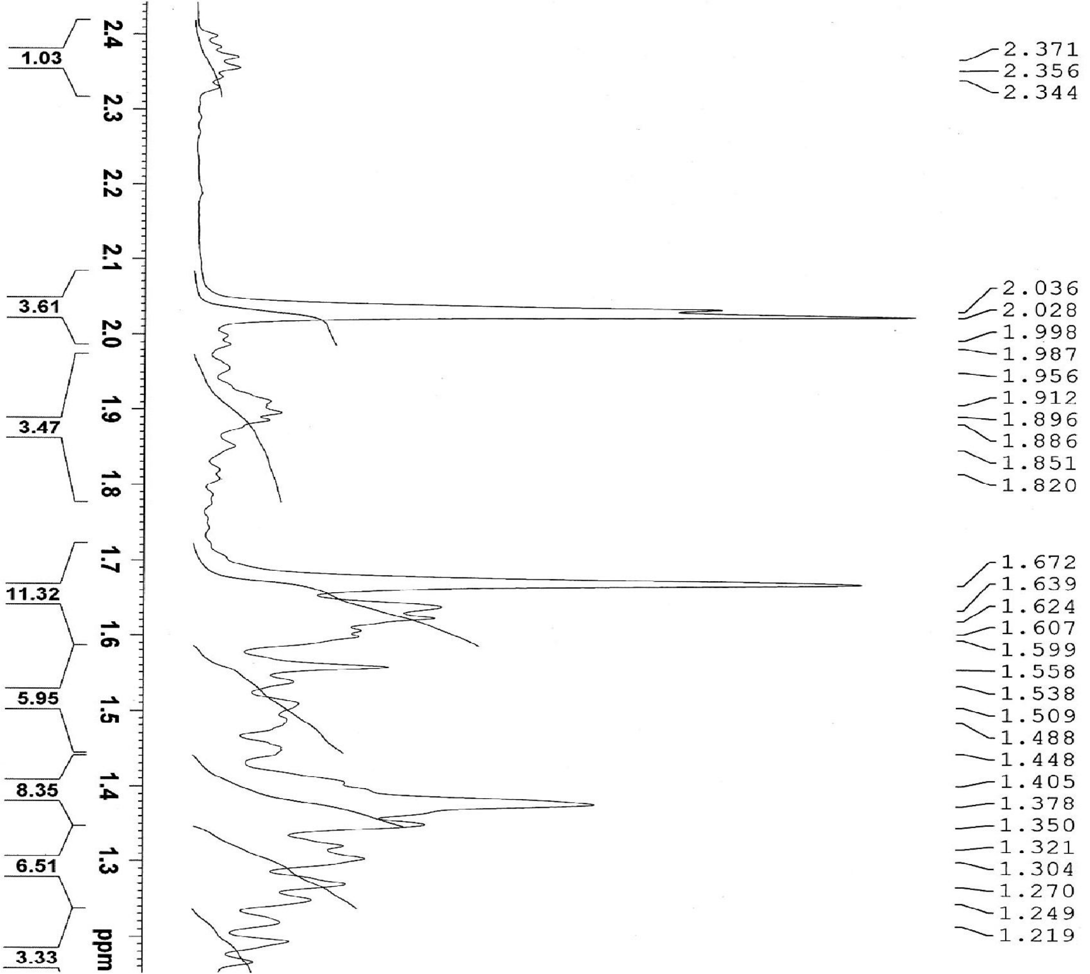

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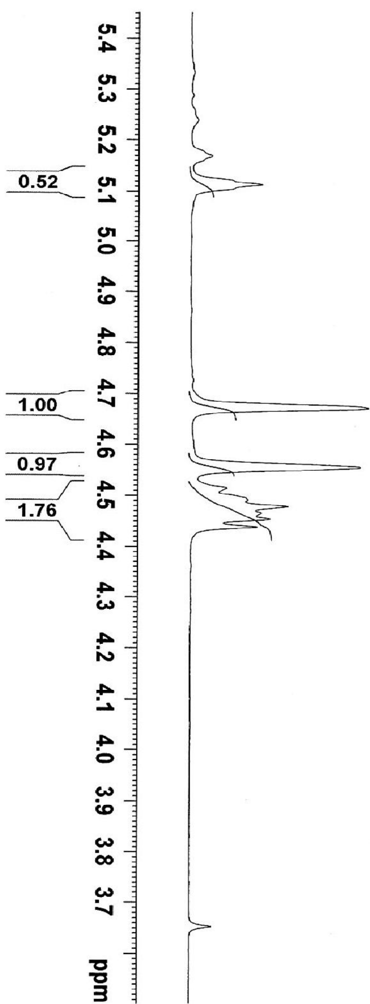
Fig 3.3: ^{1}H NMR Spectra (Expansion-2) of compound-3



