

**ISOLATION AND CHARACTERISATION OF
SOME COMPOUNDS FROM
*CENTELLA ASIATICA***

GIFT

**A
DESSERTATION
IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR
THE DEGREE OF MASTER OF PHILOSOPHY**

**SUBMITTED BY
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EXAMINATION ROLL NO.1
REGISTRATION NO. MPhil. 47
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March , 1997

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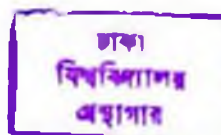
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**Isolation and characterisation of some
compounds from *Centella asiatica***

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**DEDICATED
TO
MY CHILDREN**

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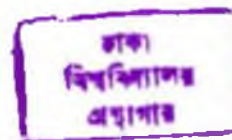
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CHAPTER ONE

INTRODUCTION

INTRODUCTION

1.1 GENERAL INTRODUCTION

Traditional system of medicine still provide therapeutic remedies sought by majority of the sick people in the developing countries. To a great extent mankind is dependent on the plant kingdom for their existence. Plants supply us food, shelter, raw materials for clothings and provide us with medicine. Plant produce within their cells, through metabolic activities, not only food materials but also certain other substances such as glycosides, alkaloids, resins, essential oils, tannins, colouring materials and a host of other chemicals. Some of them are harmful for animal life and some have found tremendous therapeutic uses as successful remedies for many diseases.

The remedies of the traditional systems are popular in Bangladesh and a large percentage of its population use these traditional medicine with great faith. The main reason for such popularity of traditional medicine is because they are cheap and readily available specially in the Indian subcontinent. Besides, they have no noticeable side and toxic effects and suitable alternatives are not available in modern medicine. Treatment with modern medicine is getting more expensive and the traditional materia medica is a storehouse of valuable experience acquired over the ages by hundreds and thousands of practitioners. Traditional system of treatment is based on empirical observations. The most puzzling thing in the study of a traditional medicine is that the drug is always a complicated mixture of a number of compounds, the individual compounds having diversified chemical structures, consequently possessing different physical, chemical and biological

properties. Therefore, any single isolated constituent may not exhibit the physiological activity which is exhibited by the parent drug. The isolation of a truly active principle from parent plant drug is very much of a challenging job.

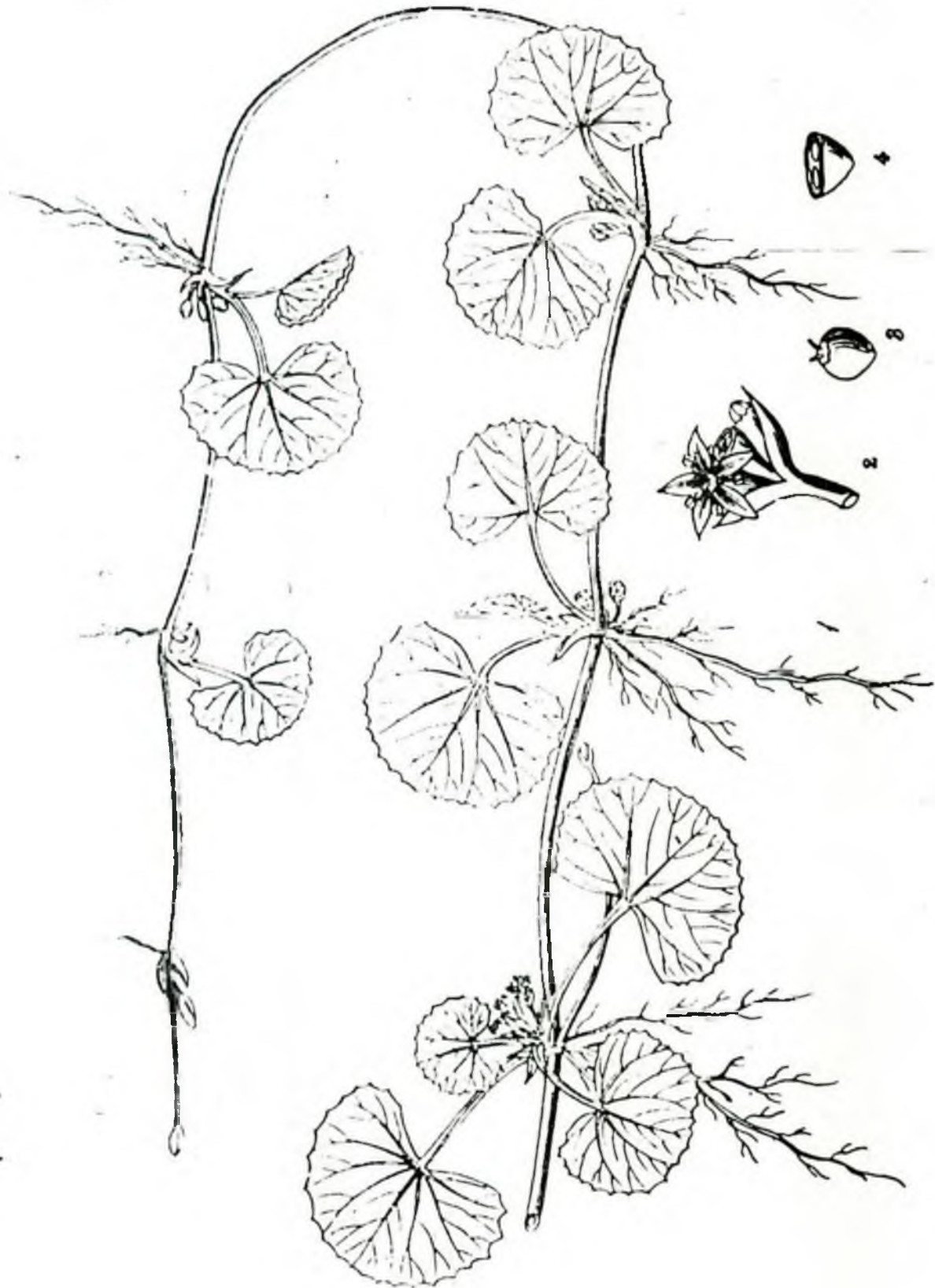
The medicinal plants of Bangladesh are distributed over a vast area of the country in the jungles, hillocks and gardens. Hakims, Vaidays and Kavirajs have been using many of these plants as successful medicines of various diseases since the ancient time.

1.2 GENERAL DESCRIPTION OF *CENTELLA ASIATICA*

(Synonym: *HYDROCOTYLE ASIATICA*)

Centella asiatica belongs to the family Umbelliferae. The Hindi name is Brahmamanduki, the English name is Indian Pennywort and the Bangla name is Thankuni. It grows extensively in tropical countries like India, Bangladesh, China, Philippines, Brazil and South Africa.

Centella asiatica is a perennial herb growing in moist places. Stem long, prostrate and creeping biliform, redish and with long internodes, rooting at the node. Leaves, 1.3 to 6.3 cm diameter, generated from each node of the stems having elongated petioles, orbicular reniform, broader, more or less cupped. It is glabrous on both sides and with numerous slender nerves from a deeply cordate base. The petioles are very variable in length, 7.5 to 15 cm or more. The stipule are short, adnate to the petioles. Flowers are very small and pink in colour. Peduncles are glabrous, short, pink. Petals are minute, pink, ovate, acute and concave. Fruits 4 mm long, longer than broad, ovoid, hard with thick end pericarp, often crowned by the persistent petals. Flowers and fruits grow in the spring.



HYDROCOTYLE ASIATICA, LINN.

1.3 MEDICINAL USES OF *CENTELLA ASIATICA*

In Yunani and Ayurveda practice, different parts of *Centella asiatica* have been used for the treatment of various diseases. The plant is considered as a useful alternative and tonic in the diseases of the skin, nerve and blood. In some parts of India the people are in the habit of taking the powdered dried leaves with milk for improving their memory. The leaves are said to be useful in syphilitic skin diseases, both externally and internally and on the Malabara Coast, the plant is one of the remedies for leprosy. Dr. A Hunter¹, after trying it in the Madras Leper Hospital, came to the conclusion that it had no claim for consideration as a specific drug in leprosy, but found it most useful in ameliorating the symptoms and improving the general health. In Mumbai¹, it is a popular remedy for the slight dysentric derangements. In the Konkan, one or two leaves are given every morning to cure stuttering and the juice is applied to skin eruptions supposed to arise from heat of blood. In Java, the leaves are taken as tonic and blood purifier and also for indigestion, nervousness and dysentery. In Indo China¹ the plant is considered diuretic. It is used internally as an alternative tonic, and externally as an stimulant.

According to the Ayurveda and Yunans this plant is used as diuretic , alesciteric and antipyretic. It improves appetite, clears the voice and the brain, is sedative to the nerves and it cures leucoderma, anemia, urinary discharges, diseases of the blood, bronchities, inflammations, asthma, smallpox and headache.

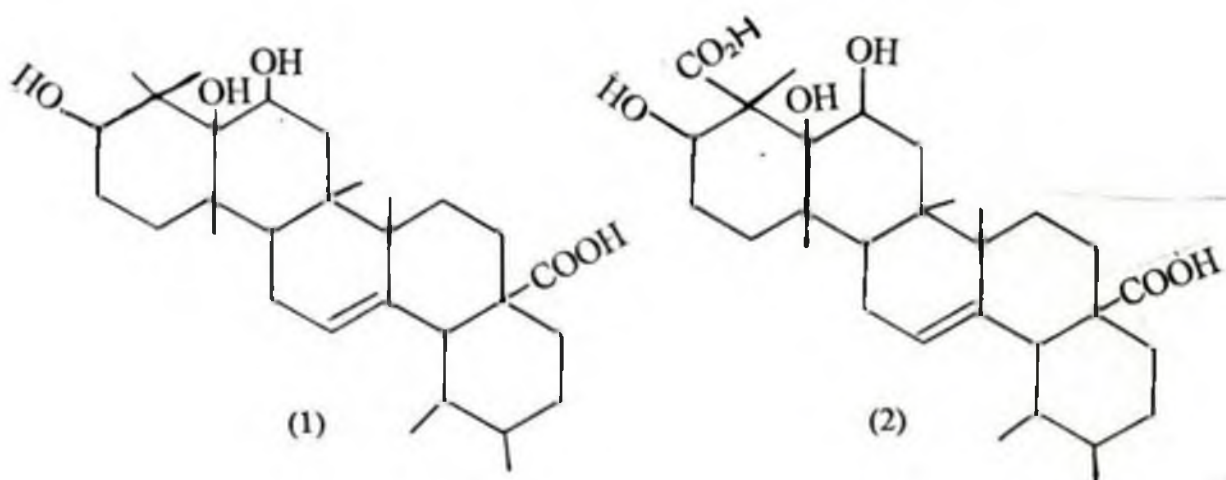
1.4 CHEMICAL INVESTIGATION OF *CENTELLA ASIATICA*

Considering the importance of *Centella asiatica* plant, a lot of work has been done on it for testing biological activities as well as for isolation of chemical compounds from it. Wali and Tumun Katti

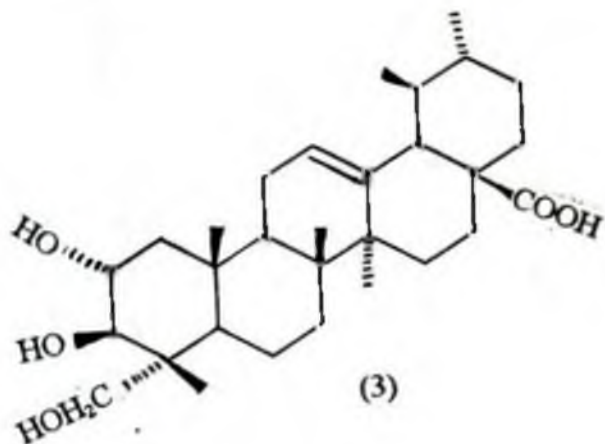
reported² that the alcohol extract of the plant was found to contain a green essential oil containing oleic, linoleic, linolenic, palmitic, stearic and lignoceric acids. In 1956 Bhattacharyya reported³ that *Centella asiatica* contains salts, sugars, essential oils, pectin and a mixture of triterpenic acids. Mathotra and co-workers extracted⁴ dried powder of *Centella asiatica* with various solvents. They detected alkaloids, glycosides, sterols, tannins, sugars and inorganic salts in the extract. Aspartic acid, glycine, glutamic acid, α -alanine and phenylalanine were also identified. The saponifiable portion of the pet-ether extract gave palmitic and stearic acids and the unsaponifiable portion, a phytosterol, identified as β -sitosterol. Glucose, KCl, and K_2SO_4 were separated from the aqueous extract and the total ash contained Cl, SO_4 , PO_4 , Fe, Ca, Mg, Na and K ions. Suryaprakasa et. al. examined⁵ *Centella asiatica* from 7 locations for their chemical contents. Alcohol and water extract of *Centella asiatica* were successively extracted with ligroine, Et_2O , EtOAc and BuOH. The ligroine extract contained wax, carotenoids and chlorophyll. The Et_2O extract contained saponins, chlorophyll, kaempferol, quercetin and flavonoids. The EtOAc extract contained kaempferol, aglycones, glucose and rhamnose. The BuOH extract contained saponins, madecassoside, asiaticoside, brahmoside, brahminoside and madecassate. George and Gnanarethnam observed⁶ that glutamate, serine and alanine were present in the leaf, petiole and stolon of *Centella asiatica*. Aspartate, glutamate, serine, threonine, alanine, lysine, histidine and aminobutyrate were present in roots especially in greater quantities. Begum and Ahmed reported⁷ the digestive protein value of *Centella asiatica* as 5.7%. Chemical investigation on *Centella asiatica* was done by Castellani⁸ et. al. They observed that there were many compounds including amino acids, flavonols, fatty acids, alkaloids, sterols, sugar-triterpenes. Special attention was given to the triterpenes, asiaticoside, asiatic acid and madecassic acid which

were principal pharmacologically active components. Castillo et. al. reported⁹ the phytochemical study of *Centella asiatica*. Dried plant was subjected to successive extraction in a Soxhlet apparatus with solvents of different polarity (pet-ether, Et₂O, CHCl₃, MeOH, and BuOH). The extracts of the reflux treatment yielded in the BuOH phase a triterpenoid saponin, and the same was found in the Et₂O-soluble fraction. Preliminary tests on crude extracts showed the presence of saponins, terpenoids, tannins and polyphenols. In 1982 Asakawa et. al. observed¹⁰ the sesquiterpenoid constituent of *Centella asiatica*. It has been reported⁵⁷ that *Centella asiatica* contains β -carotene.

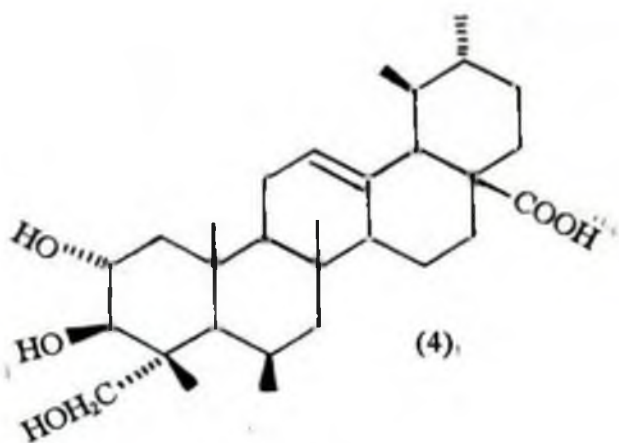
In 1956 Bhattacharyya isolated^{11,12} centic acid (1), decm-250-5°C, $\alpha_D^{18} + 38.1^\circ$ (EtOH) and centillic acid, decm-256-61°C, $\alpha_D^{19} + 43.51^\circ$ (EtOH) from alcohol and water extract, respectively. From the residue of the water extract, another compound was isolated which melted at 234.5°C, $\alpha_D^{19} + 35.72^\circ$ (EtOH). Bhattacharyya isolated¹¹ centoic acid and he gave the structure of centic acid C₃₀H₄₈O₃ (1), centoic acid C₃₀H₄₈O₆ (2), and centellic acid.



Centellic acid^{12,11} is believed to be a structured isomer of centic acid differing in the configurations of glycol linkage. Bhattacharyya also isolated³ triterpinic acid, indocentoic acid and a glycoside indocentelloside from *Centella asiatica*. Boiteau and et. al. isolated¹³ asiatic acid 2,3,23-trihydroxy-12-13-ursene-28-oic acid (3). The hot alcoholic extract⁴ of *Centella asiatica* after treatment with pet-ether, Et₂O and CHCl₃ yielded a glycoside fraction which on hydrolysis gave an aglycon corresponding to asiatic acid. Asiatic acid¹³ decreased wound area of skin.

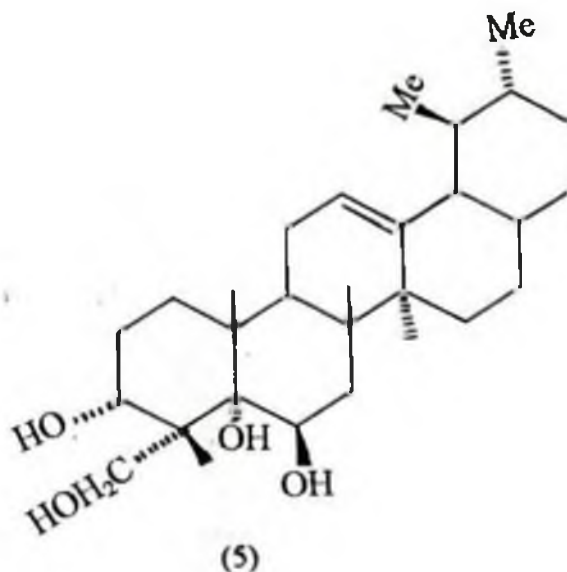


Rastogi et. al. studied¹⁶ *Centella asiatica* leaves. They reported two triterpene acids, brahmic acid C₃₀H₄₈O₆, m.p. 293°C, α_D^{20} 24 (pyridine), and isobrahmic acid m.p. 263°C and two saponins, brahmoside, m.p. 242°C and brahminoside m.p. 223°C, from the alcohol extract of air-dried plant powder of *Centella asiatica*. In 1968 the structure of brahmic acid was elucidated¹⁷ to be 2- α , 3- β , 6- β , 23-tetrahydroxy-urs-12-en-28-oic acid(4) from physical and chemical data.