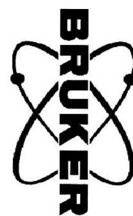


Fig 3.4: ^{1}H NMR spectra (Expansion-3) of compound-3

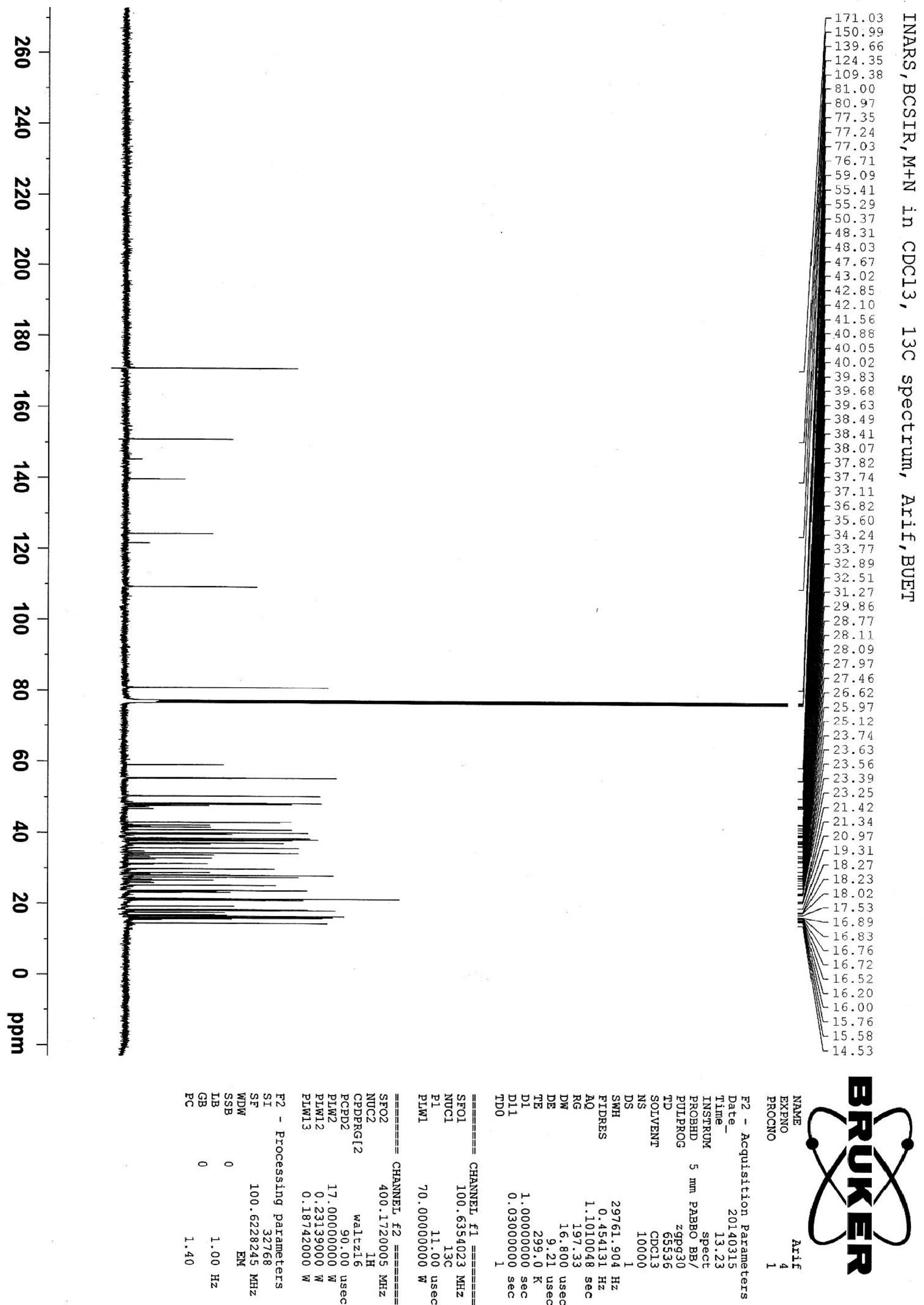


Fig 3.5: ^{13}C NMR Spectra of the compound-3

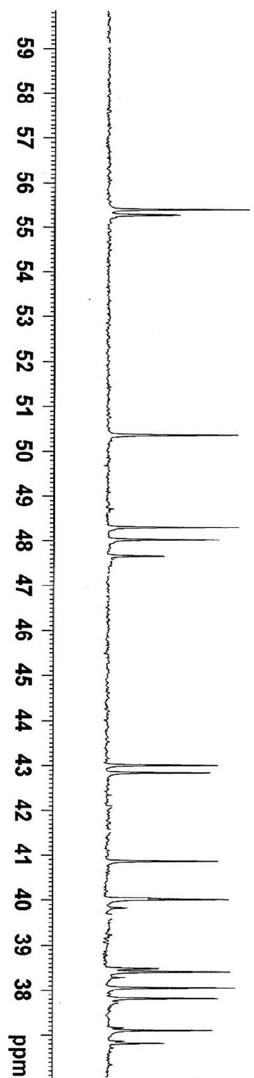


Fig 3.6: 13C NMR spectra (Expansion-1) of compound-3

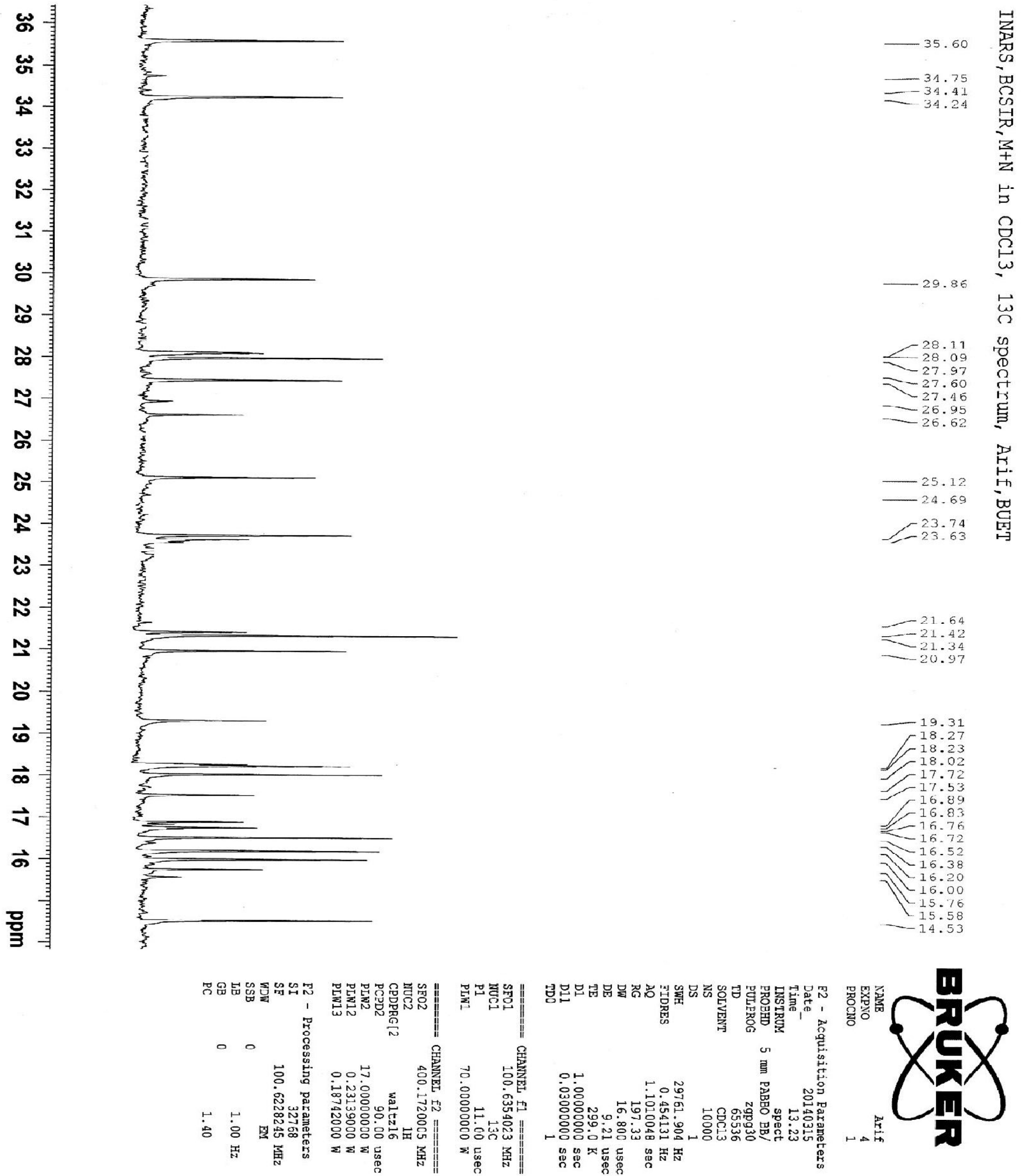
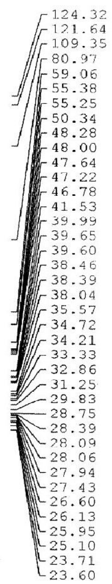
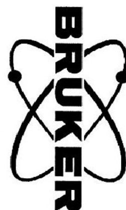


Fig 3.7: 13C NMR Spectra (Expansion-2) of compound-3





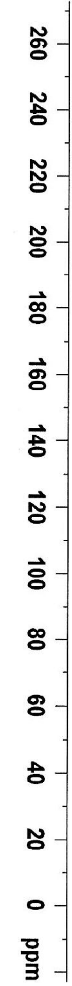


Fig 3.8: DEPT 135-1 spectra of the compound-3

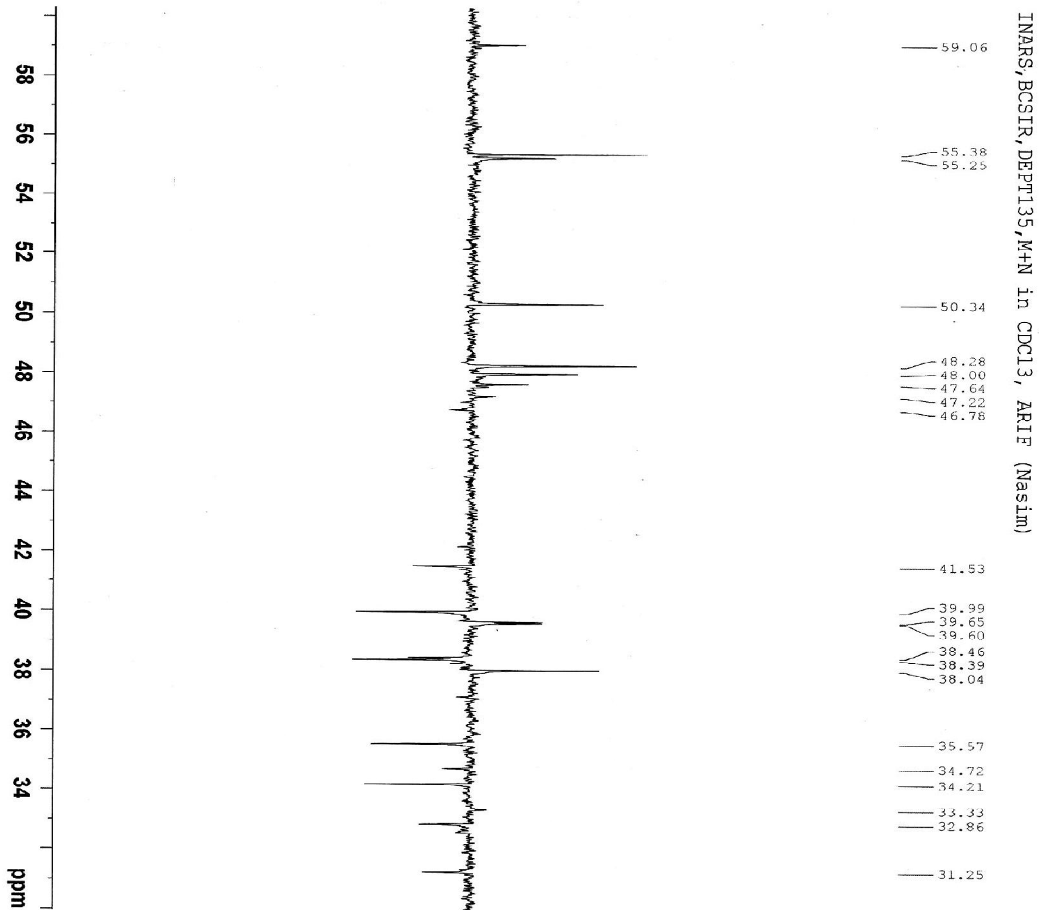


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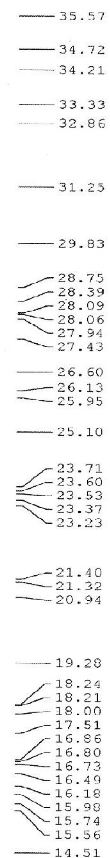
Fig 3.9: DEPT135-2 spectra of the compound-3



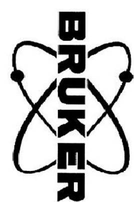
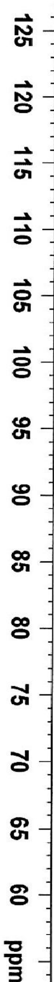


Fig 3.10: DEPT 135 -3 spectra of the compound-3



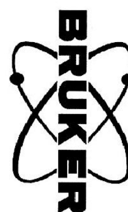


Fig 3.11: DEPT 135-4 spectra of the compound-3

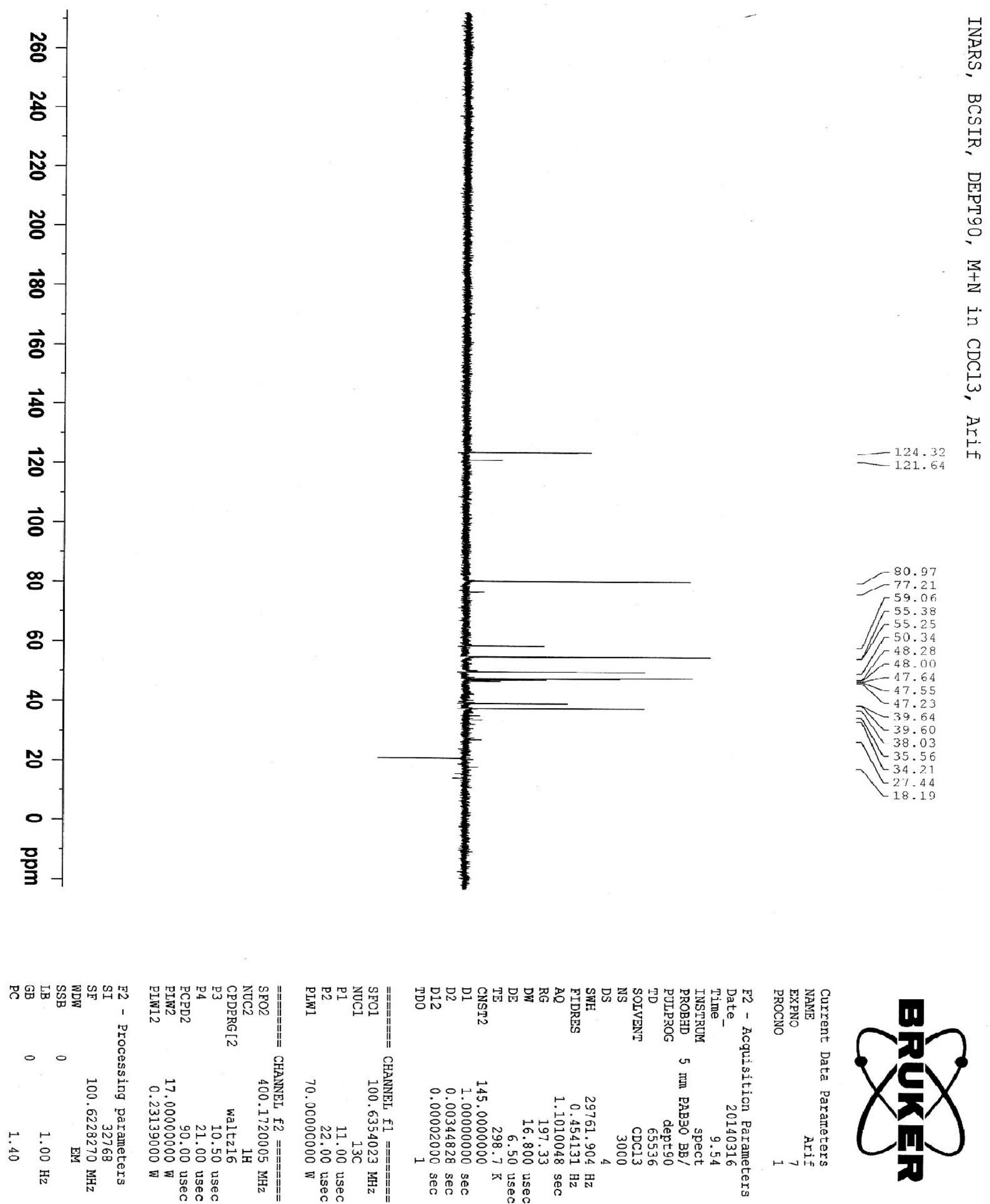
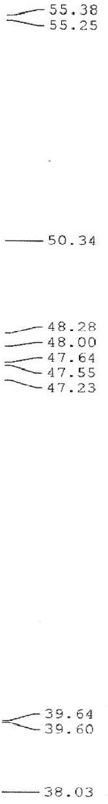