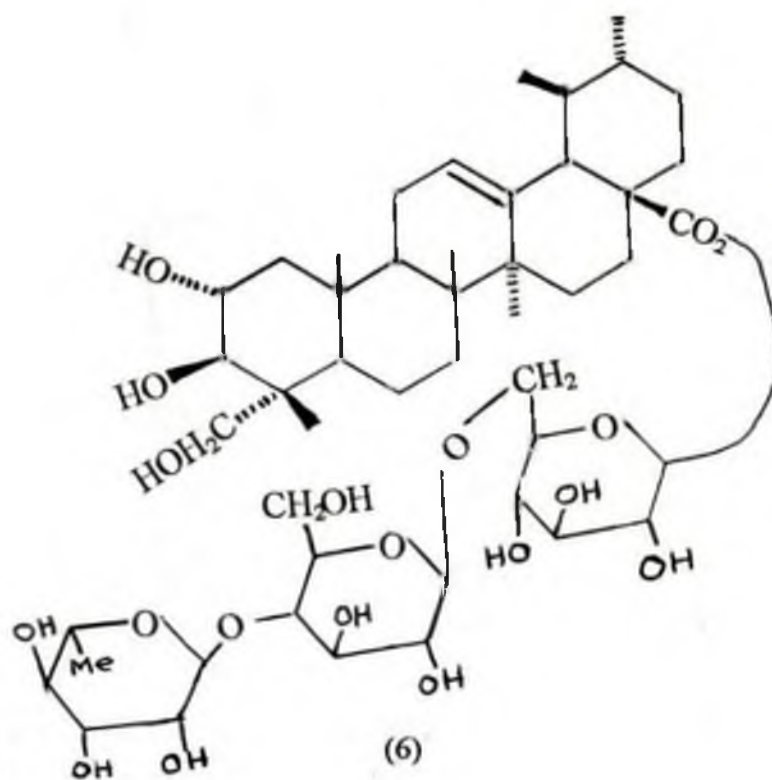


In 1963 Rastogi and Dhar chromatographically separated¹⁸ two saponins with different H₂O-EtOH mixture as eluents and identified brahmoside C₄₇H₇₈O₁₉, m.p. 242-6°C, $\alpha_D^{32} -38^\circ$ (C₅H₅N) and brahminoside, C₅₃H₈₈O₂₄, m.p. 221-3°C, $\alpha_D^{32} -19^\circ$, (C₅H₅N).

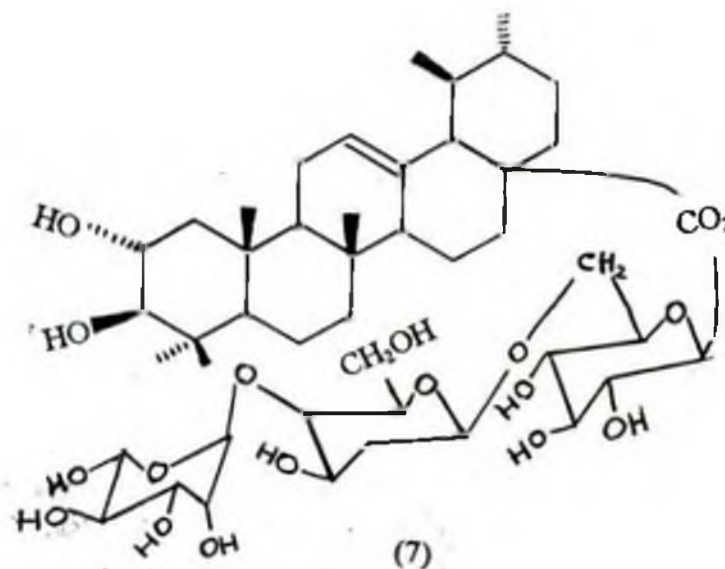
Dutta and Basu isolated¹⁹ a new type of triterpenoid glycoside, thakuniside, C₃₀H₄₇O₈, m.p. 239-43°C, $\alpha_D^{32} -18^\circ$, (70% EtOH), and a triterpene acid thankunic acid, C₃₀H₄₈O₈, m.p. 314-18°C, $\alpha_D^{32} +23.83^\circ$, (EtOH). In 1967 Tapan and Basu²⁰ identified asiatic acid from *Centella asiatica*. Tapan and Basu observed²¹ that the crude extract of *Centella asiatica* and products from it, namely isothankuniside and another compound (Me 5-hydroxy-3,6-diketo-23 or 24 norurs-12-en-28-oate), were active as oral antifertility agents in albino mice. Isothankuniside²² contained glucose and rhamnose linkage. Isothankuniside on hydrolysis afforded a new triterpene acid aglycone, isothankunic acid C₃₀H₄₈O₈ (5) and its configuration was established as 3- α ,5- α ,6- β , 24-tetrahydroxy-urs-12-en-28-oic acid belonging to the α -amyrin series.



Bozena et al isolated²⁶ asiaticoside from *Centella asiatica* in the following way-clean raw benzene insoluble plant material was refluxed with EtOH and the EtOH extract was concentrated, cooled and extracted with Me₂CO. The Me₂CO soluble material was repeatedly extracted with Et₂O and the extract was treated with Amberlite IR-120. The supernatant solution was decanted and treated with absolute EtOH in excess causing the raw asiaticoside fraction settling out. Rahandrah et. al. worked²⁷ on asiaticoside. They prepared an ester named anthrone asiaticoside, m.p. 253°C, $\alpha_D - 11.5^\circ$, (MeOH) by the treatment of anthrone reagent in concentrated H₂SO₄. Hydrolysis of asiaticoside with 20% EtOH yielded asiatic acid. Urban and Chin isolated²⁸ asiaticoside (6) from *Centella asiatica*, a herbal medicine used to promote wound healing, which as a crystalline compound was identified by mass and NMR spectroscopy.

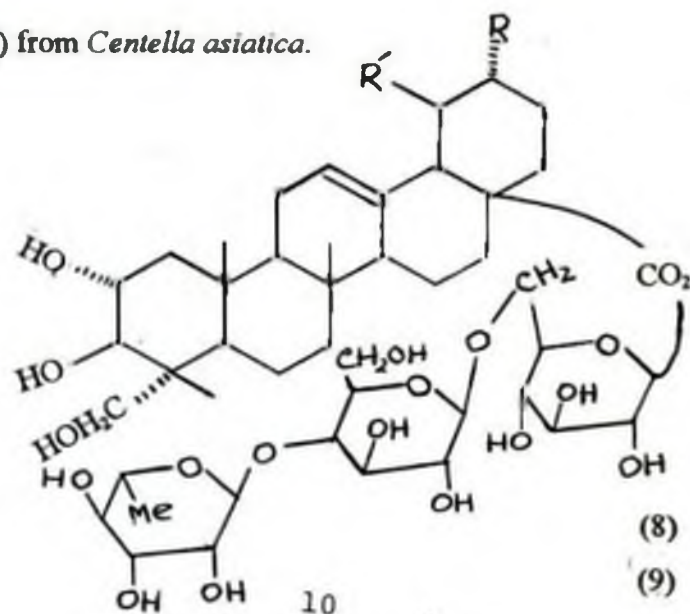


Mahato Niranjana determined²⁹ the molecular geometry of asiaticoside (7) by single crystal x-ray analysis.



Zhengmu and Yuenian reported³³ the asiaticoside as an active constituent in *Centella asiatica*. It was isolated by using double developed TLC plates on silica gel G, CM-cellulose on Na plates with $\text{CHCl}_3 : \text{MeOH} : \text{H}_2\text{O}$ (7 : 3 : 0.5). The spots were visualized with 5% H_2SO_4 -EtOH spray and were scanned by dual wavelength TLC scanner.

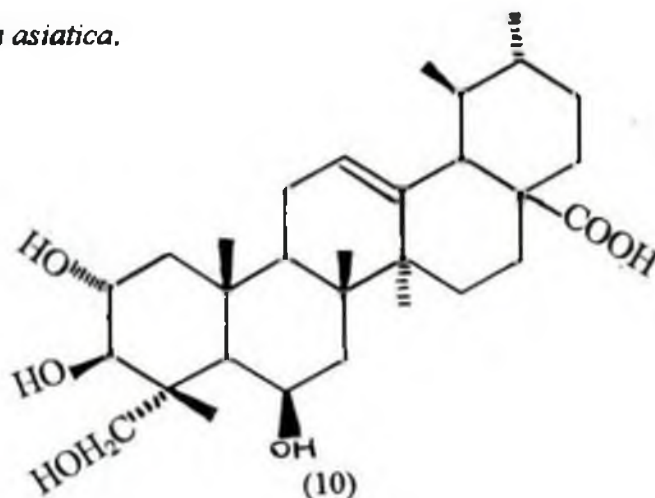
Niranjana and Roy isolated³⁴ the two new triterpenoid trisaccharides asiaticoside A(8) and asiaticoside B(9) from *Centella asiatica*.



(8) $\text{R} = \text{H}, \text{R}' = \text{Me}$

(9) $\text{R} = \text{Me}, \text{R}' = \text{H}$

In 1987 Madagascar et. al. isolated³¹ madecassic acid (10) m.p. 265-8°C, and anhydromadacassic acid from *Centella asiatica*.



In China Siqi and Hunifang separated³² two compounds, asiaticoside and madecassoside, from *Centella asiatica*. Tsurumi et. al. isolated³⁰ madecassol, an ester from *Centella asiatica*. Bhagirath and Rastogi isolated³³ asiatic acid, asiaticoside, meso-inositol and a new oligosaccharide centellose. The periodate oxidation product of asiatic acid was reinvestigated and its structure was assigned as a hemiacetal derivative.

Schulte et. al. isolated³⁵ some polyacetylenes from *Centella asiatica* by extraction with Et₂O-ligroin. They reported the isolation of the following (1-5) compounds and nine other acetylenes of which partial structure were elucidated.

- (1) $\text{Me}(\text{CH}_2)_4\text{CH}=\text{CHCH}_2-\text{C}\equiv\text{C}-\text{C}\equiv\text{C}-\text{CH}=\text{CHCH}_2\text{OAC}$
- (2) $\text{Me}(\text{CH}_2)_4\text{CH}=\text{CHCH}(\text{OAC})-\text{C}\equiv\text{C}-\text{C}\equiv\text{C}-\text{CH}(\text{OAC})\text{CH}=\text{CH}_2$
- (3) $\text{Me}(\text{CH}_2)_4\text{CH}=\text{CHCH}(\text{OAC})-\text{C}\equiv\text{C}-\text{C}\equiv\text{C}-\text{CH}(\text{OH})\text{CH}=\text{CH}_2$
- (4) $\text{Me}(\text{CH}_2)_4\text{CH}(\text{OAC})\text{CH}=\text{CH}-\text{C}\equiv\text{C}-\text{C}\equiv\text{C}-\text{CH}(\text{OH})\text{CH}=\text{CH}_2$
- (5) $\text{Me}(\text{CH}_2)_4\text{CH}(\text{OH})\text{CH}=\text{CH}-\text{C}\equiv\text{C}-\text{C}\equiv\text{C}-\text{CH}(\text{OH})\text{CH}=\text{CH}_2$

The structure of a polyacetylenic compound was elucidated³⁶ by Bohlmann and co-workers as falcarinol derivatives as shown below



Prum et. al. isolated³⁷ two flavonoid glycosides, 3-glucosylquercetin and 3-glycosylkaempferol from the leaves of *Centella asiatica*. Bozena and co-workers isolated²⁶ 1-asiaticate of O - α - 1 - rhamnopyranosyl - (1 $\rightarrow$ 4) - O- β -D - glucopyranosyl - (1 $\rightarrow$ 6) -O- β -D - glucopyranose from *Centella asiatica*. A pentacyclic triterpenic acid was isolated¹⁴ from the water insoluble part of the methanol extract of *Centella asiatica*

1.5 BIOLOGICAL ACTIVITIES OF *CENTELLA ASIATICA*

Boiteau and Ratsimamanga isolated¹³ asiatic acid and its glycoside asiaticoside. The asiaticoside was biologically active, it had therapeutic uses in the healing of refractory wounds, leprosy, rapid thickening of the skin and tuberculosis. They also worked²³ on asiaticoside and related compounds and observed highly vascularized mesodermic buds and favour the formation of scar tissue in the animals. It has been reported²⁴ that a cream was made containing 0.25-1.0% alcoholic extract of *Centella asiatica* which contained asiaticoside and asiatic acid. A cream contained 10-30% of alcoholic extract of *Centella asiatica* embryonated skin, brain and liver. It was claimed that it also maintained and regenerated the skin of old people. It has been reported²⁵ that asiaticoside have antileprosy properties.

Alcoholic extract³⁸ of *Centella asiatica* containing asiatic acid and its ester having mitotic activity was useful in dermatology for the treatment of pruritis and other skin disorders. It has been reported³⁰ that medecassol and its derivatives madecassic acid, asiatic acid and asiaticoside applied locally on wounds in rat promoted the proliferation of granulation and increased the tensile strength. They decreased wound area of skin necrosis induced by burn. All these 4 compounds were equally effective but had no significant therapeutic effect when administered orally. Viala et. al. observed³⁹ good percutaneous absorption of tritiated madecassic acid (I) and tritiated asiatic acid (II) in rat and mice when applied in the excipient (1% solution). In rats, plasma I and II levels increased with time for first 24 h while the plasma I and II levels of tissue and adjacent abdominal muscle were maximal by 1 and 3 h, respectively and decreased slowly thereafter. In mice, 2.1 and 2.2% of plasma I & II were observed by 3 h from the continuous oil phase and oil water emulsion,

respectively. In comparison, the radioactivity after oral administration was greatest in liver, kidney and paw muscles. It has been reported²⁶ that 1-asiaticate of O- α -L-rhamnopyranosyl-(1 $\rightarrow$ 4)-O- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-O- β -D glucopyranose, had therapeutic use in the treatment of various kinds of ulceration, extensive wounds, eczemas, tuberculous changes of the skin and alopecia areata. The pharmacological studies⁴⁰ on *Centella asiatica* in mice and rats were made by Ramaswamy et. al. It was indicated that the saponins exhibit a sedative action which has been compared to that of chlorpromazine and meprobamate. The mode of action appears to be mainly on cholinergic mechanism in the central nervous system. Poizot and Dumez observed⁴¹ the action of tritiated extract of *Centella asiatica* during of healing. In rats with iterative wounds, 2-phases of healing were observed, an initial lengthening followed by a contraction of the wound. Administration of *Centella asiatica* triterpenoid extract shortened the lengthening phase while having little effect on the contracting phase, and promoted epithelialization and a very early decrease in the wound surface area. Vecchio et. al. worked⁴² on biosynthetic activity of *Centella asiatica*. They observed that the addition of total triterpene fraction of *Centella asiatica* (TTFCA) to the culture medium of human embryo fibroblasts did not modify the growth rate at ≤ 25 μ g/ml but inhibited growth at 50 μ g/ml. TTFCA having ability to promote wound healing and stimulation of the incorporation of proline content suggested specific stimulation of collagen formation. A high incorporation of acetate into lipids and glycosaminoglycans (GAGS) were also observed. GAGS showed a stimulation of the synthesis of hyaluronic acid and chondroitin sulfate and to a lesser extent of heparan sulfate. Kailasapathy and Koneshan observed⁴³ that the loss of ascorbate was reduced when leafy vegetable of *Centella asiatica* were wilted under refrigerated condition compared with that under environmental temperatures. It has been reported⁴⁹ that Hafnia extract (glycoproteins) as well as oleyl acetate, *Centella asiatica* glycol extract, primrose oil and amino

acids are given for skin preparations. The Hafnia extract stimulates cell growth and has an anti-irritant activity. Cristoni and Magistretti observed⁴⁴ that *Centella asiatica* anthocyanosides administered orally to rats exerted a significant preventive and curative and antiulcer activity without affecting gastric secretion. An action of *Centella asiatica* anthocyanosides on metabolism of mucopolysaccharides may be the basis not only of their gastroprotective activity but also of their known healing and vasoprotective activities.

Azad and Jahirullah worked⁴⁵ on biochemical activity of the alcohol extract and the glycoside of *Centella asiatica*. They reported the alcoholic extract of the glycosidal fraction and the individual glycoside (brahmoside, brahminoside, asiaticoside, glucoside D and glucoside E) of *Centella asiatica* each has an antispasmodic activity on acetylcholine induced contraction of rat ileum. The glycoside E was the most potent antispasmodic compound. The alcoholic extract had no antibacterial activity against common diarrhoea causing organism. The plant did not show any hepato and nephro toxicities in the rats and it may have potentialities as an antispasmodic drug. Frank successfully prepared⁴⁶ cosmetic composition from *Centella asiatica* and it had medicinal application. It has been reported⁴⁷ that a composition containing *Centella asiatica* extract (glycol) was used for skin regeneration and stimulation. This composition contained tissue peptide proteolyzate, fibronectin and tubulin, and was formulated as a cream, paste, gel etc. A cosmetic composition⁴⁸ comprised unilamellar liposomes made of phospholipids and phytosterols which incorporated a suspension of organo silicon compound with *Centella asiatica*. Complexes⁵⁸ of saponins with phospholipids was prepared from *Centella asiatica* and it contained cosmetic and pharmaceutical compositions. Complexes of saponins with *Centella asiatica* are highly lipophilic and had improved bioavailability. They are used as active ingredient in pharmaceutical, dermatol

and cosmetic compositions. It has been observed⁵⁰ that the main triterpenoids from *Centella asiatica*, asiaticoside, madecassoside, asiatic acid and madecassic acid reduce granuloma weight in a dose-dependent manner when administered on rat tissue. Thorel prepared⁵¹ skin-rejuvenating cosmetic composition from *Centella asiatica* for application around the eye.

Francoise compared⁵² the biotransformation of benzyloquinoline (metabolites) by non-alkaloid producing plant of *Centella asiatica* and observed no glycosylation of alkaloidal metabolites in the cell culture suspension. It has been reported⁵⁴ that methanolic extract of *Centella asiatica* is used for stimulating hair growth and retarding hair loss and treatment of pruritus. Complexes⁵⁵ of saponins from *Centella asiatica* and terminalia with phospholipids where molar ratio of phospholipid to saponins was 0.5-2, had anti-inflammatory and antiedema effects. Thiocolchicine, a hemisynthetic substrate obtained⁵⁶ from natural colchicine was converted to monoglucosyl derivatives by cell suspension cultures of *Centella asiatica* and it had cytotoxic effect and showed the intercellular localization of glucosides.

1.6 OBJECTIVE OF THE PRESENT WORK

Centella asiatica is being used for a long time for the treatment of various diseases. A good number of compounds have been isolated from this valuable plant and some of them are physiologically very active. Special attention is given to the triterpenes e.g. asiaticoside, asiatic acid and madecassic acid which are principal pharmacologically active compounds. Asiaticoside is used in healing of refractory wounds, leprosy, rapid thickening of the skin and tuberculosis. Asiatic acid has mitotic activity and is useful in dermatology for treatment of pruritis and other skin disorder.

Asiaticate is used in the treatment of various kinds of ulceration, extensive wounds, eczemas and tuberculous changes. Isothankuniside has antifertility activity.

From the discussion so far it is understood that *Centella asiatica* is a very potent medicinal plant. Although a lot of compounds have been isolated from it and some of them showed medicinal value, research on this plant is far from complete. Literature survey indicates that many more compounds are yet to be isolated from this plant which may be pharmalogically active. So, it was decided to make attempts to isolate compounds from *Centella asiatica* which may be novel. Therefore, the main objective of the present work was to isolate pure compounds from various extracts (CHCl₃, EtOAC, BuOH and MeOH) of *Centella asiatica*. To achieve that objective, it was planned to fractionate the various extracts by different modern chromatographic techniques with the ultimate aim of isolating pure compounds which may be characterized by chemical and spectroscopic techniques.

CHAPTER TWO

EXPERIMENTAL

EXPERIMENTAL

2.1 GENERAL METHODS:

2.1.1 SOLVENTS AND REAGENTS:

All solvents and reagents used in the experiments were either of analytical or laboratory reagent grade purchased from E. Merck (Germany), BDH (England) or RDH (Sweden). All the solvents were distilled in glass distillation set before use.

2.1.2 EVAPORATION:

All evaporations were carried out under reduced pressure using rotary evaporator at a bath temperature not exceeding 40°C.

2.1.3 FREEZE-DRYING

Freeze-drying of extracts and fractions were carried out by a HETOSIC 52 model freeze-dryer. Aqueous extracts and fractions were first frozen in round bottomed flasks by means of HETOFRIG methanol freezer at -30° to -35°C and finally the materials were subjected to freeze-drying operation.

2.1.4 THIN LAYER CHROMATOGRAPHY (TLC):

(a) Preparation of plates:

For TLC, both precoated (coating on glass or aluminium sheets) and manually prepared glass plates were used. TLC plates were prepared by spreading a film of an aqueous slurry (gel: water 1:2 w/v) of silica gel G-60 (E. Merck, 7731) over the entire surface of glass plates (16 cm x 16

cmor 12 cm x 12 cm) by means of a spreading machine. The plates were then dried at room temperature and finally at 70°C in an oven for two hours.

(b) Application of samples and development:

Capillary tubes were used for application of samples on the chromatograms. TLC plates were developed by the ascending technique in glass jars or tanks. Solvent systems of different polarity were used.

(c) Location of spots:

Irrigated plates were developed by one of the following methods to detect the position of the spots.

(1) The plates were exposed to iodine vapour for five minutes.

(2) The plates were sprayed with 1% vanillin in concentrated sulphuric acid followed by heating in an oven at 120°C for 10 minutes.

(3) Examination under a UV light source with two different wave lengths (254nm and 350nm).

2.1.5 COLUMN CHROMATOGRAPHY:

For this technique glass column of different size varying from large glass tubes (90 cm X 10 cm, i.d.) fitted with rotaflow to small (30 cm X 1 cm, i.d.) burette were used. For stationary phase column grade silica gel (230-400 mesh, ASTM) was used. In some cases TLC grade silica gel was used as the packing material. Solid phase extraction (SPE) was carried out by using Sep pak C-18 cartridge. For reverse phase column chromatography grade RP-18 silica gel was employed. For flash column chromatography an aquarium air pump (EK 8000) was used to apply pressure. Different fractions were collected in conical flasks (50 ml) manually. Column chromatography

involved slurring of silica gel in the lowest polar solvent followed by packing of the column. Then the sample was introduced on the column bed and solvents with varying polarity (hexane to methanol) were used to elute the sample.

2.1.6 ULTRAVIOLET SPECTROSCOPY (UV):

For ultraviolet spectrum a Shimadzu UV 160A recording spectrometer was used. The sample was dissolved in chloroform and the solution was taken in 1 cm X 1 cm quartz cell for recording the spectrum.

2.1.7 INFRARED SPECTROSCOPY (IR):

A Shimadzu IR-470 spectrometer was used to obtain IR spectrum by making KBr pellets.

2.1.8 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY(NMR):

NMR spectra of different extracts and fractions were recorded by using a JEOL 60 MHz machine. For high resolution ^1H -(500 MHz) and ^{13}C -(101 MHz) NMR spectra, a Bruker spectrometer was used. In all cases tetramethylsilane (TMS) was used as the internal reference.

2.1.9 TEST FOR STEROIDS:

The sample was dissolved in a minimum volume of chloroform and then concentrated sulphuric acid was added to the solution. A characteristic red color indicated the presence of steroids. Finally a greenish color in the chloroform layer, when two drops of acetic anhydride was added, indicated the steroidal nature of the sample.

2.2 COLLECTION OF PLANT MATERIALS:

The leaves of *Centella asiatica* (Thankuni) were collected (about 26 kg) from the adjoining village area of Dhaka, during June-July 1995. The clean leaves were dried in an oven at 40°C and ground to powder by a grinding machine.

2.3 EXTRACTION OF *CENTELLA ASIATICA* LEAVES POWDER WITH DIFFERENT SOLVENTS:

2.3.1 EXTRACTION WITH CHLOROFORM:

The powder of *Centella asiatica* dried leaves (3 kg) was extracted in a large glass tank with chloroform (9 l) for about 12 days by changing chloroform. The chloroform extract (CE) was filtered and evaporated to dryness. The dried extract was evaporated by a membrane pump. In order to remove the residual solvent the extract was further dried in a freeze-dryer. The extract (108 g) was then stored in a reagent bottle at -22°C in a freezer.

2.3.2 EXTRACTION WITH METHANOL:

The chloroform extractive free residue (2 kg) was further extracted with methanol (7 l) at room temperature for 12 days by changing methanol. The extract (ME) was filtered and evaporated to dryness. The dried extract was evacuated by membrane pump in order to remove the solvent. The extract (ME 170 g) was then further dried in a freeze-dryer and stored in a reagent bottle at -22°C in freezer.

2.3.3 PARTITION OF METHANOL EXTRACT BETWEEN ETHYL ACETATE AND 1-BUTANOL:

(a) EXTRACTION WITH ETHYL ACETATE:

Methanol extract (170 g) was further extracted with ethyl acetate (2 l) for 12 days by changing methanol. The ethyl acetate extract (EE) was filtered and evaporated to dryness. The dried extract was evacuated by a membrane pump in order to remove the residual solvent. The extract (5.5 g) was then further dried in a freeze-dryer.

(b) EXTRACTION WITH 1-BUTANOL:

The ethyl acetate extractive-free residue was then extracted with butanol (1 l) for 12 days by changing 1-butanol. The extract (BE) was filtered and evaporated to dryness. The dried extract (30 g) was evacuated by membrane pump in order to remove the residual solvent. The extract (BE; 30 g) was then further dried in a freeze-dryer and stored in a reagent bottle at - 22° C in freezer.

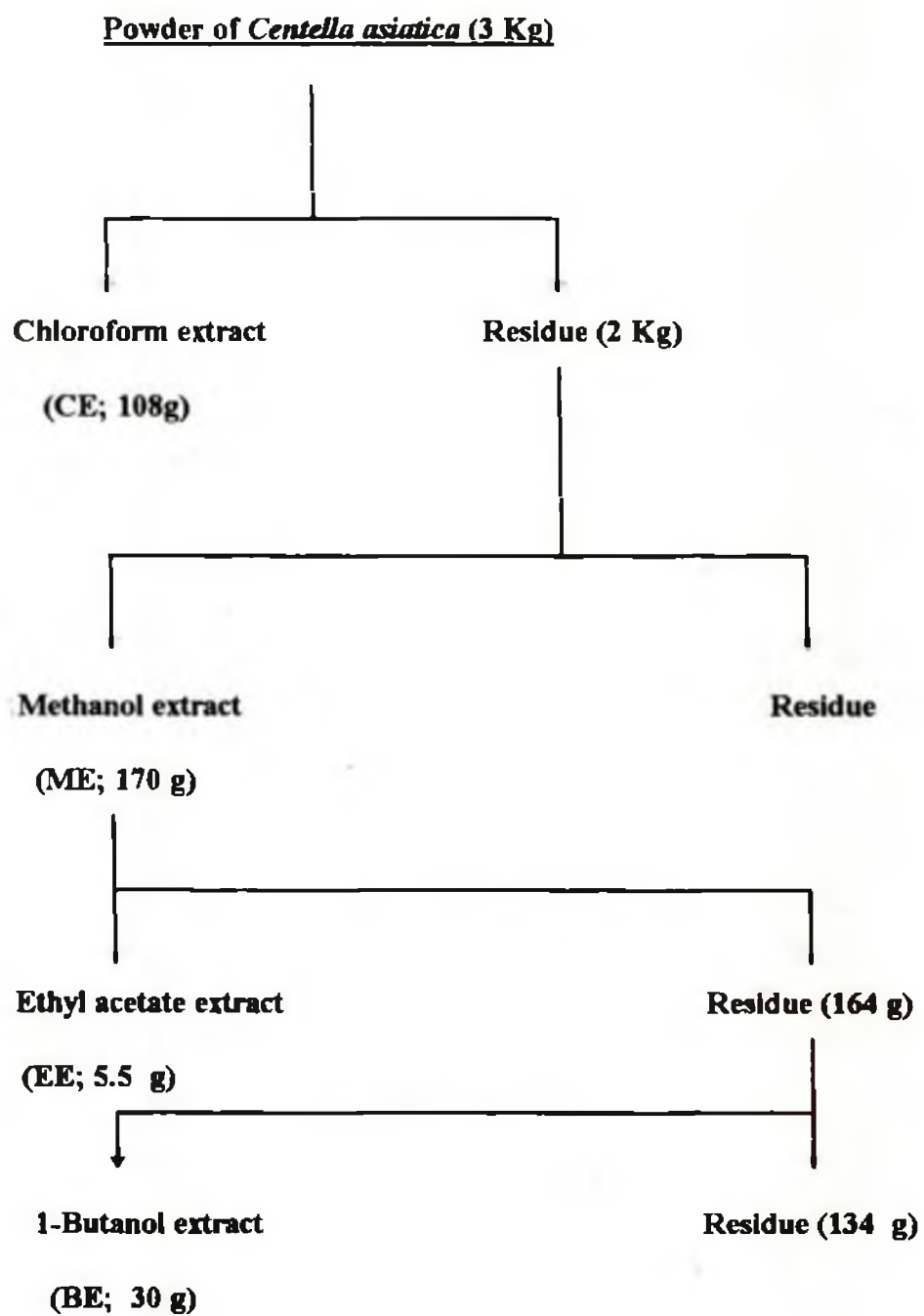
EXTRACTION SCHEME

TABLE:1 AMOUNT OF DIFFERENT EXTRACTIVES OF *CENTELLA ASIATICA* LEAVES POWDER (3 kg)

Extractives	Amount (g)
Chloroform extract (CE)	180
Methanol extract (ME)	170
Ethyl acetate extract (EE)	5.5
Butanol extract (BE)	30
Residue	134

2.5 FRACTIONATION OF CHLOROFORM EXTRACT (CE) BY COLUMN CHROMATOGRAPHY:

2.5.1 PREPARATION OF FLASH COLUMN:

A glass column (35 cm x 12 cm, i.d.) was packed with column grade silica gel (500 g, 230-400 mesh, ASTM , MERCK) using hexane as the equilibrating solvent. Then the column was flashed with three to four column volumes of hexane and finally the column bed became 24 cm in length.

2.5.2 APPLICATION OF SAMPLE AND FRACTIONATION:

Due to the viscous nature of the dried chloroform extract (62 g), it was dissolved in chloroform, mixed with silica gel (120 g) and evaporated to dryness. The dried sample was powdered in a

mortar and pestle and applied to the column. The sample was eluted by using n-hexane and the polarity was increased gradually by adding ethyl acetate. The eluted fractions (500 ml in each flask) were collected in 18 conical flasks. The solvent was evaporated by using a rotavapor and the amount of the fraction was found out. Amount of each fraction, the corresponding eluting solvent and the TLC pattern are given in Table-2:

**TABLE:2 DIFFERENT FRACTIONS OF CHLOROFORM EXTRACT (CE)
OBTAINED BY COLUMN CROMATOGRAPHY.**

Fraction	Solvent (%)			R _f values	Amount (g)	TLC pattern
	n-hexane	EtOAC	MeOH			
CE-1	100	0	0	-	0.54	Long tailing
CE-2	98	2	0	-	0.68	Long tailing
CE-3	96	4	0	-	1.45	Long tailing
CE-4	94	6	0	0.23	1.20	Single spot
CE-5	92	8	0	0.41	1.50	Single spot
CE-6	90	10	0	0.22, 0.42	2.65	Two spots
CE-7	85	15	0	0.7, 0.41	2.35	Two spots
CE-8	80	20	0	-	4.35	Mixture of
CE-9	75	25	0	-	5.62	several spots
CE-10	70	30	0	-	6.80	-DO-
CE-11	60	40	0	-	7.10	Long tailing
CE-12	50	50	0	-	5.45	Long tailing
CE-13	40	60	0	-	3.45	Long tailing
CE-14	20	80	0	0.25, 0.46,	3.20	Three spots
CE-15	0	100	0	0.66	2.35	with tailing
CE-16	0	100	0		2.35	-DO-
CE-17	0	0	100		3.9	long tailing
CE-18	0	0	100		4.	-DO-

Depending on the TLC pattern, similar fractions were combined and seven fractions were obtained. The amount of different fractions obtained and their TLC patterns are given in Table-3.

TABLE-3 DIFFERENT GROUPS OF FRACTIONS OF CE OBTAINED BY COLUMN CHROMATOGRAPHY

Fraction No.	Eluting solvent	R _f Values	Amount (g)	TLC pattern
CF ₁ (CE-1,2,3)	Hexane EtoAC 9:1	-	2.69	Long tailing
CF ₂ (CE-4,5)	Hexane EtoAC 9:2	0.5	2.70	Single spot with tailing
CF ₃ (CE-6,7)	Hexane EtoAC 9:3	0.3, 0.61	5.00	Two spots with tailing
CF ₄ (CE-8,9,10)	Hexane EtoAC 9:1	-	16.67	Mixture of several spots
CF ₅ (CE-11,1,13)	Hexane EtoAC 9:2	-	16.00	Mixture of several spots
CF ₆ (CE-14,15,16)	Hexane EtoAC 9:3	-	8.40	Long tailing
CF ₇ (CE-17,18)	Hexane EtoAC 9:4	-	8.10	Long tailing

2.6 FRACTIONS CF₁, CF₂, AND CF₃

Fraction CF₁ (2.69 g.) was an oily material which was not processed further. TLC pattern of the fraction CF₂ indicated a single spot with some tailing. The ¹H-NMR spectra of CF₂ fraction was recorded which indicated that it was a fatty material with some terpenoid compound. No further work was done on it. TLC pattern of the fraction CF₃ fraction indicated two distinct spots with tailing. But the ¹H-NMR spectra of CF₃ also indicated that it was a fatty material with small amount of terpenoid compound. So, no further work was done on it.

2.7 PURIFICATION OF FRACTION CF₄

The dark fraction CF₄ (16.67 g) contained a huge amount of chlorophyll. It was purified by column chromatography. A column (50 cm X 3 cm, i.d.) was packed with silica gel (120 g, column grade, 230-400 mesh) which was equilibrated with hexane. The sample (CF₄ ; 15 g) was applied to the column mixed with silica gel as described in Section 2.5.2. The sample was eluted by using hexane and the polarity was increased gradually by adding ethyl acetate and then methanol. The eluted fractions were collected in 50 ml conical flasks. Six different fractions were obtained according to their TLC pattern. Amount of each fraction and the TLC pattern are given in Table-4.