Fig 3.12: DEPT 90-1 spectra of the compound-3



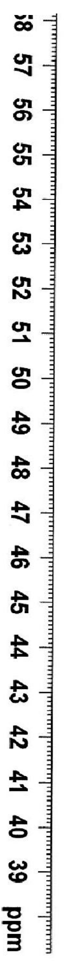
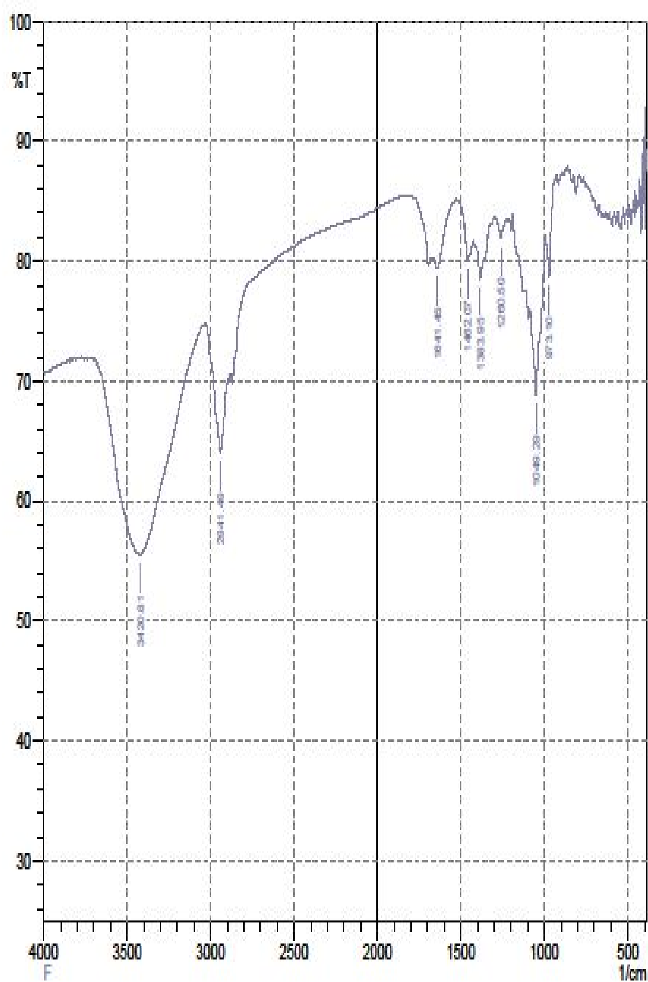


Fig 3.13: DEPT 90-2 spectra of the compound-3

SHIMADZU



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2	1049.29	68.781	9.755	1087.87	995.29	12.077	2.607
3	1260.5	81.882	1.244	1286.54	1246.04	3.347	0.116
4	1383.95	78.57	0.972	1387.81	1377.2	1.078	0.024
5	1462.07	80.064	0.49	1465.93	1460.14	0.546	0.005
6	1641.45	79.439	0.262	1657.85	1632.77	2.487	0.022
7	2941.49	63.952	8.213	3029.26	2884.59	23.448	3.305
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Fig 4.1: IR data of the H-2 or Compound-4