TABLE:4 DIFFERENT FRACTIONS OF CF₄ OBTAINED BY COLUMN CHROMATOGRAPHY

Fraction No.	Eluting solvent	R _f values	Amount (g)	TLC Pattern
CF ₄ -1	Hexane	-	1.06	Long tailing
CF ₄ -2	Hexane EtoAC 9:1	0.18, 0.4	1.56	Two spots with tailing
CF ₄ -3	Hexane EtoAC 9: 2	0.26	2.28	Single spot
CF ₄ -4	Hexane EtoAC 5: 5	-	2.08	Several spots
CF ₄ -5	Hexane EtoAC 8: 2	-	2.92	Several spots
CF ₄ -6		-	4.08	Long tailing

The fraction CF₄-3 was washed with n-hexane several times and purified by recrystallization from chloroform and was designated as C₄.

2.8 CHARACTERIZATION OF THE COMPOUND C₄:

The sample C₄ was a colourless crystalline solid having a melting point of 163-164°C. It was sparingly soluble in hexane and was readily soluble in chloroform. TLC examination showed one distinct spot in chloroform with R_f value 0.22.

U.V. SPECTROSCOPY:

UV spectrum of the compound (Fig. 1) C₄ had absorption maximum at 288 nm (CHCl₃).

IR SPECTROSCOPY:

The IR spectrum of the compound C₄ (KBr pellet) had absorbances at 3400, , 2900, 2850, 1630, 1590, 1445, 1380, 1060, and 970 cm⁻¹ (Fig. 2).

¹H-NMR (500 MHZ) SPECTROSCOPY:

The ¹H-NMR spectrum of compound C₄ (Fig. 3) gave doublet at 5.34 (1H, J_{1,2}, 2.18 Hz), two doublets of doublet at 5.15 (1H, J_{1,2}, 8.68 Hz) and 5.01 (1H, J_{1,2}, 8.72 Hz), a heptet at 3.52 (1H, J_{1,2} 4.6 Hz), and a multiplet at 2.31-2.2 ppm. There was multiplet between 2.07-1.13 ppm and six signals at 1.01, 0.84, 0.82, 0.80, 0.79 and 0.69 ppm which were assigned to six methyl protons of compound C₄.

¹³C-NMR SPECTROSCOPY:

The ¹³C-NMR spectrum of C₄ (Fig. 4) had 29 signals indicating the presence of 29 carbons. The signals were at 140.73, 138.29, 129.25, 121.67, 71.77, 56.85, 55.94, 51.22, 50.14, 42.28, 42.20, 40.48, 39.67, 37.25, 36.50, 31.88, 31.88, 31.86, 31.64, 28.90, 25.39, 24.35, 21.20, 21.06, 21.06, 19.38, 18.97, 12.23 and 12.03 ppm.

MASS SPECTROSCOPY:

The mass spectrum of C_4 (Fig. 5) had a molecular peak at m/z 412. The base peak was at m/z 83 and other intense peaks were at m/z 300, 271, 255, 159, 133, 109 and 95.

2.8 CHARACTERIZATION OF THE FRACTION CF_5

TLC and 1H -NMR (60 Mz) spectra of CF_5 fraction (16 g) indicated that it was chlorophyll with trace amount of compounds. So no further work was done on it.

2.9 PURIFICATION OF FRACTION CF_6 :

The fraction CF_6 (8.40 g ; light black material) when run with chloroform : methanol (9:1) showed at least three spots on TLC. The sample was applied on a column (50 cm x 3 cm, i.d.) by dissolving in chloroform, then the sample was eluted with chloroform followed by methanol. Eluted fractions were collected in 20 ml test tubes. The fractions were monitored by TLC and were combined on the basis of their R_f values. Five different fractions were obtained. The amount of each fraction and their corresponding TLC pattern are shown in Table-5.

TABLE-5: DIFFERENT FRACTIONS OF CF₆ OBTAINED BY COLUMN CHROMATOGRAPHY

Fraction No.	Eluting solvent	R _f values	Amount (g)	TLC Pattern
CF ₆ -1	CHCl ₃ : MeoH 9.5 : 0.5	0.38	1.852	Single spot with tailing
CF ₆ -2	CHCl ₃ : MeoH 9 : 1	0.29, 0.46	0.945	Two spots with tailing
CF ₆ -3	CHCl ₃ : MeoH 8.5 : 1.5	0.54	0.089	Single spot with tailing
CF ₆ -4	CHCl ₃ : MeoH 8 : 2	0.53	1.020	Single spot with tailing
CF ₆ -5	MeoH	-	2.565	Long tailing with two spots.

Fraction CF₆-1 was purified by washing with solvent (chloroform, methanol) and was named PC₃.

Fraction CF₆-3 was purified by washing with methanol and was named PC₁. Fraction CF₆-4 was purified by washing with methanol and was named PC₂.

2.10 CHARACTERIZATION OF THE COMPOUND PC₃

The compound PC₃ was a colourless UV inactive white solid which melted at 80°C. It was sparingly soluble in chloroform but was readily soluble in a mixture of chloroform and methanol. TLC examination showed one distinct spot in chloroform : methanol (9.5 : 0.5) with R_f value of 0.38 .

UV SPECTROSCOPY :

The UV spectrum of the compound PC₃ (Fig. 6) had absorption maximum at 320 nm.

IR SPECTROSCOPY :

The IR spectrum of the compound PC₃ (Fig. 7) had absorbences at 3400, 2900, 2850, 2700, 2140, 1615, 1540, 1450, 1370, 1280, 1120, and 960 cm⁻¹.

¹H-NMR SPECTROSCOPY :

The ¹H-NMR spectrum of PC₃ (Fig. 8) had four singlets at 5.42, 3.41, 3.31 and 1.24, one doublet at 4.60 (*J*_{1,2} 24.0 Hz) two triplets at 3.89 (*J*_{1,2} 3.48 Hz) and 0.87 (*J*_{1,2} 6.75 Hz), three doublets of doublet at 7.44 (*J*_{1,2} 3.96 Hz), 4.29 (*J*_{1,2} 4.76 Hz, and 3.31 (*J*_{1,2} 7.94 Hz) ppm. There was multiplets at 5.40-5.32 and 1.98-1.89 and a heptet at 3.99 (*J*_{1,2} 4.37 Hz) ppm and several other signals (multiplets , doublets, triplets etc.) were found between 1.7 -1.46 ppm.

¹³C-NMR SPECTROSCOPY:

The ¹³C-NMR spectrum of PC₃ (Fig. 9) had 27 signals indicating the presence of 27 carbons. The signals were at 172.21, 128.39, 128.06, 73.19, 73.11, 69.64, 69.61, 69.58, 58.92, 49.52, 32.74, 30.72, 30.53, 30.31, 29.76, 27.54, 27.52, 27.48, 27.40, 27.19, 27.18, 27.08, 24.03, 23.95, 23.09, 20.55 and 12.25 ppm.

MASS SPECTROSCOPY:

The mass spectrum of PC₃ (Fig. 10) had a molecular ion peak at *m/z* 272. The base peak was at *m/z* 63 and other intense peaks were at *m/z* at 248, 228, 221, 204, 189, 177, 149, 135, 119, 95 and 81 .

2.11 CHARACTERIZATION OF THE COMPOUND PC₁:

The compound PC₁ was a colourless UV inactive white solid which melted at 119°C. It was sparingly soluble in chloroform but was readily soluble in a mixture of chloroform and methanol.

TLC examination showed one distinct spot in chloroform : methanol (9.5 : 0.5) with R_f value of 0.29.

UV SPECTYROSCOPY:

The UV spectrum of the compound PC₁ (Fig. 11) had an absorption maximum at 293 nm.

IR SPECTROSCOPY:

The IR spectrum of the compound PC₁ (Fig. 12) had absorbances at 3300, 2900, 2840, 1615, 1530, 1460, 1065 and 960 cm⁻¹.

¹H-NMR SPECTROSCOPY:

The ¹H-NMR spectrum of PC₁ (Fig. 13) had five singlets at 3.47, 3.46, 3.24, 3.22 and 1.25, triplets at 7.90 (*J*_{1,2} 0.79 Hz), 4.60 (*J*_{1,2} 5.65 Hz) and 0.87 (*J*_{1,2} 6.74 Hz), two doublets of doublet at 7.47 (*J*_{1,2} 4.27 Hz, *J*_{1,3} 8.9 Hz) and 4.27 (*J*_{1,2} 5.95 Hz, *J*_{1,3} 19.64 Hz), two pentate at 5.37 (*J*_{1,2} 5.36 Hz) and 3.94 (*J*_{1,2} 4.57 Hz) ppm. There were multiplets between 5.33 - 5.32 , 3.74 - 3.70 and 3.65 - 3.61 ppm. There were several other signals (multiplets, doublets, triplets etc.) between 2.00 - 1.49 ppm.

¹³C-NMR SPECTROSCOPY:

The ¹³C-NMR spectrum of PC₁ (Fig. 14) had 27 signals indicating the presence of 27 carbons.

The signals were at 173.98, 129.66, 129.56, 74.89, 74.82, 71.33, 71.21, 71.17, 60.51, 50.96, 50.94, 34.12, 32.16, 32.11, 31.95, 31.88, 31.19, 29.00, 28.95, 28.91, 28.62, 25.42, 25.36, 24.56, 21.97 and 13.63 ppm.

2.12 CHARACTERIZATION OF THE COMPOUND PC₂:

The compound PC₂ was a white compound and melted at 152° C. It was soluble in a mixture of chloroform and methanol. TLC examination showed one distinct spot in chloroform : methanol (9 : 1) with R_f value of 0.3 .

UV SPECTROSCOPY:

UV spectrum of the compound PC₂ (Fig. 16) had absorption maximum at 305 nm.

IR SPECTROSCOPY:

The IR spectrum of PC₂ (Fig. 17) had absorbances at 3400, 2900, 2850, 2350, 1680, 1640, 1445, 1375, 1270, 1050 and 960 cm⁻¹.

¹H-NMR SPECTROSCOPY:

The ¹H-NMR spectrum of PC₂ (Fig. 18) had singlets at 1.05 (3 H), 0.93 (3 H), 0.92 (3 H), 0.75 (3 H) and 0.54 (3 H), five doublets at 3.30 (1H, J_{1,2} 10.7 Hz), 3.18 (1H, J_{1,2} 9.5 Hz), 3.04 (1H, J_{1,2} 10.5 Hz), 2.17 (1H, J_{1,2} 11.2 Hz) and 0.82 (3H, J_{1,2} 6.45 Hz). There was a triplet at 5.19 (1H, J_{1,2} 3.2 Hz) and multiplet at 3.49 - 3.39 ppm. Moreover there were several other signals (multiplets, doublets, triplets etc.) between 1.66 - 1.24 ppm.

¹³C-NMR SPECTROSCOPY:

The ¹³C-NMR spectrum of PC₂ (Fig. 19) had 30 signals indicating the presence of 30 carbons. The signals were at 178.35, 138.31, 124.43, 75.51, 67.40, 63.90, 52.38, 46.96, 46.94, 46.80, 45.97, 42.47, 41.73, 39.08, 38.49, 38.42, 37.28, 36.33, 32.17, 30.20, 27.50, 23.82, 23.31, 22.96, 21.09, 17.42, 17.04, 17.00, 16.88 and 13.76 ppm.

MASS SPECTROSCOPY:

The mass spectrum of PC₂ (Fig. 20) had a molecular ion peak at m/z 488. The base peak was at m/z 248 and other intense peaks were at 442, 394, 269, 219, 203, 191, 173, 163, 133, 95, 81 and 69.

2.13 PURIFICATION OF THE FRACTION CF₆-5:

Fraction CF₆-5 (Table 5) gave two close spots with tailing. It was purified by column chromatography. The sample (2.56 g) in chloroform was applied into the column and it was eluted with chloroform followed by methanol. Eluted fractions were collected in 20 ml test tubes manually. The fractions were monitored by TLC and combined on the basis of their R_f values. Three different fractions were obtained. The amount of each fraction and their TLC pattern are shown in Table-6.

TABLE 6: DIFFERENT FRACTION OF CF₆-5 OBTAINED BY COLUMN CHROMATOGRAPHY

Fraction No.	Eluting solvent	R _f values	Amount (g)	TLC Pattern
CF ₆ -1	CHCl ₃ : MeOH 9.5 : 0.5	0.38	1.852	Single spot with tailing
CF ₆ -2	CHCl ₃ : MeOH 9 : 1	0.29, 0.46	0.945	Two spots with tailing
CF ₆ -3	CHCl ₃ : MeOH 8.5 : 1.5	0.54	0.089	Single spot with tailing
CF ₆ -4	CHCl ₃ : MeOH 8 : 2	0.53	1.020	Single spot with tailing
CF ₆ -5	MeOH	-	2.565	Long tailing with two spots.

Fraction CF₆-5.2 was purified by washing with methanol and was named PC₅.

2.14 CHARACTERIZATION OF THE COMPOUND PC₅:

The compound PC₅ was a white compound with R_f value of 0.43 in chloroform : methanol (9 : 1). It was soluble in a mixture of chloroform and methanol and melted at 277° C.

UV SPECTROSCOPY:

The UV spectrum of the compound PC₅ (Fig. 21) had absorption maximum at 314 nm.

IR SPECTROSCOPY:

The IR spectrum of the compound PC₃ (Fig. 22) had absorbances at 3400, 2900, 2850, 1680, 1450, 1380, 1160, 1100, 1070 and 1020 cm⁻¹.

¹H-NMR SPECTROSCOPY:

In the ¹H-NMR spectrum of PC₃ (Fig. 23) there were nine singlets at 5.32 (1 H), 4.90, 3.32, 0.96 (3 H), 0.82 (3 H), 0.78 (3 H), 0.77 (3 H), 0.67 (3 H) and 0.65 (3 H), four doublets at 4.21 (J_{1,2} 7.84 Hz), 0.99 (3H, J_{1,2} 6.55 Hz), 0.89 (3H, J_{1,2} 6.34 Hz) and 0.80 (3H, J_{1,2} 2.98 Hz), four doublets of doublet at 5.15 (1H, J_{1,2} 8.73 Hz), 5.01 (1H, J_{1,2} 8.73 Hz), 3.63 (J_{1,2} 5.35 Hz) and 2.36 (J_{1,2} 2.38 Hz) ppm. There was a triplet at 4.90 (J_{1,2} 5.66 Hz), two multiplets at 3.50 - 3.38 and a sextet at 2.89 (J_{1,2} 4.36 Hz) ppm.

¹³C-NMR SPECTROSCOPY:

In the ¹³C-NMR spectra of PC₃ (Fig. 24) there were 51 signals indicating the presence of 51 carbons. The signals were at 140.42, 138.01, 128.79, 121.17, 121.13, 100.77, 76.89, 76.74, 76.72, 73.43, 70.05, 61.05, 56.24, 56.15, 55.41, 55.32, 50.57, 49.60, 49.58, 45.12, 41.83, 41.72, 38.29, 36.81, 36.20, 36.19, 33.33, 31.39, 31.35, 31.31, 29.24, 28.68, 28.48, 27.77, 25.41, 24.86, 23.87, 23.85, 22.58, 21.09, 20.92, 20.57, 19.69, 19.08, 18.92, 18.83, 18.60, 12.11, 11.82, 11.76 and 11.65 ppm.

MASS SPECTROSCOPY:

The mass spectrum of PC₃ (Fig. 25) had molecular ion peak at m/z 412. The base peak was at m/z 69 and other intense peaks were 396, 300, 255, 157, 145, 133, 107, 95 and 61.

2.15 PURIFICATION OF FRACTION CF₇

The fraction CF₇ (8.01 g) was a dark brown material. It was purified by column chromatography. A column (50 cm x cm, i.d.) was packed with silica gel (120 g ; column grade, 230 - 400 mesh) which was equilibrated with chloroform. The sample was applied to the column in the powdered form as described in Section 2-5.2 . The sample was eluted by using chloroform and the polarity was increased gradually by adding methanol. The eluted fractions were collected in 20 ml test tubes. Seven different fractions were obtained according to their TLC pattern. The amount of each fraction and their corresponding TLC pattern are shown in Table-7.

TABLE 7: DIFFERENT FRACTION OF CF₇ OBTAINED BY COLUMN CHROMATOGRAPHY

Fraction No.	Eluting solvent	R _f values	Amount (g)	TLC pattern
CF ₇ -1	CHCl ₃ : MeOH 9 : 1	0.52	0.360	Single spot with some tailing
CF ₇ -2	CHCl ₃ : MeOH 8.5 : 1.5	0.46	0.260	Single spot with some tailing
CF ₇ -3	CHCl ₃ : MeOH 8 : 2	0.53 , 0.69	0.380	Two spots with some tailing
CF ₇ -4	CHCl ₃ : MeOH 7.5 : 2.5	0.71	0.210	Single spot with some tailing
CF ₇ -5	CHCl ₃ : MeOH 7.5 : 2.5	0.51 , 0.71	0.398	Two spots with some tailing
CF ₇ -6	CHCl ₃ : MeOH 7 : 3	0.58	0.120	Single spot with some tailing
CF ₇ -7	CHCl ₃ : MeOH 5 : 5	-	3.000	Long tailing

Fraction CF₇-1 was purified by washing with methanol and was named L₁. Fraction CF₇-2 and compound PC₂ were identified as similar compound (From IR and ¹H-NMR data). Fraction CF₇-4 was purified by washing with methanol and was named L₃. Fraction CF₇-6 was purified by repeated column chromatography and was named L₄.

2.16 CHARACTERIZATION OF THE COMPOUND L₁

The compound L₁ was a colourless compound which melted at 133 - 134° C. It was soluble in a mixture of chloroform and methanol. TLC examination showed one distinct spot in chloroform : methanol (9 : 1) with a R_f value of 0.52.