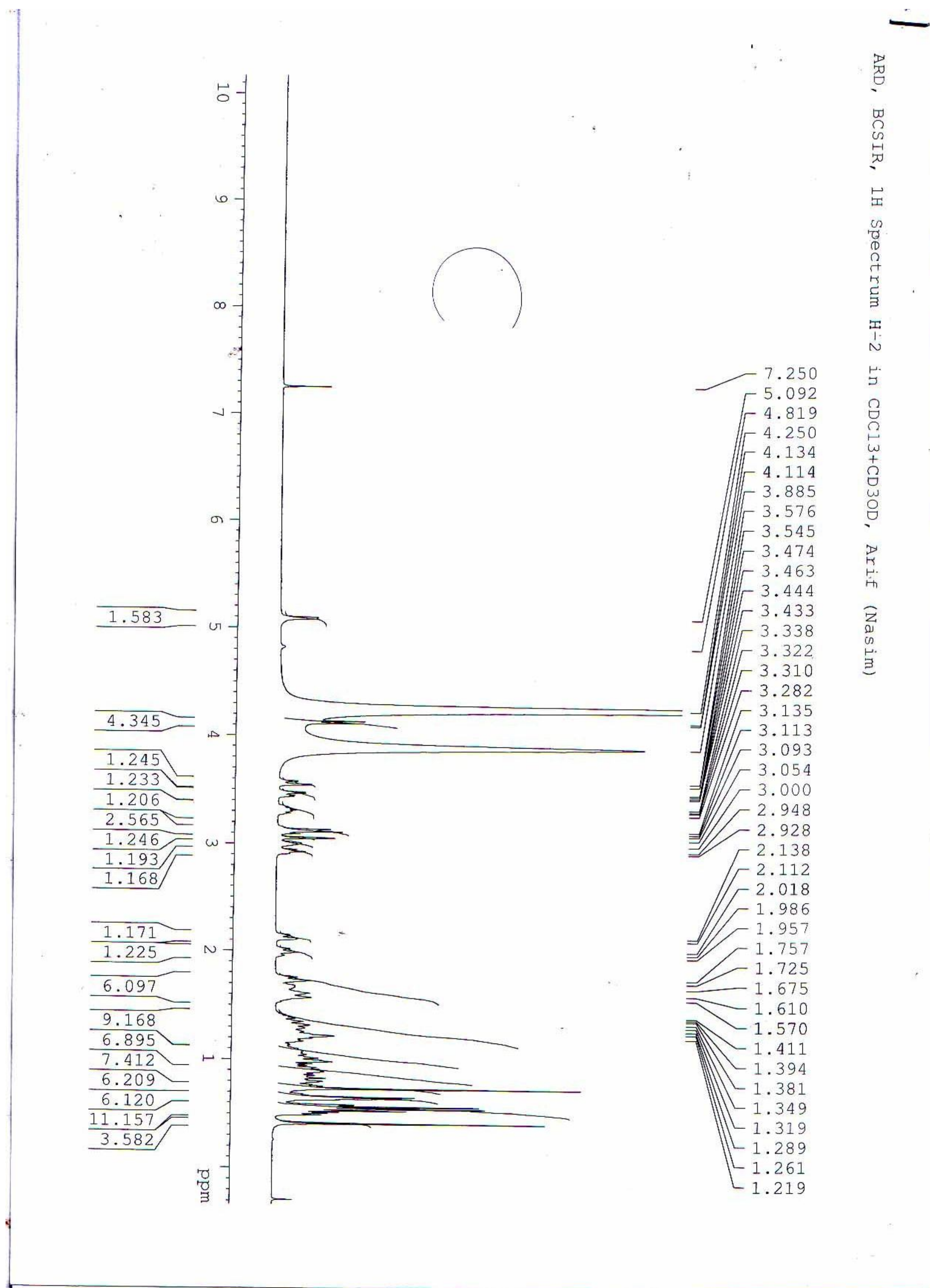


Fig 4.2: ^{1}H NMR spectra of the H-2 or Compound-4

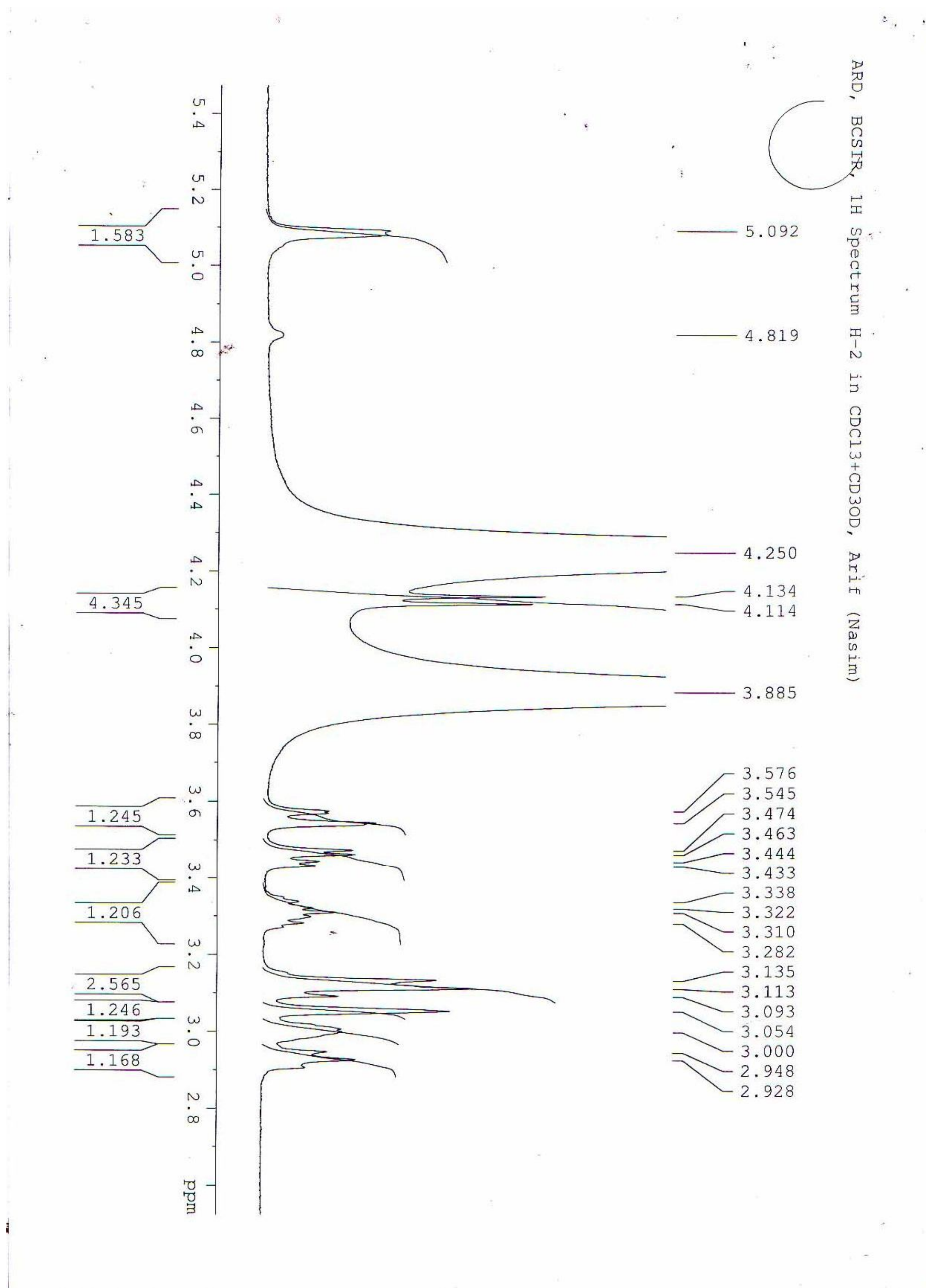


Fig 4.3: ^{1}H NMR (Expantion-1) spectra of the H-2 or Compound-4

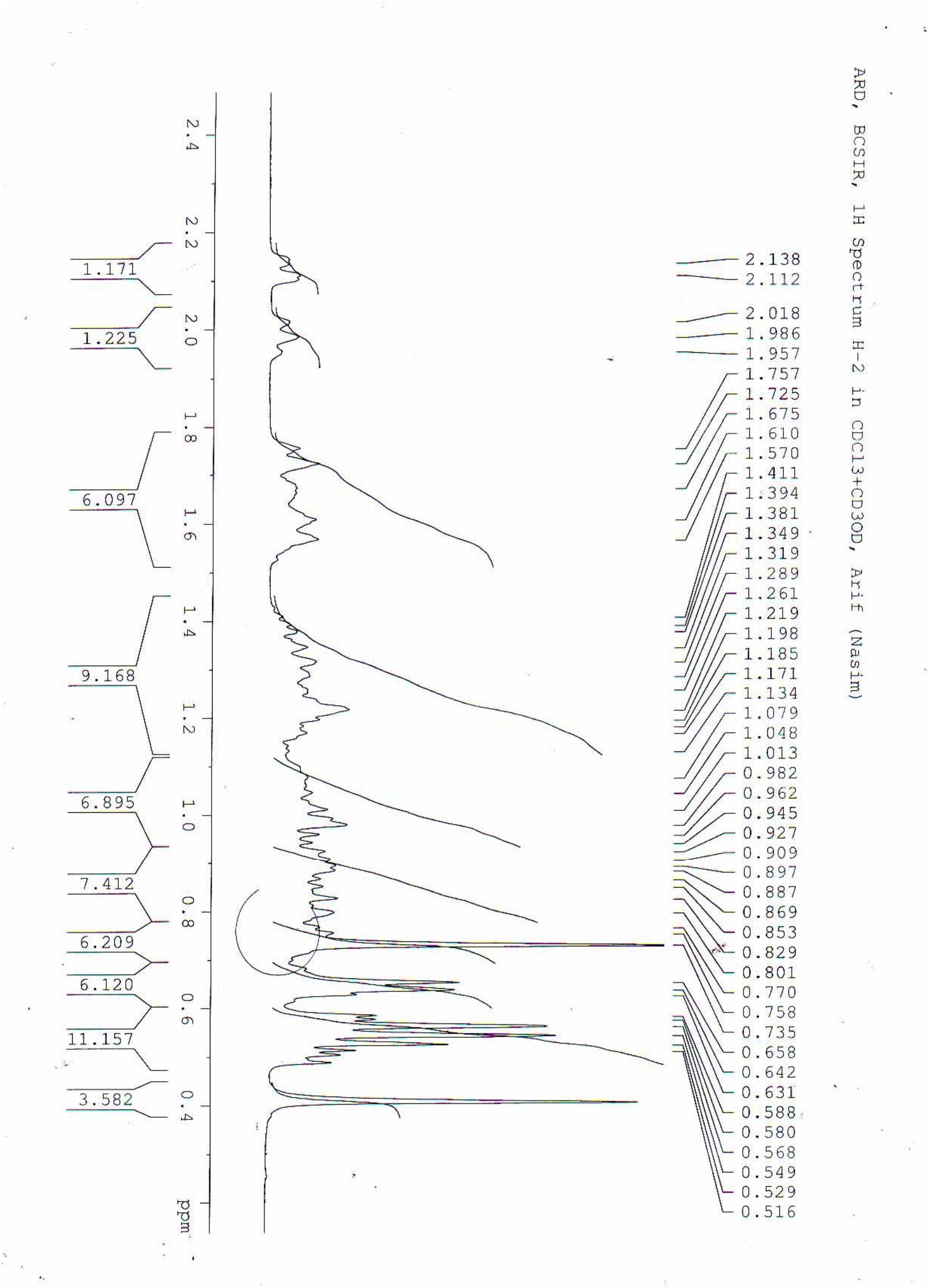


Fig 4.4: ^{1}H NMR (Expantion-2) spectra of the H-2 or Compound-4

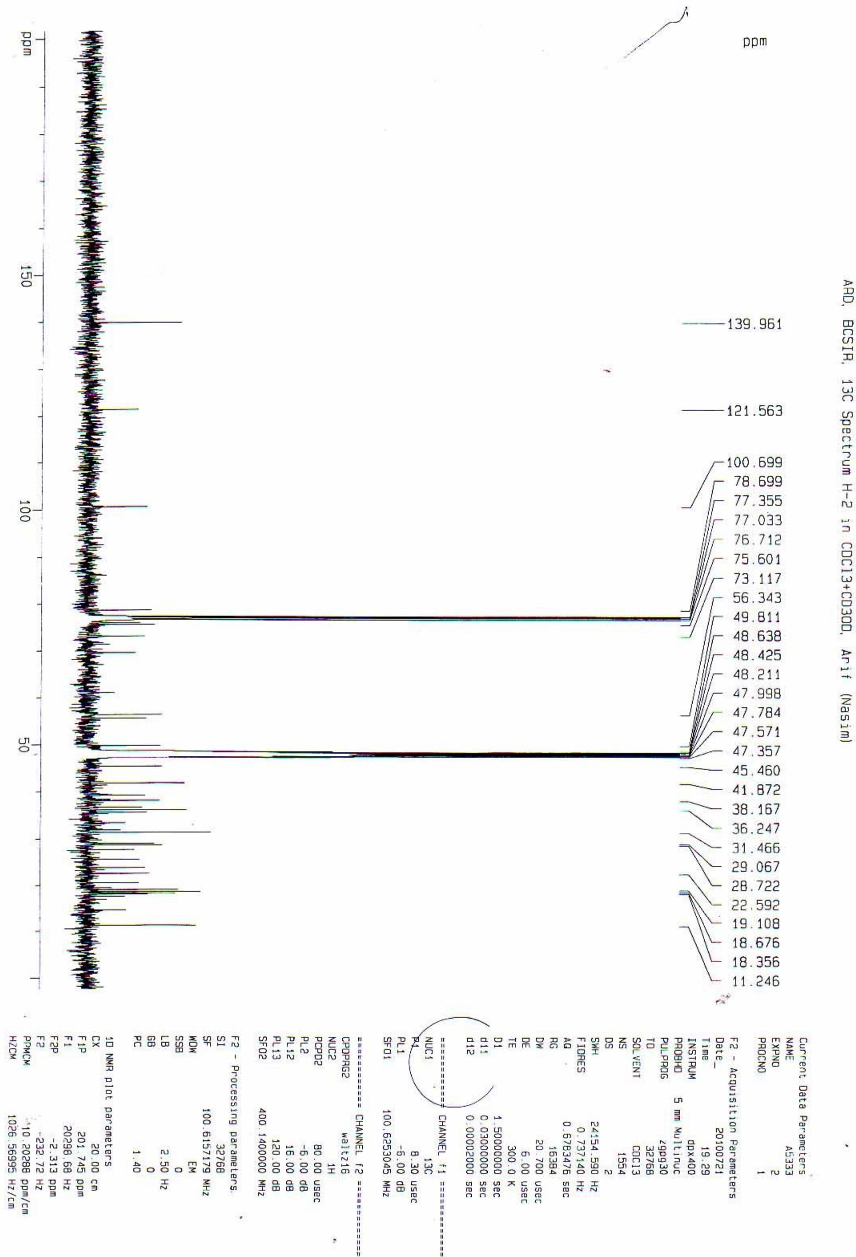


Fig 4.5: ^{13}C NMR spectra of the H-2 or Compound-4

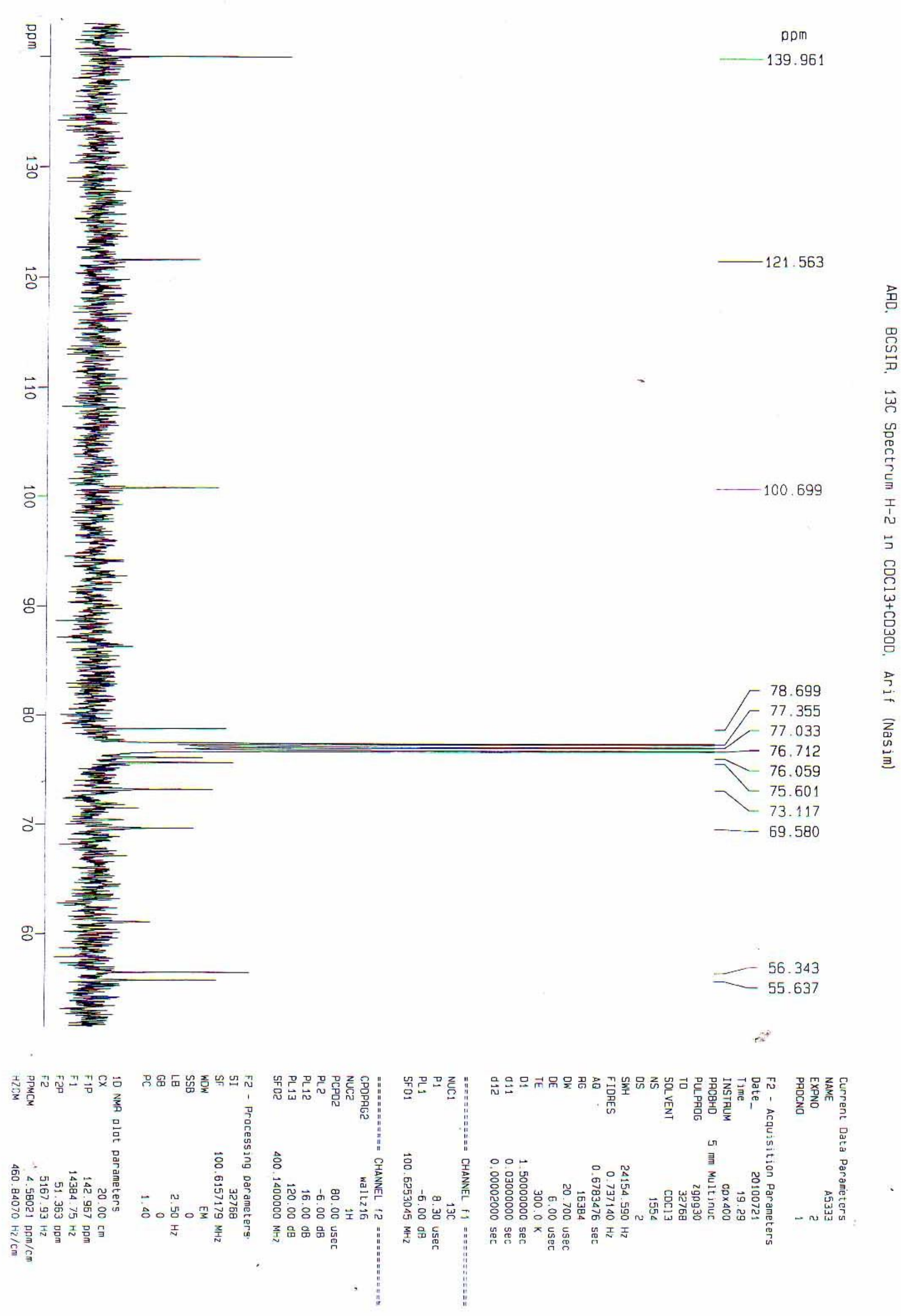


Fig 4.6: ^{13}C NMR (Expantion-1) spectra of the H-2 or Compound-4

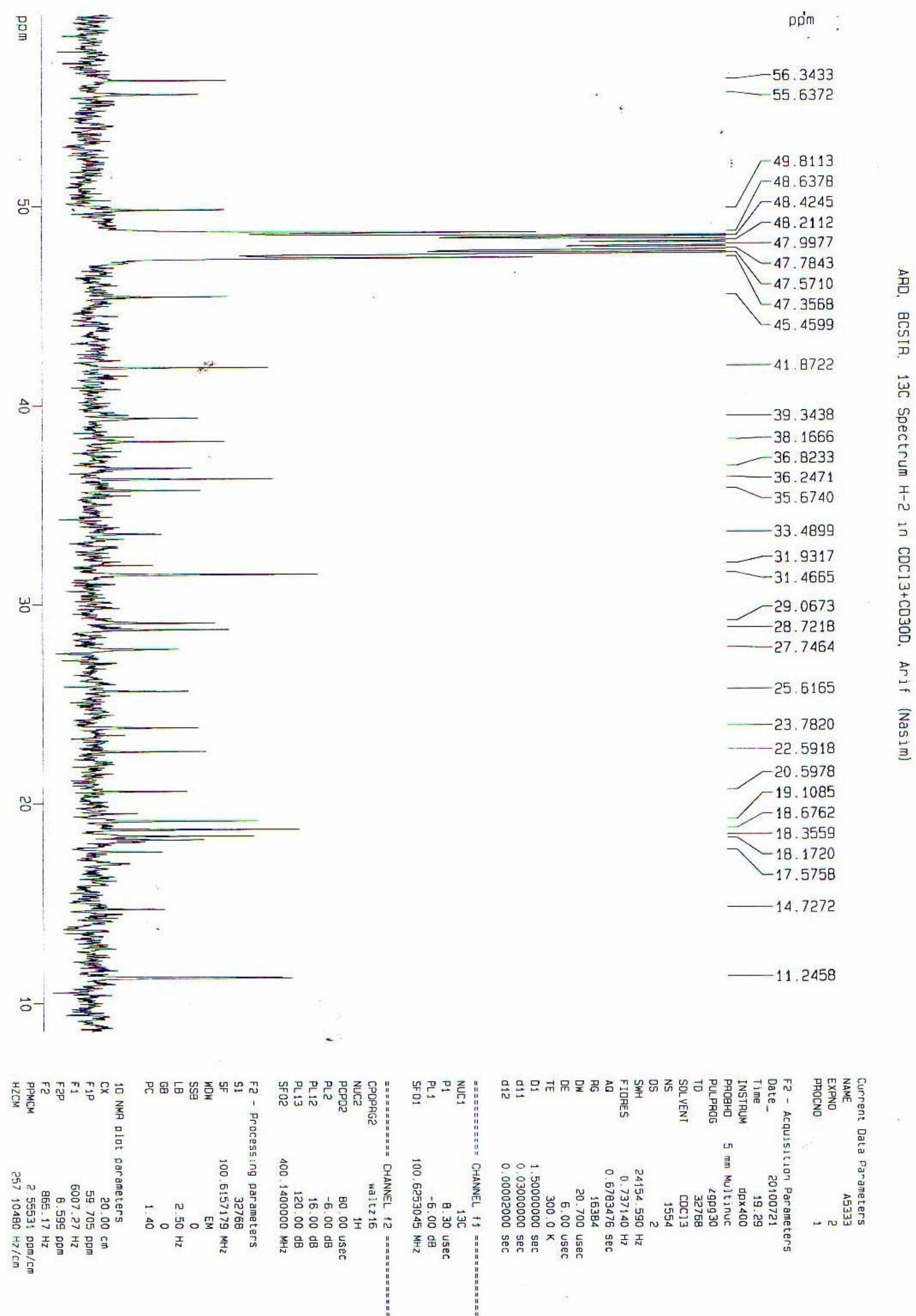
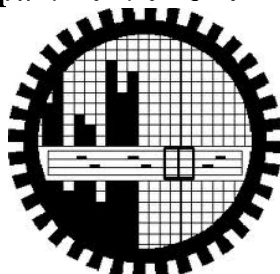


Fig 4.7: ^{13}C NMR (Expantion-2) spectra of the H-2 or Compound-4

Bangladesh University of Engineering and Technology, Dhaka, Bangladesh
Department of Chemistry



THESIS ACCEPTANCE LETTER

This thesis titled **Phytochemical and Pharmacological Investigation on the Roots** of *Asparagus rosemosus* submitted byMd. ArifulHoque, Roll No. 040803113F,Session-April 2008 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Masters of Philosophy (M.Phil) in Chemistry on March 31, 2014.

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1.1 General Consideration

Nature has been a source of medicinal agents since times immemorial. The importance of herbs in the management of human ailments cannot be over emphasized. From the beginning of civilization man has an amount of knowledge of drugs derived from various plants. It is clear that the plant kingdom harbors an inexhaustible source of active ingredients invaluable in the management of many intractable diseases. However, these complementary components give the plant as a whole a safety and efficiency much superior to that of its isolated and pure active components [1].

The Rigveda and Atharvaveda (5000 BC), the earliest Indian documents have references on health and diseases. During the vedic period the Susrutasamhita and the Charakasamhita were influential works on traditional medicine. Atharvaveda (one of the four most ancient books of Indian knowledge, wisdom and culture) contains 114 hymns or formulations for the treatment of diseases. Hundreds of medicinal plant were identified and have been traditionally used since then. Over the following centuries, ancient people developed a number of medicinal preparations and surgical procedures for the treatment of various ailments and diseases. The medicinal preparations consist mainly of plant materials in the form of powders, semi-solid preparations, elixir and distillates. Many of them also contain inorganic chemical substances, minerals and animal products. Alcoholic extracts and alcoholic solutions of the ingredients, tinctures and elixirs are also frequently used in to find out active ingredients.

Besides this, natural medicines have becomes the part of our daily diet like turmeric, cardamom, garlic, onion, ginger, tulsi, cloves, etc. Fruit, vegetable, pulses, wheat and rice grains which we consume daily provide us with essential nutrients like vitamins, carbohydrates, proteins, and different minerals required for smooth functioning of our body, and help us in fighting the various diseases, the idea here is that prevention is better than cure and to the much extent this is true.

As a herbal remedy or natural remedy we can easily remember use of 'turmeric' to treat body inflammations, burns and further use for skin beautification, 'Hing' is well known for digestive disorders, 'honey' is very popular as a home remedy for various ailments, the list of herbal or better call home remedy is very long. Further extension and use of medicinal plants and the pure isolates from them form the basis of drugs from nature.

The world health organization (WHO) estimates that 80% of the population living in the developing countries relies exclusively on traditional medicine for their primary health care needs [2] In almost all the traditional medicine, the traditional plants play a major role and constitute the backbone of the traditional medicine. Indian Materiamedica includes about 2000 drugs of natural origin almost all of which are derived from different traditional system and folklore practices. Out of these drugs, 400 are of mineral and animal origin while the rest are vegetable origin.

Coming out of the traditional age and with the development in science and technology, it was possible to identify various constituents in the medicinal extract and utilize the concentrated extract as an herbal remedy. Further, identification of the active pure principle of the herbal remedy paved way for researchers to find out the mode of action, conduct clinical trials and carry out chemical modifications to get even more potent molecules. Target based studies made us understand the mechanism of action which are now utilized to design and develop new chemical scaffolds and giving this generation a new insight into pharmacological aspects of natural drugs.

Plants became the basis of traditional medicine system throughout the world for thousands of years and continue to provide mankind with new remedies. The plant based indigenous knowledge was passed down from generation to generation in various parts of the world, especially in the Indian sub-continent and has significantly contributed to the development of different traditional systems of medicines. Beginning with the discovery of morphine as the first and active alkaloid extracted from opium poppy plant in December 1804, the quest for isolation and discovery of active principles from the traditionally used medicinal plants took the momentum.