UV SPECTROSCOPY:

The UV spectrum of the compound L₁ (Fig. 26) had absorption maximum at 321 nm.

IR SPECTROSCOPY:

The IR spectrum of L₁ (Fig. 27) had absorbances at 3300, 3200, 2900, 2850, 1610, 1530, 1450, 1120 and 960 cm⁻¹.

¹H-NMR SPECTROSCOPY:

The ¹H-NMR spectrum of L₁ (Fig. 28) had two singlets at 3.21, 1.23, two doublets of doublet at 3.89 (J_{1,2} 4.96 Hz) and 3.84 (J_{1,2} 4.97 Hz), and triplets at 4.03 (J_{1,2} 12.60 Hz), 1.92 (J_{1,2} 4.86 Hz) and 0.85 (J_{1,2} 6.65 Hz) ppm. There were multiplets at 5.40-5.31, 3.56-3.48, 3.38-3.32, 1.60-1.44 and 1.28-1.23 ppm.

¹³C-NMR SPECTROSCOPY:

The ¹³C-NMR spectrum of L₁ (Fig. 29) had 21 signals indicating the presence of 21 carbons. The signals were at 74.28, 70.80, 60.28, 51.15, 32.32, 32.06, 31.33, 29.18, 29.13, 19.11, 29.07, 29.05, 29.03, 28.96, 28.76, 28.73, 28.63, 23.66, 24.44, 22.13 and 13.91 ppm.

MASS-SPECTROSCOPY:

The mass spectrum of L₁ (Fig. 30) had the molecular ion peak at m/z 648. The base peak was at m/z 70 and other intense peaks were at m/z 620, 394, 356, 339, 262, 225, 138, 124, 111, 97 and 83.

CHARACTERIZATION OF THE COMPOUND L₃:

The compound L₃ was a colourless compound. It was soluble in a mixture of chloroform and methanol. TLC examination showed one distinct spot in chloroform : methanol (8.5 : 1.5) with a R_f value of 0.65. Its UV, IR, ¹H-NMR and ¹³C-NMR data were identical with compound PC₃ which was separated from the fraction CF₆-5 (Table-6). There was slight difference in mass spectrum. The molecular ion peak of L₃ (Fig. 31) was m/z at 414 (M⁺ of PC₃ was 412).

CHARACTERIZATION OF THE COMPOUND L₄ :

The compound L₄ was a white compound with a R_f value of 0.45 in chloroform methanol (8 : 2). It was soluble in chloroform and methanol and melted at 266° C.

IR SPECTROSCOPY :

The IR spectrum of L₄ (Fig. 32) had absorbances at 3400, 2900, 2850, 2350, 1680, 1450, 1350, 1240 and 1030 cm⁻¹.

¹H-NMR SPECTROSCOPY :

In the ¹H-NMR of L₄ (Fig. 33) there were ten singlets at 8.31, 1.28, 1.27, 1.23, 1.15, 1.05, 0.97, 0.91, 0.90 and 0.88, five doublets at 3.19 (*J*_{1,2} 8.43 Hz), 3.10 (*J*_{1,2} 9.42 Hz), 2.12 (*J*_{1,2} 1.31 Hz), 0.99 (*J*_{1,2} 4.27 Hz) and 0.82 (*J*_{1,2} 6.44 Hz), two doublets of doublet at 4.02 (*J*_{1,2} 3.28 Hz) and 2.76 (*J*_{1,2} 9.91 Hz) and four broad signals at 4.30, 4.25, 4.16 and 4.07 ppm. There were multiplets between 5.20-5.16, 1.99-1.82 and several other signals (multiplets, doublets triplets etc.) between 1.74-1.35 ppm.

¹³C-NMR SPECTROSCOPY :

In the ¹³C-NMR spectrum of L₄ (Fig. 34) there were fifty nine signals indicating the presence of fifty nine carbon. The signals were at 143.30, 137.64, 124.77, 121.67, 75.69, 75.65, 67.41, 67.39, 65.94, 65.88, 63.67, 52.44, 49.15, 48.85, 47.43, 47.29, 46.83, 46.71, 43.54, 45.68, 45.44, 43.12, 43.10, 42.13, 41.79, 40.84, 38.49, 38.44, 38.30, 38.12, 36.92, 36.82, 36.35, 33.34, 32.84, 32.14, 31.27, 30.41, 30.20, 30.41, 28.99, 28.68, 27.45, 27.12, 25.70, 23.83, 23.37, 23.36, 22.97, 22.60, 22.08, 21.07, 18.25, 18.10, 17.80, 17.00, 14.98, 14.92 and 13.94 ppm.

MASS SPECTROSCOPY :

The mass spectrum, of L₄ (Fig. 35) had molecular ion peak at *m/z* 410. The base peak was at *m/z* 73 and other intense peaks were at *m/z* 269, 248, 225, 209, 163, 153, 145, 124, 109, 95 and 83.

CHAPTER THREE
RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

3.1 COLLECTION OF LEAVES :

For the present work the leaves of *Centella asiatica* were collected from an adjoining village area of Dhaka. The leaves were dried in an oven at 40° C and were powdered in a grinding machine for effective extraction.

3.2 DIFFERENT EXTRACTIVES OF *CENTELLA ASIATICA* :

The dried and powdered leaves were first extracted with chloroform. The residue was then extracted with methanol. As a result 108 g of chloroform extract and 170 g of methanol extract were obtained (Sec 2. 3. 1, 2. 3. 2). The methanol extract was then divided into ethyl acetate soluble (EE) (Sec. 2. 3. 3a) and insoluble parts by partition with butanol (Sec. 2. 3. 3b) both the fraction was collected by evaporation followed by freeze-drying. The methanol extract contained nearly 5.5 g of ethyl acetate (EE) soluble material and 30 g of butanol soluble material. The scheme of extraction and the amount of extractives are given in Sec. 2.4 and Table 1.

3.3 CHARACTERIZATION OF THE COMPOUND C_4 :

The colourless crystalline compound C_4 was soluble in chloroform and had a melting point of 163-164° C. The IR spectrum of C_4 (Fig. 2) had a band at 3400 cm^{-1} due to -OH stretching. Absorption band at 2850 cm^{-1} was due to aliphatic -C-H stretching. Absorption band at 1630 cm^{-1} indicated C=C stretching vibration. Absorption bands at 1590 and 1445 cm^{-1} indicated -CH₂- and -CH₃ bending vibrations, respectively.

In the ^1H -NMR spectrum of C_4 (Fig. 3) had two singlets at 1.01 (3H) and 0.69 (3H) ppm. which were assigned to two angular (at position 19 and 18, respectively) methyl groups. Three doublets at 0.84 (3H), 0.80 (3H) and 0.80 (3H) ppm were due to the three methyl groups attached to methine carbons (at positions 21, 26, 27, respectively) and a triplet at 0.82 (3H) ppm indicated the presence of a methyl group (at position 29) adjacent to a $-\text{CH}_2-$ group. A distorted triplet at 5.34 (1H, $J_{1,2} = 2.18$ Hz) ppm indicated the presence of an olefinic proton. The low coupling constant (2.18 Hz) indicated that the double bond was in the ring carbon. Two doublets of doublet at 5.15 (1H, $J_{1,2} = 8.68$ Hz) and 5.01 (1H, $J_{1,2} = 0.72$ Hz) ppm. indicated the presence of two olefinic protons in the side chain. One heptet (each signal splitted into two) at 3.52 (1H, $J_{1,2} = 4.6$ Hz) ppm indicated the presence of a methyne carbon having adjacent $-\text{CH}_2-$ groups and an alcoholic proton. The signals (multiplets, doublets, triplets etc.) between 2.07-1.13 ppm were due to other methylene and methyne protons.

The ^{13}C -NMR spectrum of C_4 (Fig. 4) had 29 signals indicating that the compound contained 29 carbons. The ^{13}C -NMR data of the compound C_4 was compared with those data of known steroidal compounds published earlier³⁹. It was found that the data fitted well with the published data of stigmasterol. The comparison of the ^{13}C -NMR signals of C_4 and stigmasterol are given in Table-8.

Table:8 THE COMPARISON BETWEEN C¹³-NMR SPECTRAL DATA OF STIGMASTEROL⁵⁹ AND COMPOUND C₄

Carbon No.	Types of Carbon	Chemical shift in ppm	
		Stigmasterol	C ₄
1	-CH ₂ -	37.31	37.25
2	-CH ₂ -	31.69	31.64
3	>CH-OH	71.81	71.77
4	-CH ₂ -	42.35	42.20
5	-C-	140.80	140.73
6	=CH	121.69	121.67
7	-CH ₂ -	31.94	31.86
8	-C-H	31.94	31.88
9	-C-H	50.20	50.14
10	-C-	36.56	36.50
11	-CH ₂ -	21.11	21.20
12	-CH ₂ -	39.74	39.67
13	-C-	42.35	42.28
14	-CH	56.91	56.85
15	-CH ₂ -	24.39	24.35
16	-CH ₂ -	28.96	28.90
17	-C-H	56.06	55.94
18	-CH ₃	12.07	12.03
19	-CH ₃	19.42	19.38
20	-C-H	40.54	40.48
21	-CH ₃	21.11	21.06
22	=CH	138.37	138.29
23	=CH	129.32	129.25
24	-CH	51.26	51.22
25	-C-H	31.94	31.88
26	-CH ₃	21.26	21.06
27	-CH ₃	19.02	18.97
28	-CH ₂ -	25.44	25.39
29	-CH ₃	12.29	12.23

```

*** PEAK-PICK ***
-- PEAK -- -- VALLEY --
No.  λ      ABS      λ      ABS
-----
1  288.0  1.398    790.0  0.034
2    220.0 -0.181

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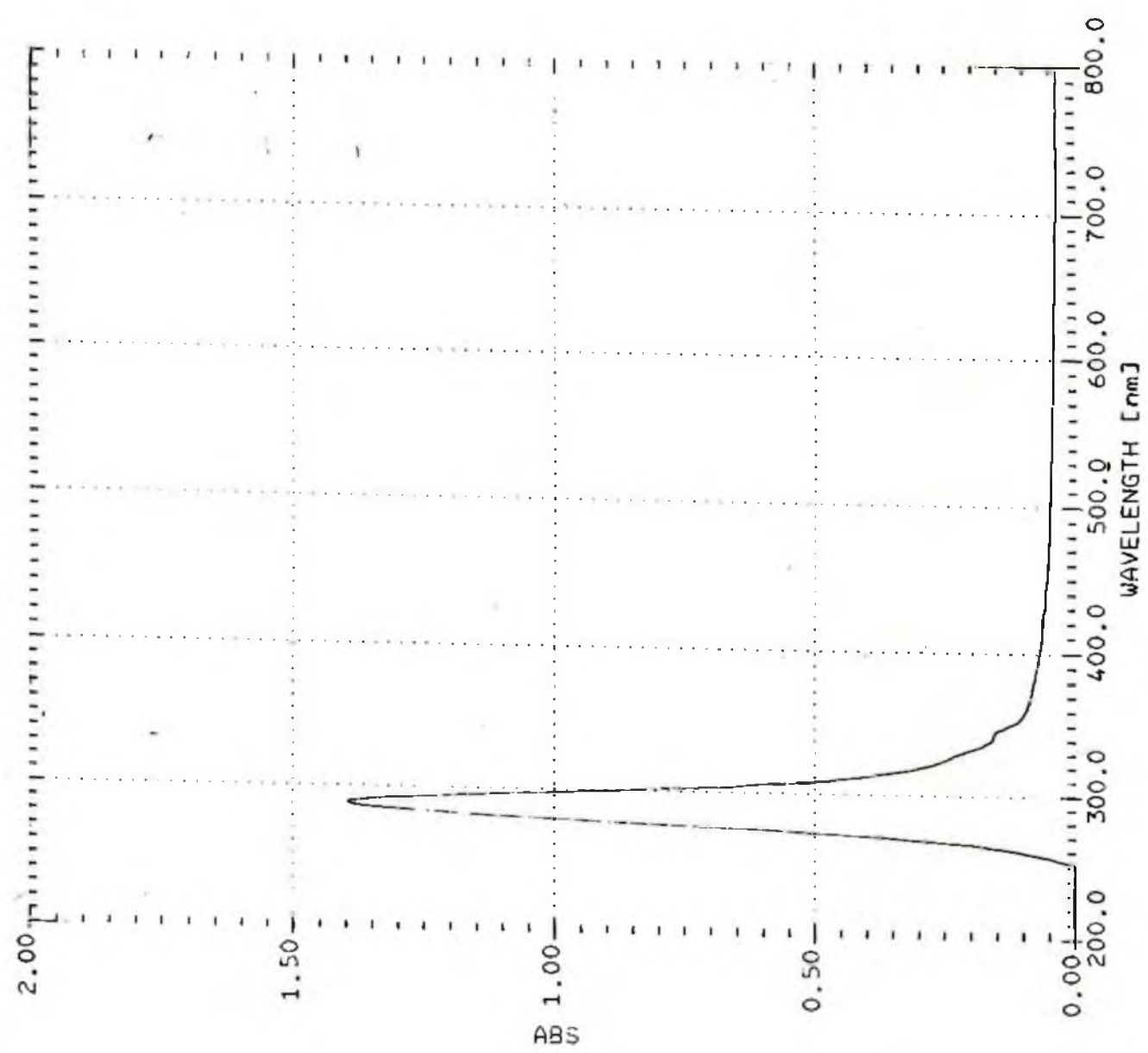


Fig.1: UV spectrum of compound C₄

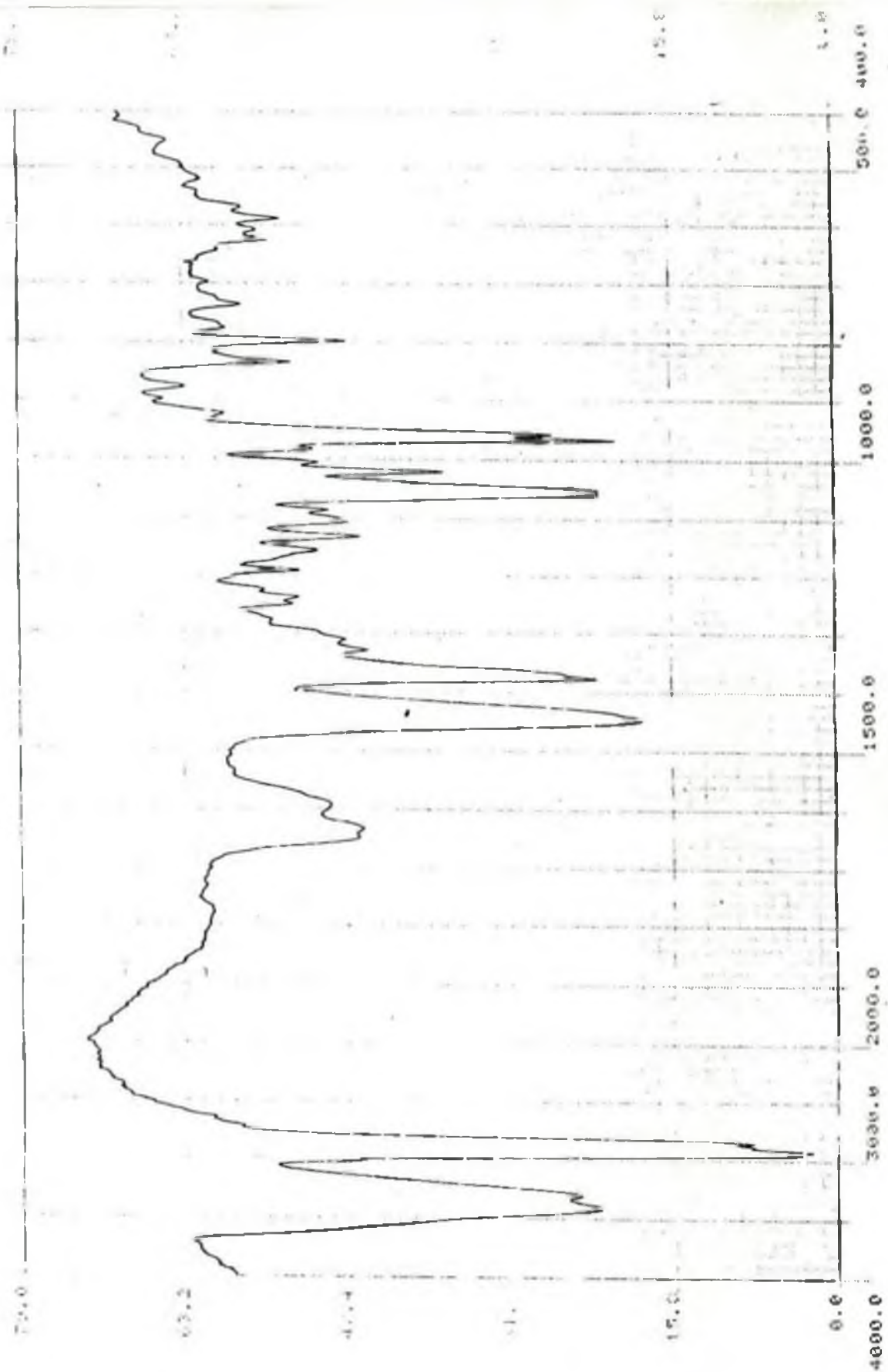


Fig 2 : IR spectrum of compound C4

```

mccloud/mos(huzzaman
C-4 (18mg/200ul)
tgm2d3
COC13
MOMO
Experiment : H1 s2pu1
Temperature : 27 C
Date : Oct 15 96
Probe : Nalorac M0500-3mm
exp6 s2pu1

SAMPLE 15 96 dfrq 499.880 DEC. A VT
date Oct COC13 dfrq 499.880 H1
file COC13 exp 30 H1
ACQUISITION exp 30 dfr -1044.8
sfrq 499.882 dm n
tn H1 dm 200
at 5.002 dfr 200
np 55024 dseq
sw 6499.8 dres 1.0
fb 3500 homo 27.0
bs 16 temp
== 1 PROCESSING 0.30
tpr 60 lb
pw 5.0 wf file
dl 5.000 proc ft
tof 300.0 fn 131072
nt 32 math y
ct 32 verr
a lock n wexp svf( "/homo-
gain n wexp e/vmr1/arch/vc/mc-
flags n n cloud/tgm2d3")
ll n n wnt wft
ln n n
dp n n
bs n n

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sp -452.5
wp 6499.8
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wc 250
hz 0.10
fs 36720.03
rf1 452.5
rfp 0
th 4
ins 41.824
nm ph

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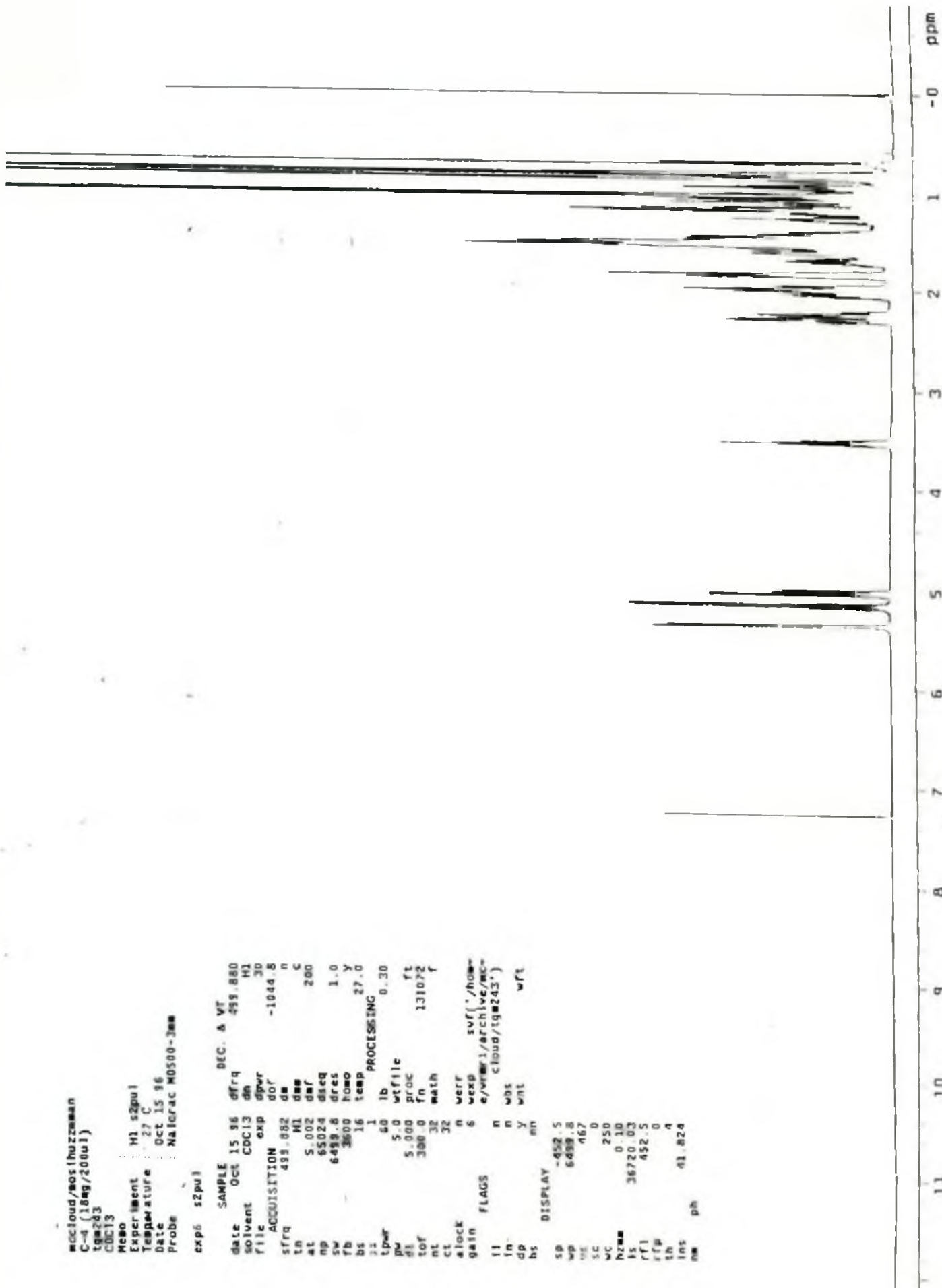


Fig.3: ¹H-NMR spectrum of compound C₁

mccloud/mosshuzzaman
 C-4 (18mg/200ul)
 180223
 CDCl₃
 Name :
 Experiment : H1 s2pul
 Temperature : 27 °C
 Date : Oct 15 96
 Probe : Nalorac HD500-3mm



Fig.3a: Partial ¹H-NMR spectrum of compound C₄ (5.5-0 ppm)

acc1006/mos1huzzaman
C-4 (10mg/200ul)

199243

CDCl₃

Memor

Experiment : H1 s2p01

Temperature : 27 C

Date : DEC 15 96

Probe : Nalorac MDS00-3mm

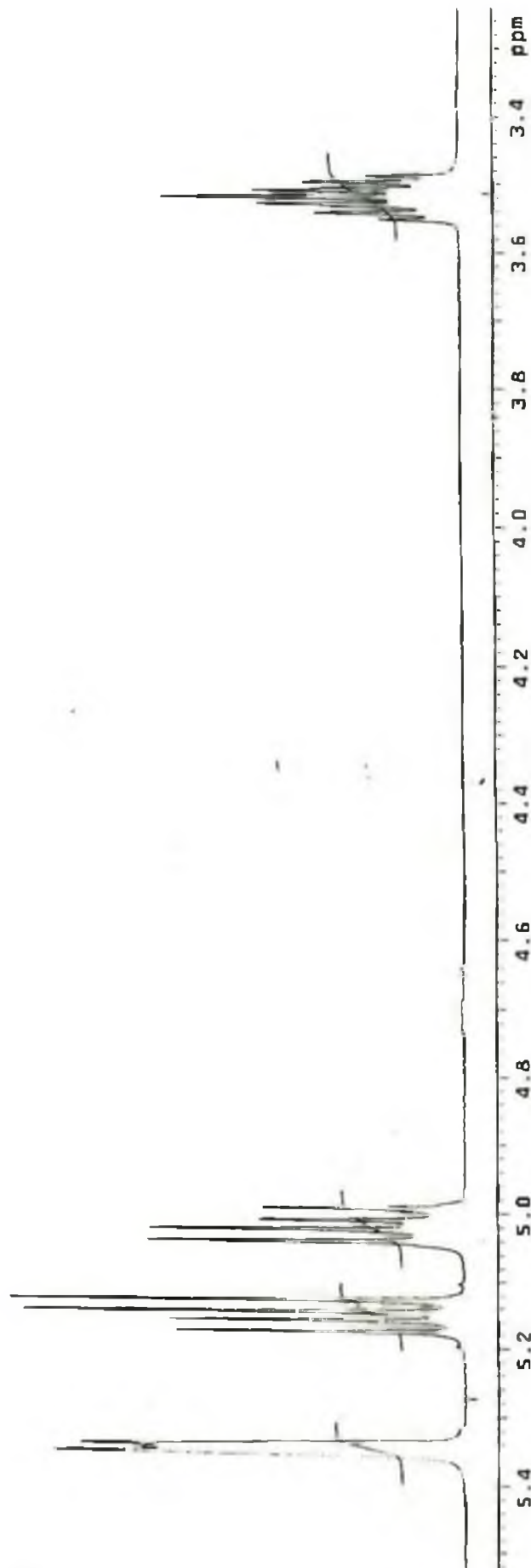


Fig.3b: Partial ¹H-NMR spectrum of compound C₄ (5.4-3.4 ppm)

mccloud/mos thuzassan
 C-4 (18mg/200ul)
 tpe243
 CD-13
 Meso :
 Experiment : H1 s2pu1
 Temperature : 27 C
 Date : Oct 15 96
 Probe : Nalorac MD500-3mm

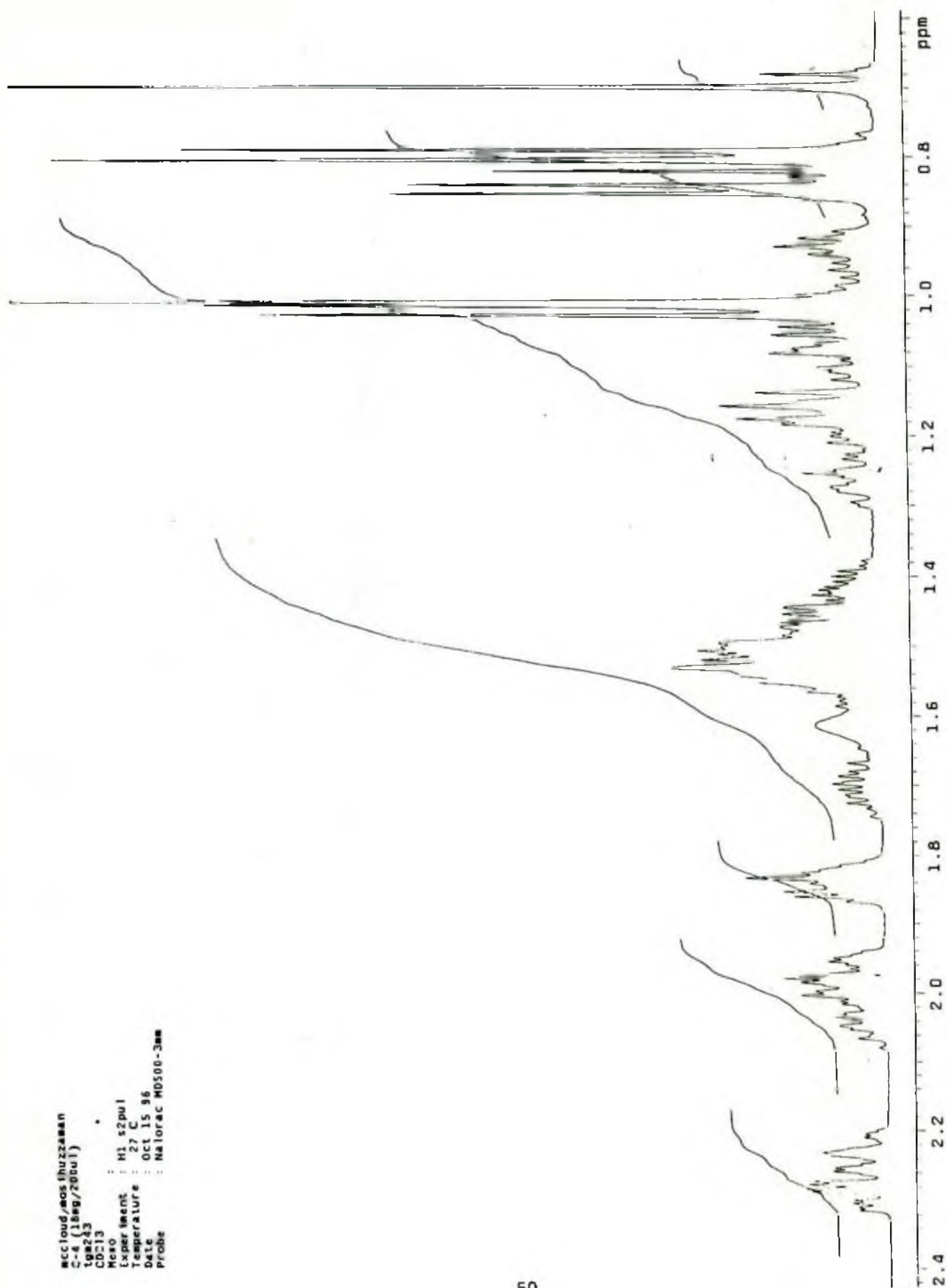


Fig.3c: Partial ^1H -NMR spectrum of compound C_4 (2.4-0.0 ppm)

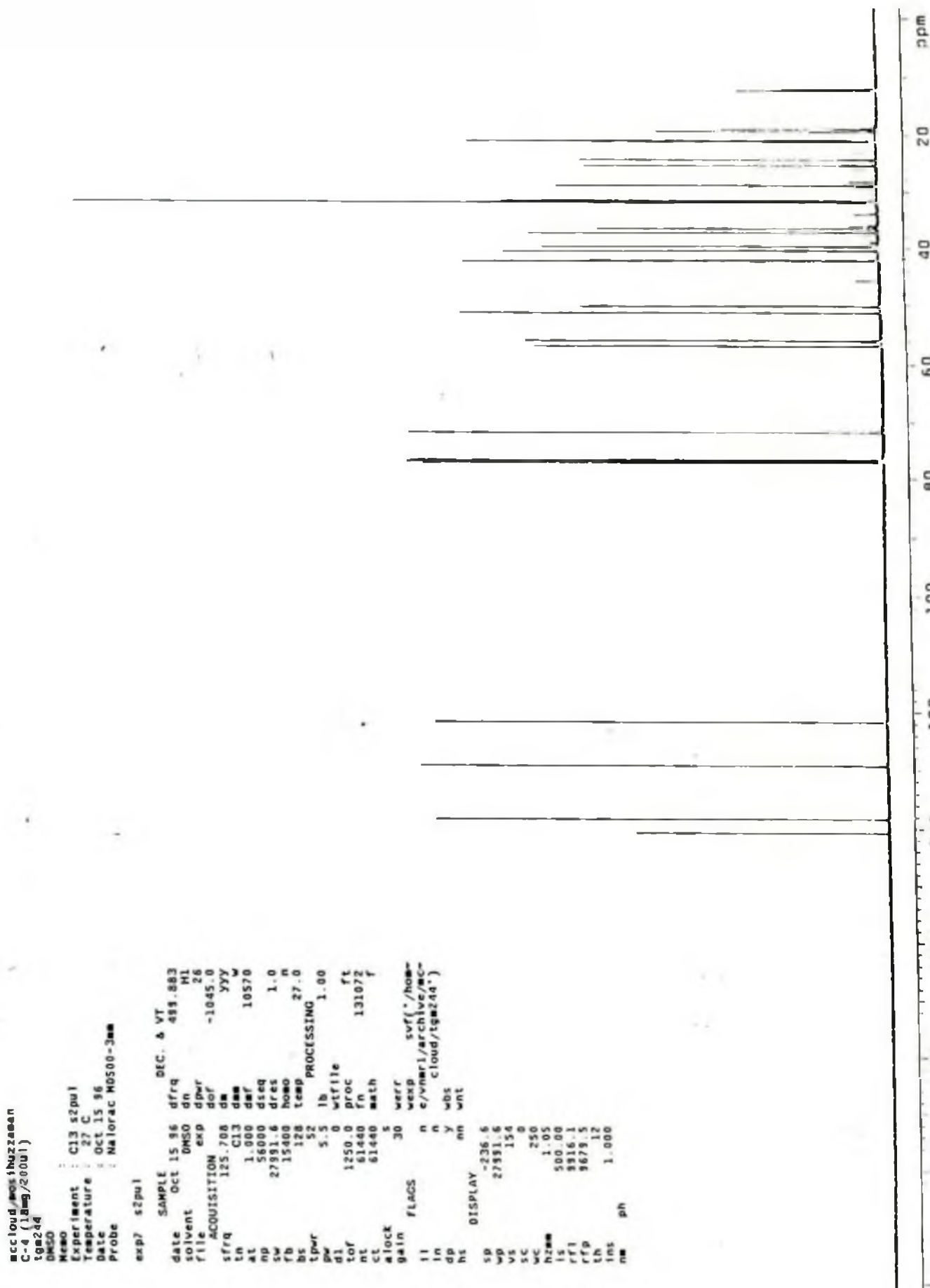


Fig.4: ¹³C-NMR spectrum of compound C₄

@ccloud/mosituzaan
 C-4 (18ag/28bu)
 19a244
 DMSO
 Name : C13 s2bu1
 Experiment : 27 C
 Temperature : Oct 15 96
 Date :
 Probe : Nalorac MDS00-3mm