Quinine from the bark of the cinchona tree remained the first choice of treatment for malaria until 1940's, later it was replaced by other drugs. Reserpine isolated from the dried roots of *Rouwolfia serpentina* known as '*Sarpaganda*' had been used for centuries in India for the treatment of insanity, fever and snakebites and also it was utilized as antipsychotic antihypertensive drug to control high blood pressure. Guggulsterones were later on identified as the active principles of gum of *Commiphor amukul* which was traditionally used for the treatment of increased cholesterol levels. Another example is Shatavarins obtained from the roots of the plant *Asparagus racemosus* popularly known as 'shatavari'. Roots of this plant are highly reputed in Ayurveda as galactogogue (increase in female milk production) and it was recommended for overall female health. Recent research has shown it to be an immunomodulator with antioxidant, healing and adaptogenic properties. For that reason, it has been often referred to as the "Indian Ginseng". It is used in several indigenous drug preparations for maintaining health as well as treatment of several disease conditions. The active principles of this plant were later identified as Withanolides (Withaferin A, the first withanolide to be isolated from this plant). Bacosides obtained from *Bacopamonnieri*, locally known as 'brahmi' were another significant discovery of natural product chemistry. This plant has been used since centuries particularly in India as a brain tonic , supposedly enhancing memory development, learning, and concentration.

Now natural product could be defined as single chemical compound that occurs naturally. This term is typically used to refer to an organic compound of limited distribution in nature. More precisely, any biological molecule is a natural product, but the term is usually reserved for secondary metabolites which are small molecules produced by an organism that are not strictly required for the survival of the organism, unlike the more prevalent macromolecules such as proteins, starch, nucleic acids that make up the machinery for the more fundamental processes of life.They have a wide range of chemical structures and biological activities. They are derived by unique biosynthetic pathways from primary metabolites and intermediates. Some researchers refer the production of the secondary metabolites to be a potent chemical defense system against herbivorous, deterrents, pathogens, etc. The immobility of plants in diverse and changing physical environment, along with the danger of attack by herbivores and pathogens, has led to the development of numerous chemical and biochemical adaptations for protection and defense, plants for example can produce highly toxic compounds or compounds mimicking substances

normally produced by a herbivore, for example growth hormones or pheromones. Different classes of compounds produced by plants chiefly include alkaloids, terpenoids, flavonoids and many new classes originating from them.

It may be sufficient for the plant to produce compounds that are unpleasant, odorous, or distasteful, or that possess digestibility reducing properties, i.e. compounds that decrease the uptake of nutrients, thus preventing over-browsing of the plant. One example of a compound with strong antifeedant activity against numerous insects is azadirachtin (highly functionalized terpenoid), produced by the Indian neem tree (*Azadirachta indica*). The tree has been used for centuries to protect other plants and clothes from insects. The structure of the active compound is very complex and could not be proven until 1985. Due to its potency and selectivity against insects this compound has been commercialized as antifeedent. Indeed many plants do not keep permanent stores of their defense compounds, but manufacture them in response to predation.

Drug discovery from the natural sources broadly involves two approaches, first one is chemically driven (finding biological activities for purified compounds) and second is biologically driven (bioassay-guided approach beginning with crude extracts) or it can be combination of the both. Beginning in the 1970´s through today, the majority of the academic-based research efforts have become essentially ‘biologically driven’ i.e., the object of the search has shifted to discover natural products with biological activity. The biological activities include exploring their potential as agrochemicals and pharmaceuticals, as well as their possible chemical ecological roles.

With bioassay-guided approach to purify the compounds responsible for the activity of the extract, NMR and chromatographic techniques are used to isolate the chemically most interesting substances. Ideally the structurally unusual or novel compounds are also responsible for the activity of the extract. This approach works well when the active compounds are present in high concentration and the assay turnaround time is longer than a couple of weeks. This approach is indeed productive with respect to isolating numerous new compounds, at least some of which usually express some of the activity observed for the crude extract, but is obviously not the best method to identify the most active compounds if they are present in low concentrations.

Natural product chemistry is one of the oldest branches of the chemical sciences, its origin dating back to the first decades of the 19th century, or even earlier. Presently after almost 200 years of study, this is still vibrant and evolving. Today, the modern specific and sensitive screening methods can detect bioactive molecules present in as low as 1μg/L concentration. The difficulty in purification and the identification are now eliminated with the use of advanced HPLC (High Performance Liquid Chromatography), high resolution 2D-NMR (Two Dimensional Nuclear Magnetic Resonance) and spectroscopic methods.