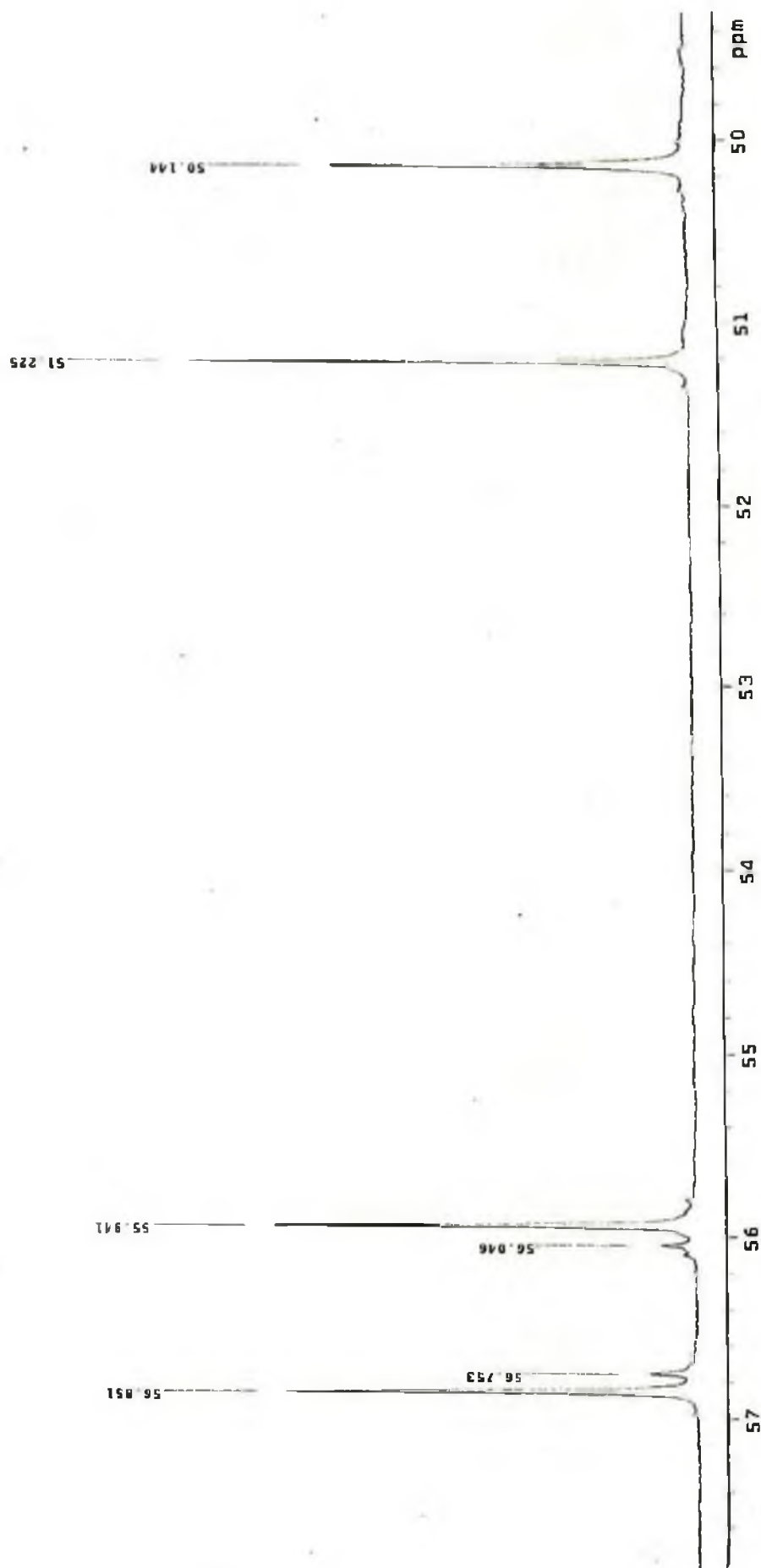
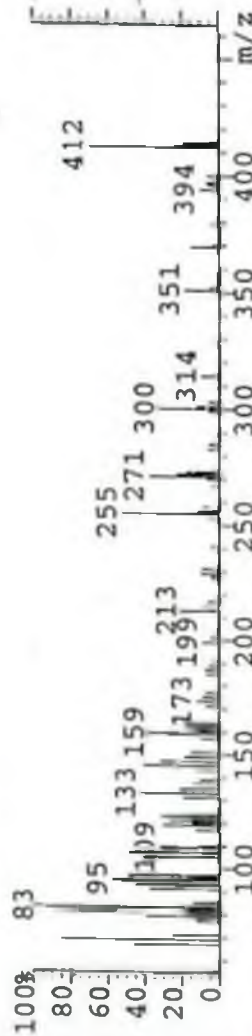


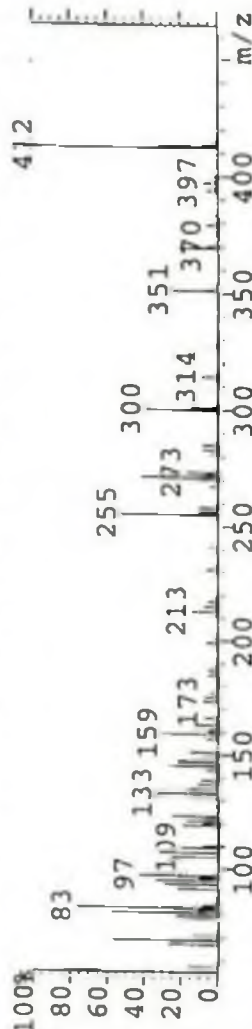
Fig.4a: Partial ^{13}C -NMR spectrum of compound C_4 (57-50 ppm)

Libraries 1: NIST (62235) 2: NISTREP (12593)

Peaks: 8 5 6 7 4 3 2 1 0
 Matches: 0 1 4 1 34 157 1634 15284 57706
 Total: 0 1 5 13 47 204 1838 17122 74828
 Normal Search: 27 of 47 Rank: Purity Mass Weighting: On Presearch Minimum: 20
 File Text: C-4 File: E96705 Ident: 14 Mer Def 0.30 Acquired: 11-SEP-1996 13:00:08 +1:35
 70S EI+ Function: Magnet TIC: 12622327



Rank: 1 NISTREP #12094 Purity: 825 Mixture: 836 Matched: 7 MF: C29H48O MW: 412 CAS: 83-48-7 NIST: 48824
 Name: Stigmasterol SMILES: CCC(C=CC(C)C3CCC4C2CC=CC(C)O)CCC1(C)C2CCC34C)C(C)C



Rank: 2 NISTREP #12093 Purity: 823 Mixture: 833 Matched: 6 MF: C29H48O MW: 412 CAS: 83-48-7 NIST: 49793
 Name: Stigmasterol SMILES: CCC(C=CC(C)C3CCC4C2CC=CC(C)O)CCC1(C)C2CCC34C)C(C)C

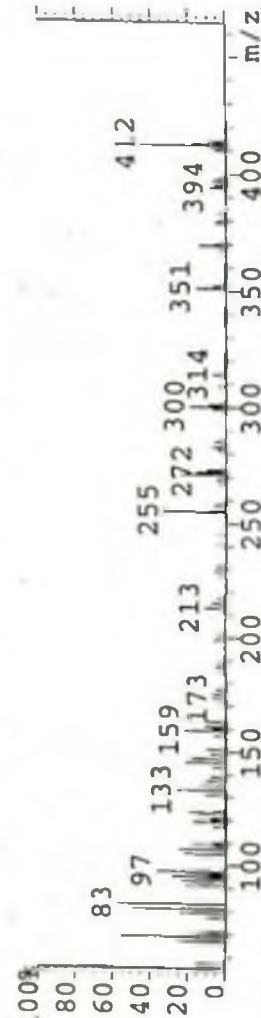
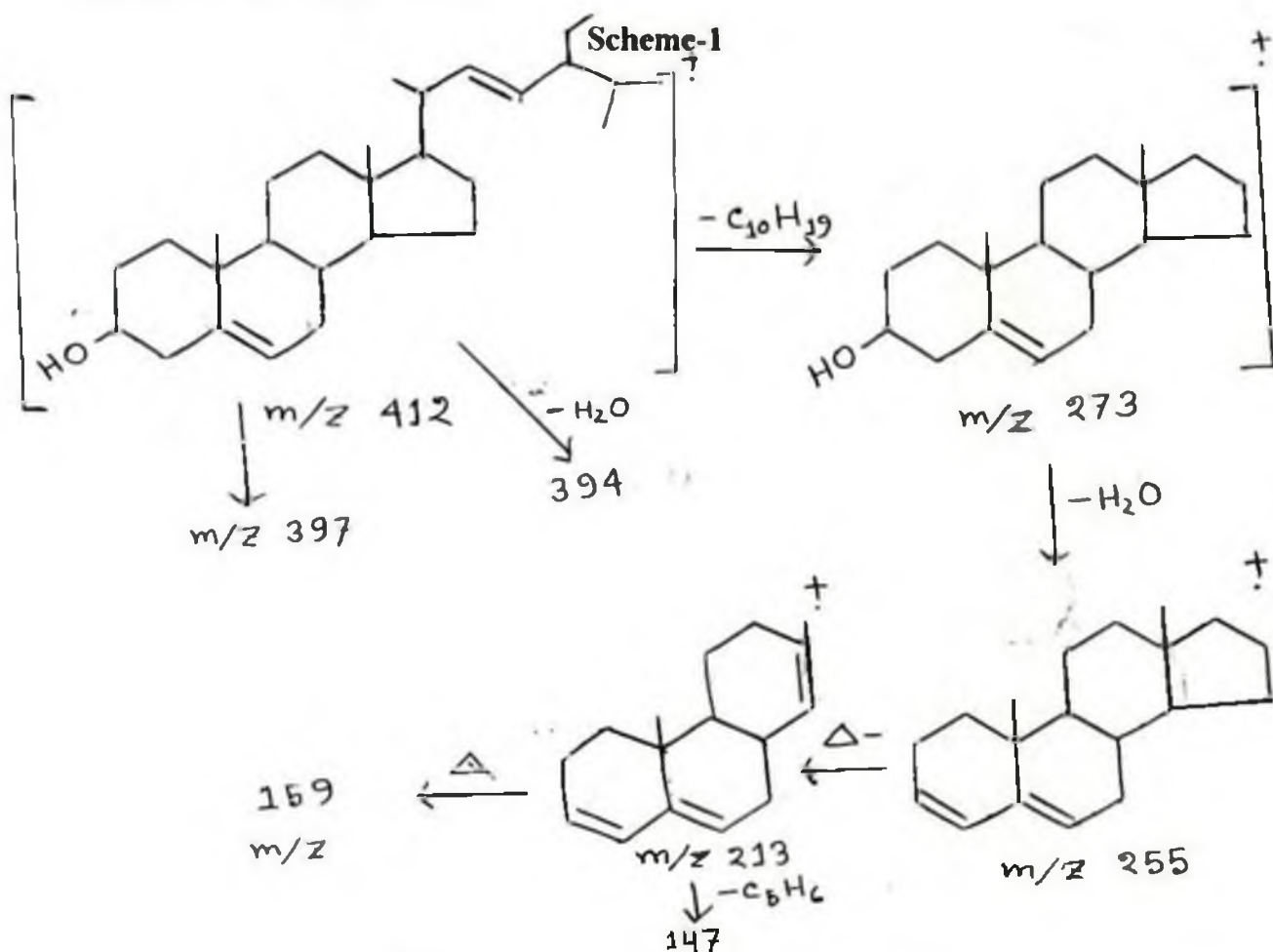


Fig. 5: Mass spectrum of compound C1

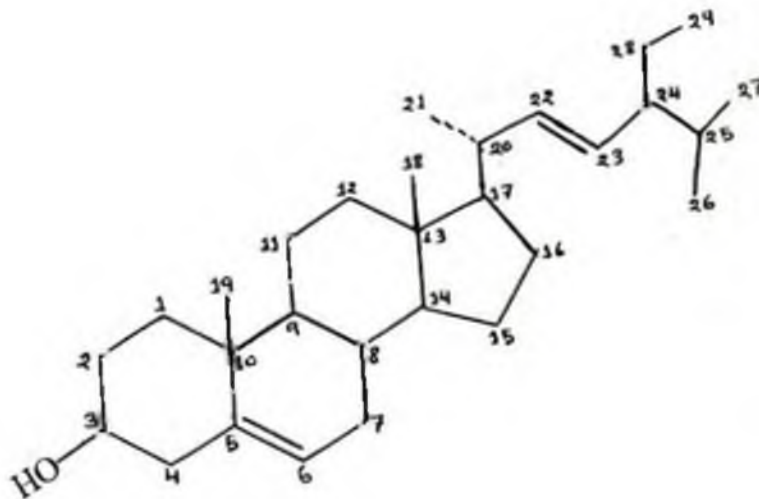
The signals at 140.73, 121.67, 138.37 and 129.32 ppm were assigned to four carbons of two olefinic double bonds at position C-5, C-6, C-22 and C-23. The signals at 71.77 ppm was assigned to a methyne carbon attached to -OH group at C-3. The signals at 21.06, 21.06, 19.38, 18.97, 12.23 and 12.03 ppm were due to methyl groups at C-26, C-21, C-19, C-27, C-29 and C-18, respectively. The signals at 50.14, 56.85, 59.54, 40.48, 51.22, 31.88, and 31.88 ppm were assigned to methyne carbons at C-9, C-14, C-17, C-20, C-24, C-8 and C-25, respectively. Again the signals at 42.20, 39.67, 37.25, 31.88, 31.86, 28.92, 25.39, 24.35 and 21.20 were assigned to methylene carbons at C-4, C-12, C-1, C-7, C-2, C-16, C-28, C-15 and C-11, respectively. The signals at 42.28, and 36.50 ppm were due to the quaternary carbons at C-13 and C-10, respectively. The structure was further supported by mass spectrum (molecular ion peak 412) fragmentation pattern (Scheme-1).



Scheme-1: Possible mass fragmentation of C_4

3.5 STRUCTURE OF THE COMPOUND C₄

From the ¹H-NMR and ¹³C-NMR and UV spectral data, and by comparison with published spectral data the following structure is given for the compound C₄ (11). It is known as stigmasterol.



(11)

Although stigmasterol is a known compound but it was not reported earlier from *Centella asiatica*

3.6 CHARACTERISATION OF THE COMPOUND PC₂

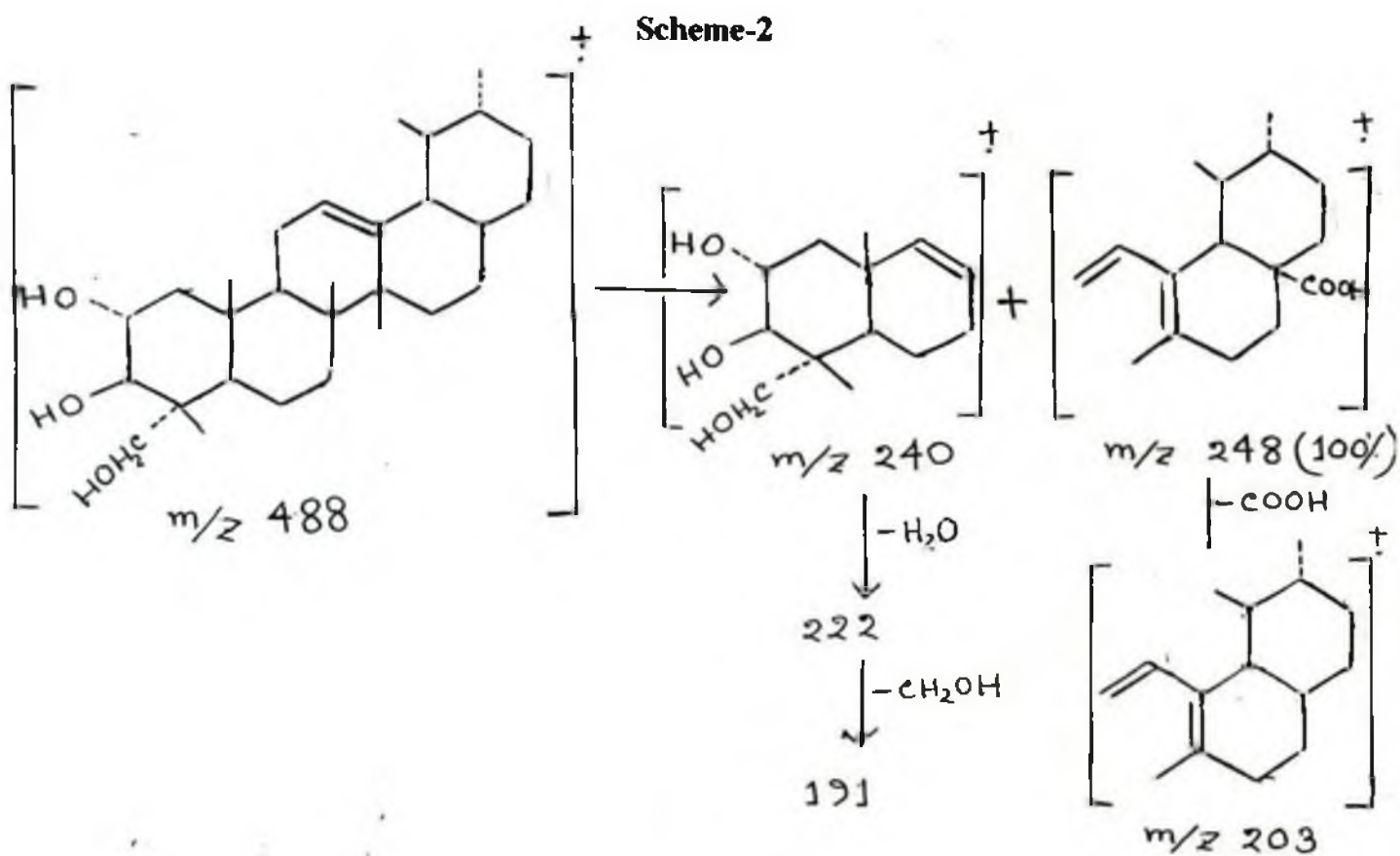
The colourless compound PC₂ was sparingly soluble in chloroform and was readily soluble in a mixture of chloroform and methanol. The IR spectrum (Fig. 17) of PC₂ had absorption at 3400 cm⁻¹ due to OH stretching, 2900 and 2850 cm⁻¹ were due to the aliphatic C-H stretching. Absorption band at 1680 cm⁻¹ was assigned to C=O stretching. Absorption band at 1640 and 1630 cm⁻¹ indicated C=C stretching vibration. Absorption bands at 1445 and 1375 cm⁻¹ indicated -CH₂- and -CH₃- bending vibrations, respectively.