Drug discovery from natural product is very tedious process involving identification of plant material, preliminary screening of the crude extract, then isolation of various secondary metabolites, elucidation of their structures and finally evaluation of their biological activity (*in vitro* and *in vivo*). If the molecule is really interesting with its strong pharmacological properties then further preclinical studies are conducted on the molecules such as toxicity studies, stability and solubility studies, pharmacokinetic studies and mechanism studies where the plausible mode of action are predicted. After undergoing these studies if it is found that molecule is more active than the presently used drug then processes are developed for its economical and easy isolation from the source so that it can be easily available for therapeutic use.

Molecule may or may not go for clinical trials, all depends on the bioavailability. In most of the cases natural product as a drug are limited to the active extract (containing mixture of compounds) herbal formulations due to their poor bioavailability and high input costs. The last ordeal is clinical trials where the candidate molecule is tested for its safety and efficacy on several group of human volunteers. Counting pre-clinical and clinical trials time, it takes almost a decade for new candidate drug to get approval for launch in market. So development of a new drug from natural source is a time and money consuming process, similar with that of a synthetic molecule.

Molecules which are of limited use are subjected to chemical modifications, partial derivatization or its structure may be used for the development of synthetic analogs to increase its therapeutic use. Natural product chemist may also develop feasible synthetic schemes to produce the lead active molecule having poor bioavailability. A lot of pain and patience is involved with the drug development besides money.

Natural Products act as lead molecules for the synthesis of various potent drugs. The plant-derived compounds have a long history of clinical use, better patient tolerance and acceptance. There are several plants derived compounds, which have recently undergone development and have been marketed as drugs includes Taxol from the bark of Pacific vew tree *Taxus brevefolia* for lung, ovarian and breast cancer, Artemisinin from the leaves of traditional Chinese plant *Artemisia annua*to combat multidrug resistant malaria, Forskolin extract produced from the root of *Coleus forskohlii*, an ancient Ayurvedic plant containing a unique substance known as forskolin used in the treatment of allergies, respiratory problems, cardiovascular diseases, glaucoma, psoriasis, hypothyroidism and weight loss, Silymarin (the mixture of flavonolignans) extracted from the seeds of *Silybummari anum* or milk thistle for the treatment of liver diseases, and Picroliv, another hepatoprotective agent (an iridoid glycoside mixture containing 60% picroside I and kutoside in the ratio of 1:1.5) obtained from the plant *Picrorhizakur rooa* (root and rhizome). Natural products entities depend not only on plants but also on other form of life like algae, fungus, sponges, mollusc, etc. Microorganisms have been extensively screened for antibiotics since Fleming's discovery of the antibacterial activity of *Penicilliumchryso genum*, which is the source of several β-lactam antibiotics, most significantly penicillin. Many antibiotics have been identified from the bacteria especially belonging to *streptomyces* genus. Streptomycin from *Streptomyces griseus*, neomycin from *Streptomyces fradiae*, tetracycline from some species of streptomyces have been the pioneers in the antibiotic treatment. Marine natural products with their unique structural features and pronounced biological activities continue to produce lead structures in the search for new drugs from nature. Invertebrates such as sponges, tunicates, shell-less molluscs and others that are either sessile or slow moving and mostly lack morphological defence structures have so far provided the largest number of marine-derived secondary constituents including some of the most interesting drug candidates. Ziconotide (*Prialt*) which is a aminoacid peptide isolated from the venom of the marine snail *Conus magus* is now available in the market as a potent analgesic for severe chronic pain, its analgesic effect is comparable to the opioid analgesics (e.g. Morphine). Several marine-derived compounds have generated considerable interest scientifically and commercially, and from public and health point of view, these include palytoxin, ciguatoxin, brevetoxins, okadaic acid, tetrodotoxin, saxitoxin, calyculin A, manoalide and kainic acid. This is widely accepted to be true when applied to drug discovery in 'olden times' before the advent of

high-throughput screening and the post-genomic era: more than 80% of drug substances were natural products or inspired by a natural compound. The trend continues even today as can be seen on the comparison of information presented on sources of new drugs from 1981 to 2007 indicate that almost half of the drugs approved since 1994 are based on natural products. Thirteen natural-product- related drugs were approved from 2005 to 2007. Recently approved natural-product-based drugs include compounds from plants (including apomorphine, elliptinium, galantamine and huperzine), microbes (daptomycin) and animals (exenatide and ziconotide), as well as synthetic or semi-synthetic compounds based on natural products (e.g. tigecycline, everolimus, telithromycin, micafungin and caspofungin), even though many pharmaceutical companies have discontinued their programs of drug discovery from natural sources. These new drugs have been approved for the treatment of cancer, neurological diseases, infectious diseases, cardiovascular and metabolic diseases, immunological, inflammatory and related diseases, and genetic disorders, which encompass many of the common human diseases. Over a 100 natural-product-derived compounds are currently undergoing clinical trials and at least 100 similar projects are in preclinical development. Most are derived from leads from plants and microbial sources. There is, however, also a growing interest in the possibility of developing products that contain mixtures of natural compounds from traditionally used medicines (as discussed previously) has been approved by the FDA and has recently come on the market.

The practice of using natural substances from the plants continues today also because of its biomedical benefits, culture beliefs in many parts of the world, readily availability, economic viability and comparatively safe than synthetic drugs. In spite of the numerous past successes in the development of plant derived products, it has been estimated that only 5-15% of the 2,50,000 existing species of higher plants have been systematically surveyed for the presence of biologically active compounds. As, many secondary metabolites are genus or species specific, the chances are therefore good to excellent that many other plant constituents with potentially useful properties remain undiscovered. The high throughput screening of natural sources will greatly facilitate the discovery and development of new drugs.

It could be concluded that natural products discovered so far have played a vital role in improving the human health and have been the drugs of choice despite facing a tough competition from their synthetic counterparts, due to their safe and long lasting effects.

1.2 Medicinal importance of plant materials

Plants not only provide man with food, shelter, and medicine, but also the sustaining oxygen. From ancient time to modern age the human has been successfully used plants and plant products as effective therapeutic tools for fighting against diseases and various health hazards.

Although with the advent of synthetic drugs the use and procurement of plant derived drugs have declined to a large extent, a large number of drugs of modern medicine are obtained from plant sources. According to some generous estimates, almost 80% of the present day medicines are directly or indirectly obtained from plants.

As therapeutic use of plants continued with the progress of civilization and development of human knowledge, scientists endeavored to isolate different chemical constituents from plants, put them to biological and pharmacological tests and thus have been able to identify and isolate therapeutically active compounds, which have been used to prepare modern medicines. In course of time their synthetic analogues have also been prepared. In this way, the discovery of vincristine (**1**) was done from *Catharanthus roseus*, which is, used in the treatment of cancer.

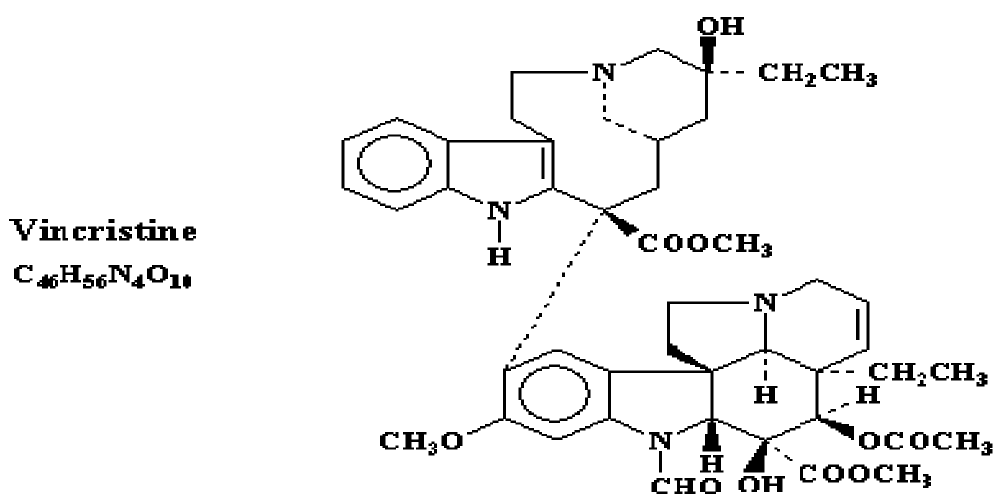


Fig: 1.1 Vincristine (**1**)

Calanolidea (**2**) is a reverse-transcriptase inhibitor isolated from the Malaysian rainforest tree, *Calophyllum langerum* by the US NCI. It has exhibited synergistic anti-HIV activity in combination with nucleoside reverse-transcriptase inhibitor, including AZT, ddI and ddC. Medichem pharmaceuticals, Inc., and the state of Sarawak, Malaysia have begun clinical development of Calanolide A as a potential treatment for AIDS and HIV infections.

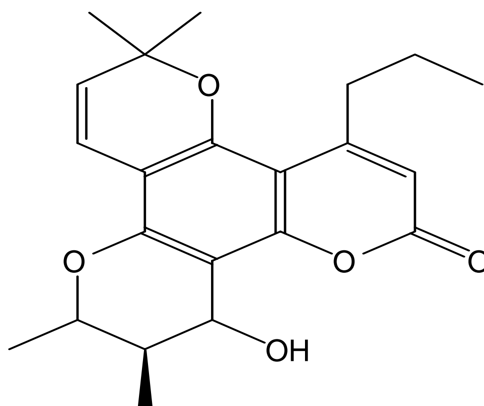


Fig:1.2. Calanolide A (**2**)

Ancient Chinese medical texts written 2000 years ago describe the herb *Artemisia annua* as a remedy for malaria. From this indication, in the late 1960s Chinese researchers initiated evaluation of various extracts of this herb. Bioassay guided isolation yielded the new anti-malarial compound artemisinin (**3**) which is effective in treating chloroquine resistant cases and other severe cases without major toxicity.

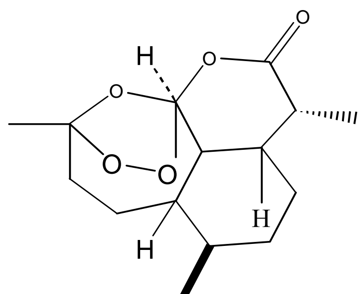


Fig: 1.3 Artemisinin (**3**)

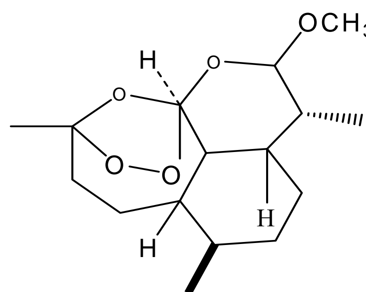


Fig: 1. 4 Artem ether (**4**)

Artemether (**4**), a synthetic analogue of artemisinin has been developed in the People's Republic of China. Two recent clinical studies suggested that artemether is as effective as quinine in the treatment of severe malaria.

For several centuries, elderly people in some parts of Mainland China have brewed tea from the leaves of the club moss(*Huperziaserrata*) for improvement of their memory. In the early 1980s, Chinese scientist isolated huperzine (**5**) from the plant Club moss as a potent, reversible and selective inhibitor of acetycholinesterase. A total synthesis has been developed due to very low levels in nature, and the product is found to be a promising candidate for the treatment of cholinergic related neurodegenerative disorders such as Alzheimer's disease (AD). In a prospective, multicenter, double-blind trial with 103 patients, huperzine A was found to be safe and superior to placebo and induced improvement in memory cognition and behavior in about 58% of patients with AD.

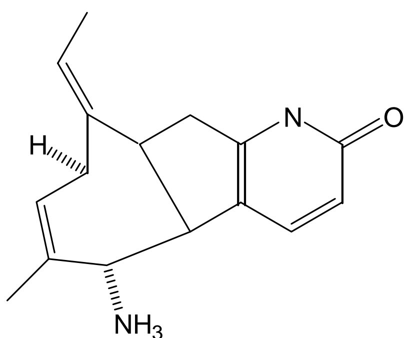


Fig: 1.5 Huperzine (**5**)

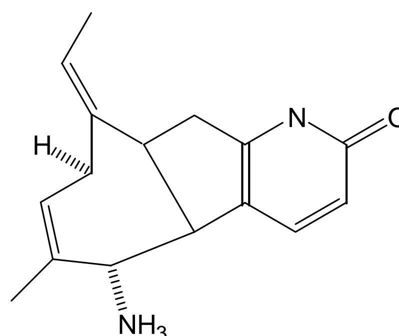
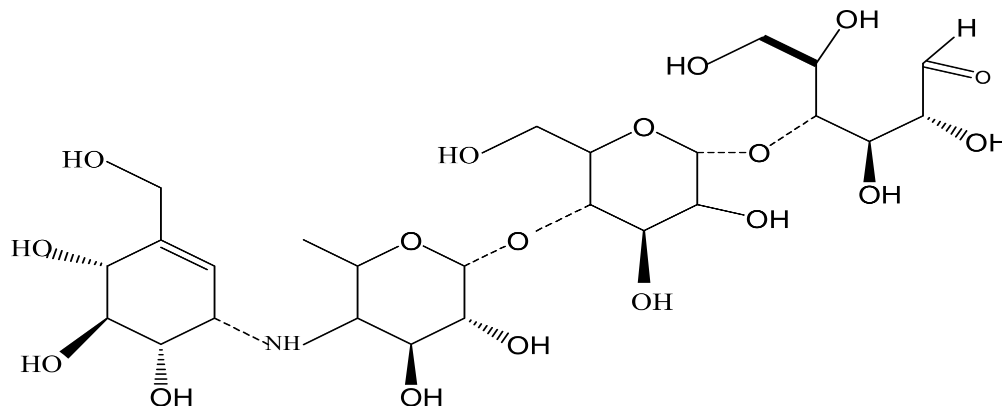


Fig: 1.6 Galathmine (**6**)

Galanthamine (**6**) is a long acting, centrally active competitive cholinesterase inhibitor; a natural product isolated from *Galanthusnivalis* in the 1950s. Galanthamine under the name of Nivalein is marketed in Austria for AD and in Germany for other indication such as facial neuralgia.

In the antidiabetes area, the past decade has witnessed the market introduction of several α-glucosidase inhibitors derived from natural products. Acarbose (**7**), a complex oligosaccharide one of them, was isolated from *Actinoplanes* sp. At Bayer from a search for α-glucosidase enzyme inhibitors. By inhibiting α-glucosidase, acarbose decreases the release of glucose from ingested carbohydrate and slows the increase of food-induced blood glucose levels. Acarbose is

now approved in Germany, Japan, the US and other countries and has been used as adjuvant therapy in diabetes.



Acarbose (**7**)

Fig: 1.7 Carbose (**7**)

Forskolin (Colforsin) is a diterpene natural product isolated from the Indian plant *Coleus forskohlii* at Hoechsts research labs in India and have blood pressure lowering and cardioactive properties. Later Forskolin was found as a potent adenylatecyclase activator. Colforsindaproate (NHK-477, **8**) is a semisynthetic product of forskolin derivative and was then brought into phase III clinical trials in Japan for treatment of cardiac insufficiency and phase II trials for treatment of asthma.

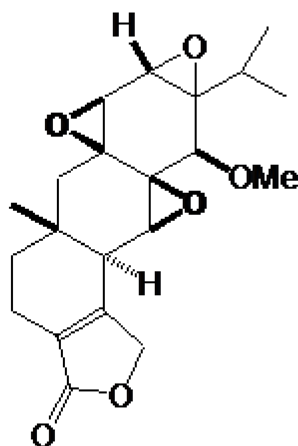


Fig: 1.8 Triptolide(8)

Triptolide (8) is an active component isolated from the Chinese plant *Tripterygiumwilfordii*, a plant traditionally used for the treatment of rheumatoid arthritis. Varieties of formulations were developed in Mainland China and are shown to be effective in the treatment of inflammatory and autoimmune diseases. Triptolide was demonstrated to significantly inhibit arthritis in animal modal and have potent cytotoxicity.

The Chinese tree *Ginkgo biloba* has been used therapeutically for thousands of years. More recently, extracts of the leaves have become available in many European countries as over-the counter products for the treatment of cerebral vascular insufficiency and tinnitus. Ginkgolides, a class of unique diterpene cage like molecules were isolated from the leaves of *Ginkgo biloba* and represent a group of highly selective platelet activity factor (PAF) receptor antagonists. Among them, Ginkgolide B (BN-52021, **10**) has been advanced to phase III clinical trials for the treatment of septic shock in patients with severe sepsis caused by Gram positive bacterial infections and also good result in inflammatory and autoimmune disorders.

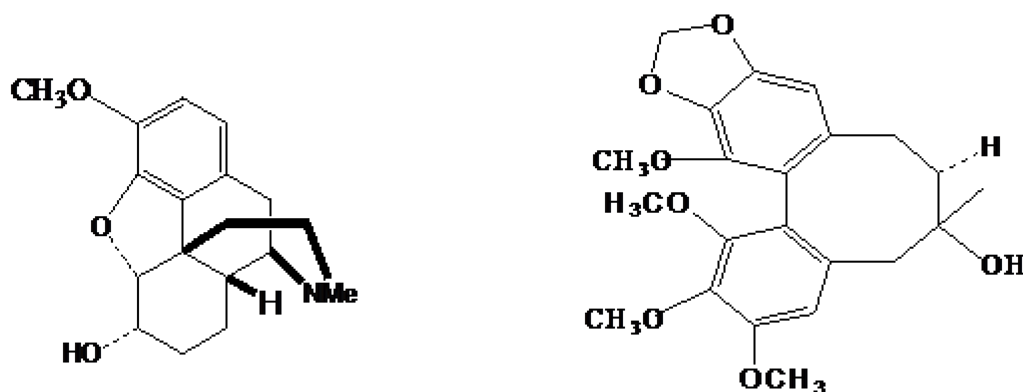


Fig: 1.9 Dextromethorphan (12) Fig: 1.10Gomisin (11)

Gomisin A (**11**) is a lignin derivative isolated from the dry fruit of *Schisondrachinensis*, a traditional Chinese medicine used for the treatment of liver intoxication. Gomisin A was found to be hepatoprotectant and protect liver damage in various animal models.

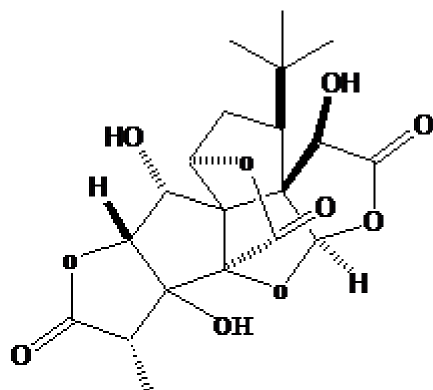
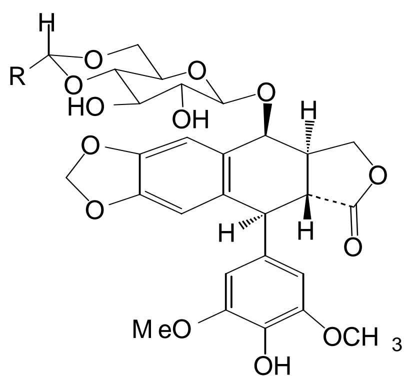


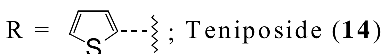
Fig: 1.11Ginkgodide (12)

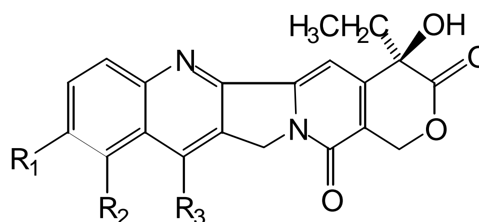
Morphine was first isolated by Serturner in 1806 followed by Codeine in 1832 by Robiquet and then the non-morphine alkaloid papaverine by Merck in 1848 from the seeds of *Poppy*. Dextromethorphan (**12**) is a semisythentic product of morphine used in most cough syrup today.

Two active anti-tumor agents' etoposide (**13**) and teniposide (**14**) was isolated from the root of various species of the genus *Podophyllum*. These plants possess a long history of medicinal use by early American and Asian cultures, including the treatment of skin cancer and wart.



R = Me; Etoposide (**13**)





Camptothecin (**15**) $R_1 = R_2 = R_3 = H$;

Toptican (**16**) R_1 = OH, $R_2 = CH_2N(CH_3)_2$, $R_3 = H$;

Fig: 1.12Camptothecin&Toptican

Camptothectin (**15**) was isolated from the Chinese ornamental tree *Camptothecaacuminata* by Wani and Wall. It was advanced to clinical trial by NCI in the 1970s but was dropped because of severe bladder toxicity. But toptican (**16**) is a modified camptothecin was approved for use in the USA in 1996. The discovery of quinine (**17**) was done from *Cinchona* bark, which is, used in the treatment of malaria by French scientist Caventon and Pelletier.

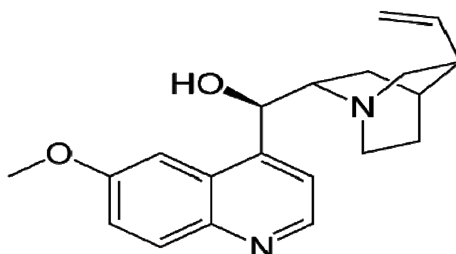


Fig: 1.13 Quinine (**17**)

Uses of folk or traditional medicine represent the way of shortcut discovery of modern medicine. An inventory of medicinal plants compiled by WHO on the basis of literature from 91 countries including the classical text on Ayurvedic and Unani medicine list 21000 species of "medicinal plants". According to WHO, around 80% of the world's 5.76 billion populations in the developing world rely on herbal remedies for their basic health care. The use of medicinal plants as a source for relief from illness can be traced back over five millennia from written documents of the early civilizations in China, India and near east but it is doubtless an art as old as mankind. Even today, plants are the almost exclusive source of drugs for the majority of the world's population. In industrialized countries, medicinal plant research has had its ups and downs during the last decades.

Plants will continue to be extremely important as source of new drugs as evidenced by recent approvals in the United States of several new plant derived drugs based on the secondary metabolites of plants. For example, in the treatment of refractory ovarian cancer, new drug has recently been approved in the United States from taxol, an anti cancertaxanediterpenoid derived from the relativity scarce pacific western Yew tree, *Taxusbrevifolia* Nutt. A relatively new semi-synthetic antineoplastic agent based on podophylotoxin is etoposide a constituent of the Mayapple *Podophyllum petatum*, which is useful in the chemotherapeutic treatment of refractory

testicular carcinomas, small cell lung carcinomas, non-Hodgkin's lymphoma and non-lymphocytic leukemia.

The list of modern medicine derived from medicinal plants is very long now. Some of them are is as follows

Table 1.1: Important drugs/chemicals from plant source and their actions/chemical uses.

Drug/Chemical	**Action/Clinical use**	**Plant source**
Betulinic acid	Anticancerous	*Betula alba*
Camptothecin	Anticancerous	*Camptotheca acuminata*
Chymopapain	Proteolytic, mucolytic	*Carica papaya*
Cissampeline	Skeletal muscle relaxant	*Cissampelo spareira*
Colchiceine amide	Antitumor agent	*Colchicum autumnale*
Colchicine	Antitumor agent, anti-gout	*Colchicum autumnale*
Cynarin	Choleretic	*Cynarascolymus*
Danthron	Laxative	*Cassia species*
L-Dopa	Anti-parkinsonism	*Mucunasp*
Etoposide	Antitumor agent	*Podophyllum peltatum*
Glaucarubin	Amoebicide	*Simarouba glauca*
Glycyrrhizin	Sweetener, Addison's disease	*Glycyrrhiza glabra*
Hesperidin	Capillary fragility	*Citrus species*

Drug/Chemical	**Action/Clinical use**	**Plant source**
Irinotecan	Anticancer, antitumor agent	*Camptotheca acuminata*
Lapachol	Anticancer, antitumor	*Tabebuia sp.*
Menthol	Rubefacient	*Mentha species*
Pilocarpine	Parasympathomimetic	*Pilocarpus jaborandi*
Papain	Proteolytic, mucolytic	*Carica papaya*
Quinidine	Antiarrhythmic	*Cinchona ledgeriana*
Quinine	Antimalarial, antipyretic	*Cinchona ledgeriana*
Rutin	Capillary fragility	*Citrus species*
Sennosides A, B	Laxative	*Cassia species*
Stevioside	Sweetner	*Stevia rebaudiana*
Taxol	Antitumor agent	*Taxusbr evifolia*
Teniposide	Antitumor agent	*Podophyllum peltatum*
a Tetrahydrocannabinol (THC)	Antiemetic, decrease occular tension	*Cannabis sativa*
Theobromine	Diuretic, vasodilator	*Theobroma cacao*
Topotecan	Antitumor, anticancer agent	*Camptotheca acuminata*
Vincristine	Antitumor, Antileukemic agent	*Catharanthus roseus*

1.3 Status of medicinal plants in Bangladesh

The number of medicinal herbs included in the *Asparagus rosemosus*of traditional medicine in this subcontinent at present stands at about 2000. About 450 to 500 of such medicinal herbs have so far been enlisted as growing or available in Bangladesh. Almost 80% of rural population is dependent on medicinal plants for their primary health care. Herbal medicine is widely used in Bangladesh in traditional healthcare system such as Ayurvedic, Unani, Hekimi and other form of folk treatments.

Picture 1: The roots of *Asparagus rosemosus* (Shotomulli)

Picture 2: the *Asparagus rosemosus*(Shotomulli)

Picture 3: dry roots of *Asparagus rosemosus*

Picture 4: close look of the roots of Shotomulli

1.4 Description of *Asparagus racemosus* (Satavar, Shatavari, or Shatamulli).

Shatavari means “who possesses a hundred husbands or acceptable to many”. It is considered both a general tonic and a female reproductive tonic. Shatavari may be translated as “100 spouses”, implying its ability to increase fertility and vitality. This amazing herb is known as the “Queen of herbs”, because it promotes love and devotion. Shatavari is the main Plant medicine rejuvenate tonic for the female, as is with anima for the male. Asparagus racemosus (family Asparagaceae) also Known by the name Shatavari is one of the well-known drugs in Ayurveda, effective in treating madhurrasam, madhurvipakam, seet-veeryam, somrogam, chronic fever and internal heat [1,2]. This herb is highly effective in problems related with female reproductive system. Charak Samhita written by Charak and Ashtang Hridyam written by Vagbhata, the two main texts on Ayurvedic medicines, list Asparagus racemosus (A. racemosus) as part of the formulas to treat women’s health disorder [3-6]. A. racemosus is a well-known Ayurvedicrasayana which prevent ageing, increase longevity, impart immunity, and improve mental function, vigor and addvitality to the body and it is also used in nervous disorders, dyspepsia, tumors, inflammation, neuropathy, hepatopathy. Reports indicate that the pharmacological activities of A. racemosus root.

Extract include antiulcer, antioxidant, and antidiarrhoeal, antidiabetic and immunomodulatory activities. A study of ancient classical Ayurvedic literature claimed several therapeutic attributes for the root of A. racemosus and has been specially recommended in cases of threatened abortion and as a galactogogue. Root of A. racemosus has been referred as bitter-sweet, emollient, cooling, nervinetonic,constipating, galactogogue, and aphrodisiac, diuretic, rejuvenating, carminative, stomachic, antiseptic and as tonic. Beneficial effects of the root of A. recemosus are suggested in nervous disorders, dyspepsia, diarrhoea, dysentry, tumors, inflammations, hyper dipsia, neuropathy,hepatopathy, cough, bronchitis, hyperacidity and certain infectious diseases [7,8]. The major active constituents of A. racemosus are steroidal saponins (Shatavarins I-IV) that are present in the roots. Shatavarin IV has been reported to display significant activity as an inhibitor of core Golgi enzymes transferase in cell free assays and recently to exhibit immuno-modulation activity against specific T-dependent antigens in immuno compromised animals [9].

1.5.Scientific classification

Kingdom:	Plantae
Division:	Angiosperms
Class:	Monocots
Order:	Asparagales
Family:	Asparagaceae
Sub-Family:	Asparagoideae
Genus:	Asparagus
Species:	Asparagus rosemosus.
Synonym:	Satavar, shatavari, Shatamulli.

1.6 Characteristics of A. racemosus

A. racemosus is a woody climber growing to 1-2 m in height. The leaves are like pine needles, small and uniform and flowers are white and have small spikes. This plant belongs to the genus Asparagus which has recently moved from the sub family Asparagae in the family Liliaceae to a newly created family Asparagaceae.

1.7 Habitat

Its habitat is common at low altitudes in shade and in tropical climates throughout Asia, Australia and Africa. Out of several species of Asparagus grown in India, *Asparagus rosemosus* is most commonly used in indigenous medicine [14].

1.8 Phytochemicals

Shatvari is known to possess a wide range of photochemical constituents which are mentioned below.

a) Steroidal saponins, known as shatvarins. Shatvarin I to VI are present. Shatvarin I is the major glycoside with 3-glucose and rhamnose moieties attached to sarsapogenin [15-18];

b) Oligospirostanoside referred to as Immunoside [19];

c) Polycyclic alkaloid-Aspargamine A, a cage type pyrrolizidinealkaloid [20-22];

d) Isoflavones-8-methoxy-5, 6, 4-trihydroxy isoflavone-7-0-beta-D-glucopyranoside [23];

e) Cyclic hydrocarbon-racemosol, dihydrophenantherene [24, 25];

f) Furan compound-Racemofuran [26];

g) Carbohydrates-Polysacharides, mucilage [27];

h) Flavanoids-Glycosides of quercitin, rutin and hyperoside are present in flower and fruits [28];

1.9. Pharmacological activity

1.9.1. Galactogogue effect

The root extract of A. rosemosus is prescribed in Ayurveda to increases milk secretion during laction [36]. A. rosemosus is combination with other herbal substance in the form of Ricalax tablet (Aphali pharmaceutical Ltd. Ahmednagar) has been shown to increase milk production in females complaining of dificient milk secretion [37]. Gradual decrease in milk secretion, on withdraw of the drug suggested that the increase in milk secretion was due to drug theraphy only and not due to any psychological effect. In the form of a commercial preparation, lactare (TTK Pharma, Chennai) is reported to enhance milk output in women complaining of scantly breast milk, on the 5th day after delivery [38].A significant increase in milk yield has also increase growth of mammary glands, alveolar tissue and acini in guinea pigs [39]. Patel et al. have shown galactogogue effect of A. racemosus in buffaloes [40]. However, Sharma et al. did not observed any increase in prolectin level in females complaining of secondary lactational failure with A.racemosus, suggesting that it has no lactogenic effect [41]. In other study, the aqueous fraction of the alcoholic extract of the roots at 250 mg/kg, administratered intramuscularly, was shown to cause both on increase in the weight of mammary gland lobuloaveolar tissue and in the milk yield of oestrogen primed rats. The activity has attributed to the action of released corticoteroids or an increase in prolactin [42].

1.9.2. Anti-secretory and anti-ulcer activity

Efficacy of A. rosemosus was evaluated in 32 patients by administrating the root powder 12 g/d in four doses, for an average duration of 6 weeks. Shatavari was found to relive most of the symptoms in majority of the patients. The ulcer healing effect of the drug was attributed to a direct to a direct healing effect, possible by potentiating intrinsic protective factor as it has neither anti-secretory activity nor antacid properties, by strengthening mucosal resistance, prolonging the lifespan of mucosal cells, increasing secretion and viscosity of mucous and reducing H^+ ion back diffusion. It has been found to maintain the continuity and thickness of aspirin treated gastric mucosa with a significant increase in mucosal main. As A. rosemosus

heals duodenal ulcers without inhibiting acid secretion, it may have cytoprotective action similar action to that of prostaglandin other binding of bile salts [43-45].

1.9.3 Anti-tussive effect

Methanolic extract of roots, at dose of 200 and 400 mg/kg, showed significant antitussive activity on sulphur dioxide- induce cough in mice. The cough inhibition of 40% and 58.5%, respectively, was comparable to that of 10-20 mg/kg of codeine phosphate, where the inhibition observed 36% and 55.4%, respectively [46].

1.9.4. Adaptogenic activity

Aqueous extract was administered orally to experimental animals of biological, physical and chemical stressors. A model of cisplatin induced alteration in gastrointestinal motility was used to test the ability of extract to exert a normalising effect, irrespective or direction of pathological change. The extract reversed the effects of cisplatin on gastric emptying and also normalized cisplatin-induced intestinal hyper motility [47].

1.10 Aim of the project

Bangladesh is a good repository of medicinal plants belonging to various families, including **Asparagaceae**. The **Asparagaceae** plants contain wide range of chemical and unique pharmacologically active compounds.

Though a large number of **Asparagus** species have been investigated but locally a little attention was given to this particular species. Therefore, an attempt has been taken to study the chemical constituents and biological activities of *Asparagus rosemosus*.These investigations may provide some interesting compounds, which may be pharmacologically active. If significant results are obtained, these can be used as remedies for the treatment of some diseases. Since this plant is available in Bangladesh and a lot of herbal health centers & herbal industries are using such related herbal plants for treatments, so if the constituents of this plant can be studied thoroughly, this may be a cost- effective treatment. So, the objective is to explore the possibility of developing new drug candidates from this plant for the treatment of various diseases.

1.11 Present study protocol

The present study was designed to isolate pure compounds as well as to observe biological activities of the isolated pure compounds with crude extract and their different fractions. The study protocol consisted of the following steps:

- Cold extraction of the powdered roots of the plant with ethanol.
- Partition of the crude methanol extract by n-hexane, dichloromethane, ethyl acetate and 1-butanol successively.
- Fractionation of each partitioned extract by vacuum liquid chromatography (VLC)/Column chromatography (CC).
- Isolation and purification of the pure compounds from different column fractions by various chromatographic techniques.
- Determination of the structure of the isolated compounds with the help of spectroscopic methods.
- Observation of *in vitro* antimicrobial activity of crude extracts, column fractions and pure compounds.