The ^1H -NMR spectrum (Fig. 18) had three singlets at 1.05 (3H), 0.75 (3H) and 0.93 (3H) ppm which were assigned to three angular methyl groups (at positions 27, 26, 25, respectively). A doublet at 0.82 (3H, J 6.4 Hz) ppm and a singlet at 0.92 (3H) ppm were assigned to two methyl groups attached to methine carbons (at positions 29, 30 respectively). Another singlet at 0.54 (3H) ppm indicated the presence of methyl group (at position 24) attached to a quaternary carbon. A triplet at 5.19 (1H, $J_{1,2}$ 3.2 Hz) ppm, indicated the presence of an olefinic proton (at position 12). The low coupling constant (3.2 Hz) indicated that the double bond was in the ring carbon. A multiplet at 3.49 (1H) and doublet at 3.18 (1H, $J_{1,2}$ 9.5 Hz) ppm indicated the presence of two protons which were attached to carbons containing hydroxy group (at position 2 and 3, respectively). Two doublets at 3.30 (1H, $J_{1,2}$ 10.7 Hz) and 3.04 (1H, $J_{1,2}$ 10.5 Hz) ppm indicated the presence of two protons on carbon attached to primary hydroxy group (at position 23).

The ^{13}C -NMR spectrum of PC_2 (Fig. 19) had 30 signals indicating that the compound contained 30 carbons. The signals at (Table-9) 13.76, 16.88, 17.00, 23.31, 17.04 and 21.09 ppm were due to six methyl group at positions C-24, C-25, C-26, C-27, C-29 and C-30 respectively. The signals at 46.94, 17.42, 32.17, 22.96, 27.50, 23.82, 30.20, 36.33 and 63.90 ppm were assigned to methylene carbons at the positions C-1, C-6, C-7, C-11, C-15, C-16, C-21, C-22 and C-23 respectively. The signals at 67.40, 75.51, 45.97, 46.96, 124.43, 52.38, 38.42 and 38.49 ppm were assigned to methyne carbons at the positions C-2, C-3, C-5, C-9, C-12, C-18, C-19 and C-20 respectively. The signals at 42.47, 39.68, 37.28, 138.31, 41.73, 46.80 and 178.35 ppm were due to the quaternary carbons at the positions C-4, C-8, C-10, C-13, C-14, C-17 and C-28 respectively. Among them the downfield signals at 124.43 and 138.31 ppm were assigned to two carbons of one olefinic double bonds at positions C-12 and C-13 respectively. The signals at 67.40 and 75.51 ppm were assigned to methyne carbons attached to -OH group at position C-2 and C-3 and signals at 63.90 ppm. was assigned to methylene carbon attached to -OH group at C-23.

More information were available from HMBC and HMQC data in table-9a and COSY data in table-9b which enable the unambiguous identification of the compound. In the HMBC experiment, the proton at 5.13 (H-12) ppm showed $3J$ correlation to the methine carbon at 52.38 ppm and

methylene carbon at 22.96 ppm which could be assigned, therefore, to C-18 and C-11 respectively. The proton 3.49 (H-2) ppm showed 2/ 3/ HMBC correlation to the methine carbons at 75.51 ppm and methylene carbons at 46.94 and 63.90 ppm which could be assigned, therefore, to C-3, C-1 and C-23 respectively. The proton at 3.18 (H-3) ppm showed similar 3/ 2/ HMBC correlation to C-1 (46.94 ppm) , C-2 (67.40 ppm), C-23 (63.90 ppm) and C-24 (13.76 ppm) . Thus PC2 identified as 2, 3, 23 - Trihydroxy -12 - ursene - 28 - oic acid, a known compound. The structure was further supported by mass spectrum (molecular ion peak 488 fragmentation pattern (Scheme-2).



Scheme:2 Possible mass fragmentation of PC₂

The ^{13}C -NMR data of the compound PC₂ was compared with ^{13}C -NMR of known asiatic acid published earlier^{60,61,62}. The comparison of the ^{13}C -NMR signals of PC₂ and asiatic acid are given in Table-9.

Table: 9 THE COMPARISON BETWEEN ^{13}C -NMR SPECTRAL DATA OF ASIATIC ACID⁶⁰⁻⁶² AND THE COMPOUND PC₂

Carbon No.	Types of carbon	Chemical shift ppm	
		Asiatic Acid	PC ₂
1	-CH ₂ -	46.5	46.94
2	OH-CH	68.9	67.40
3	OH-CH	80.5	75.51
4	-C-	42.7	42.47
5	-CH	49.2	45.97
6	-CH ₂ -	18.5	17.42
7	-CH ₂ -	32.8	32.17
8	-C-	39.7	39.08
9	-CH	47.6	46.96
10	-C-	38.3	37.28
11	-CH ₂ -	23.5	22.96
12	=CH	125.4	124.43
13	=C-	138.4	138.31
14	-C-	42.3	41.73
15	-CH ₂ -	28.2	27.50
16	-CH ₂ -	24.4	23.82
17	-C-	48.2	46.80
18	-CH	53.0	52.38
19	-CH ₂ -	39.2	38.42
20	-C-	39.0	38.49
21	-CH ₂ -	30.8	30.20
22	-CH ₂ -	36.8	36.33
23	OH-CH ₂	70.4	63.90
24	-CH ₃	13.0	13.76
25	-CH ₃	17.1	16.88
26	-CH ₃	17.2	17.00
27	-CH ₃	23.9	23.31
28	-C-OH	178.2	178.35
29	-CH ₃	17.3	17.01
30	-CH ₃	21.3	21.09

Table 9a: HMQC AND HMBC CORRELATION DATA OF PROTONS AND CARBONS IN PC₂

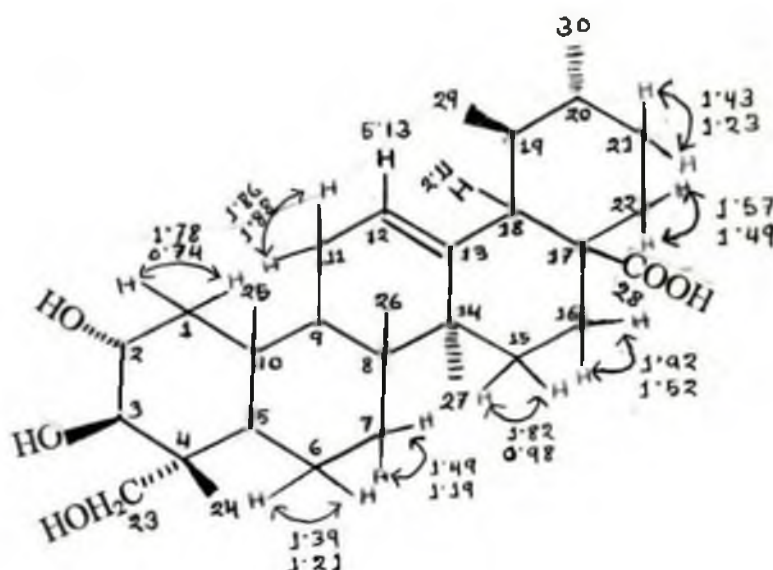
Assignment area	HMBC	HMQC	Delta (ppm)
CO ₂ H	2.11, 1.93, 1.52	-	178.35 (1)
C=	2.11, 1.86, 1.04, 0.98sm	-	138.31 (2)
CH=	2.11, 1.86	5.13	124.43 (3)
CHOH	3.49, 3.30, 3.04, 1.78, 0.73, 0.54	3.18	75.51 (4)
CHOH	3.17, 3.04sm, 1.78, 0.73	3.49	67.40 (5)
CH ₂ OH	3.17, 0.54	3.04, 3.31	63.90 (6)
CH	5.14, 0.82	2.11	52.38 (7)
CH	-	1.50	46.96 (8)
CH ₂	5.14, 0.74	1.78, 0.74	46.94 (9)
C	3.17, 2.11, 1.51, 0.93	-	46.80 (10)
CH	3.30, 0.41, 1.78, 1.19, 0.93, 0.54	1.21	45.97 (11)
C	3.30, 3.17, 3.04, 1.19, 0.54	-	42.47 (12)
C	5.14, 2.11, 1.50, 1.04, 0.74	-	41.73 (13)
C	1.50, 1.19, 1.04, 0.74	-	39.08 (14)
CH	-	0.92	38.49 (15)
CH	2.11, 0.91, 0.82	1.296	38.42 (16)
C	1.77, 1.50, 1.19, 0.93, 0.73	-	37.28 (17)
CH ₂	-	1.57, 1.49	36.33 (18)
CH ₂	0.74	1.49, 1.19	32.17 (19)
CH ₂	0.92	1.43, 1.24	30.20 (20)
CH ₂	1.93, 1.04	1.82, 0.98	27.50 (21)
CH ₂	2.11, 1.81	1.92, 1.52	23.82 (22)
Me	1.17	1.04	23.31 (23)
CH ₂	5.14, 1.50	1.88, 1.86	22.96 (24)
Me	-	0.92	21.09 (25)
CH ₂	1.19	1.39, 1.21	17.42 (26)
Me	-	0.82	17.04 (27)
Me	-	0.75	17.00 (28)
Me	2.11, 1.78, 1.50, 1.19, 0.73	0.93	16.88 (29)
Me	3.30, 3.17, 3.04, 1.19	0.54	13.76 (30)

Table: 9b COSY CORRELATION DATA OF PROTON PROTON IN PC₂

Assig	Area	Over hause	Cosy	Delta (ppm)	Multi (J, Hz)
H	1	I	2.11wk, 1.88, 1.86, 1.82wk, 1.50wk	5.14 (1)	cm
H	2.7	I	-	4.7-3.9 (34, 35, 36)	vbcm's
H	1	I	3.18, 1.78, 0.74	3.49 (2)	d(4.5), d(9.5), d(11.2)
H	1	I	3.04, 0.54wk	3.30 (3)	d(10.7)
H	1	I	3.49, 1.78wk, 0.74vwk	3.17 (4)	d(9.5)
H	1	I	3.30, 1.39wk, 0.54vwk	3.04 (5)	d(10.5)
H	1	II	1.88vwk, 1.86vwk, 1.52, 1.30, 0.82vw k	2.11 (6)	d(11.2)
H	1	II	1.82, 1.52, 0.98	1.93 (7)	d(4.3), torq(13.1)
H	1	II	1.86, 1.52, 1.48	1.88 (8)	cm
H	1	II	1.88, 1.52, 1.48	1.86 (9)	cm
H	1	II	1.52, 1.05, 0.98	1.82 (10)	d(4.8), d(13.9)
H	1	II	0.74	1.78 (11)	d(4.6), d(12.4)
H	1	II	D	1.57 (12)	cm
H	1	II	E	1.52 (13)	cm
H	1	II	F	1.50 (14)	cm
H	1	II	G	1.49 (15)	cm
H	1	II	O	1.49 (16)	cm
H	1	II	H	1.43 (17)	cm
H	1	II	I	1.39 (18)	cm
H	1	II	J	1.296 (19)	cm
H	1	II	K	1.24 (20)	cm
H	1	II	L	1.21 (21)	cm
H	1	II	M	1.21 (22)	cm
H	1	II	N	1.19 (23)	cm
Me	3	III	A	1.05 (24)	s
H	1	III	1.53	0.98 (25)	cmd (13.4)
Me	3	III	C	0.93 (26)	s
H	1	III	0.74	0.92 (27)	cm
Me	3	III	1.28	0.92 (28)	s
Me	3	III	1.30	0.82 (29)	d(6.4)
Me	3	III	1.50	0.75 (30)	s
H	1	III	3.50, 1.78, 0.92	0.74 (31)	d(11.3), d(12.4)
Me	3	III	H	0.54 (32)	s

3.7 STRUCTURE OF THE COMPOUND PC₂

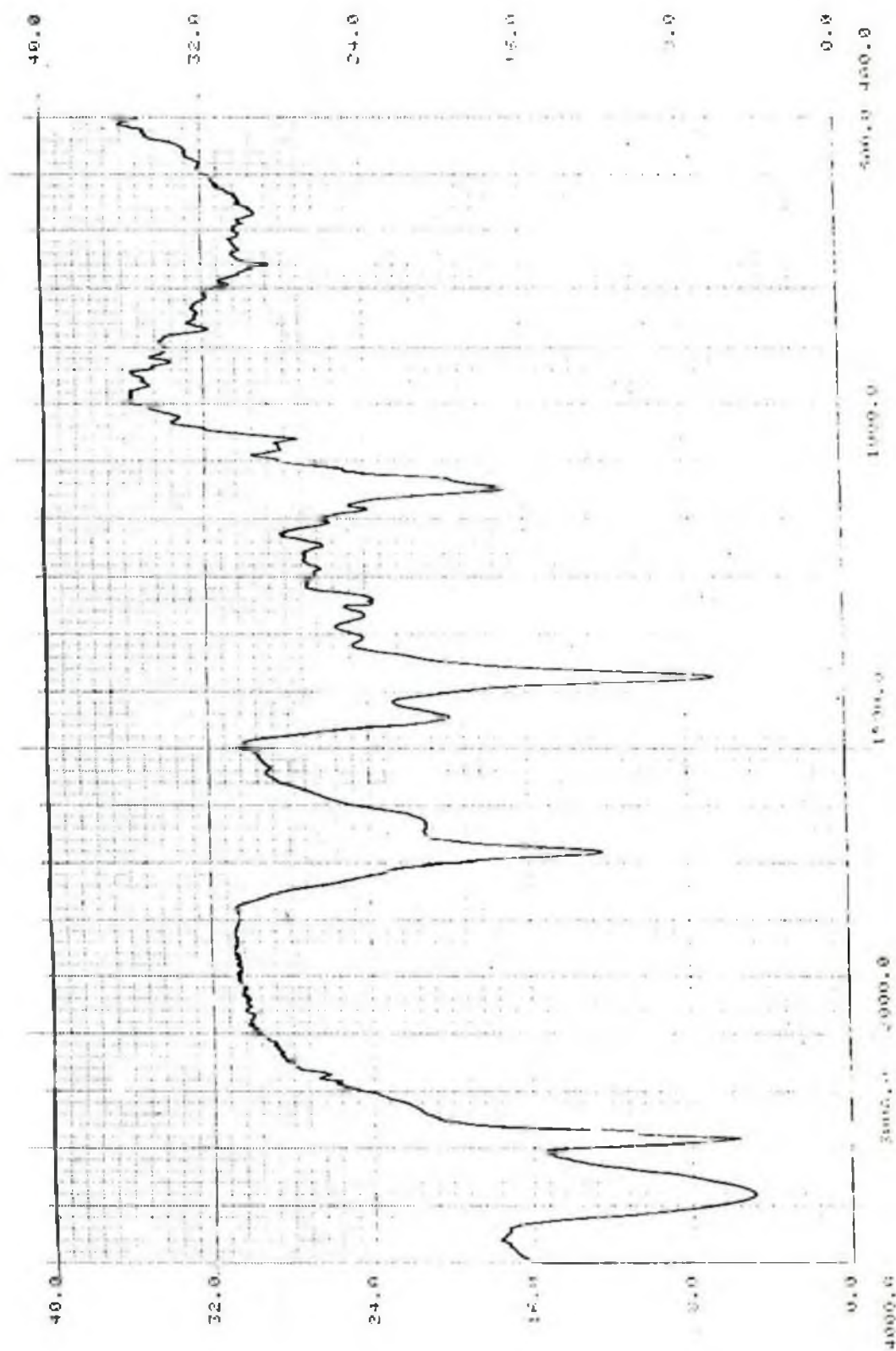
The comparison (Table:9) holds out quite well, but several carbons are at noticeable different shifts. The ¹H-NMR data of compound PC₂ was compared with those ¹H-NMR data of asiatic acid.^{63,64,65} It was found that in asiatic acid there were four singlets and two doublets for six methyl groups. But compound PC₂ had five singlets and one doublet for six methyl groups. It may be ¹H-NMR data of PC₂ did not resolve well or this compound is some isomer of asiatic acid. However ¹H-NMR, ¹³C-NMR IR, UV, HMBC, HMQC and COSY spectral data of compound PC₂ is tentatively assigned the structure 2 α , 3 β , 23- trihydroxy-12-ursene-28-oic acid (asiatic acid), the following structure is given (Fig. 12, with COSY interactions). Confirmation of the structure needs further spectroscopic studies.



3.8 CHARACTERIZATION OF THE COMPOUND CP₃

The compound PC₃ was a white compound with a R_f value 0.43 in chloroform : methanol 9:1 . It was soluble in mixture of chloroform and methanol and melted at 277° C. The UV spectrum of the compound PC₃ (Fig. 21) had absorption maximum at 314 nm indicating the presence of non-conjugated double bond. The IR spectrum (Fig. 22) of PC₃ had absorption band at 3400 cm⁻¹ due to -OH stretching. Absorption band at 2900 and 2850 cm⁻¹ were due to the aliphatic -C-H stretching. Absorption band at 1680 cm⁻¹ indicated C=C stretching vibration.

The ¹H-NMR spectrum of PC₃ had four singlets at 0.96 (3H), 0.78 (3H) , 0.67 (3H) and 0.65 (3H) ppm which were assigned to four angular methyl groups. The five doublets at 0.99 (3H, J_{1,2} 6.55 Hz), 0.89 (3H, J_{1,2} 6.34 Hz), 0.82 (3H) , 0.82 (3H) and 0.77 (3H) were due to the five methyl groups attached to the methyne carbons. A broad peak at 5.32 ppm indicated the presence of an olefinic proton. two doublets of doublet at 5.15 (J_{1,2} 8.73 Hz) and 5.01 (J_{1,2} 8.73 Hz) ppm indicated the presence of two olefinic protons on the side chain. A doublet at 4.21 (J_{1,2} 7.84 Hz) ppm indicated the presence of an anomeric proton. The multiplets at 3.65-3.62 and 3.50-3.38 ppm indicated the presence of protons from sugar residue. The signals (multiplets, doublets, triplets etc.) between 1.66-1.02 ppm were due to the other methylene and methine protons in the molecule.



SHIMADZU CORPORATION CHART 2 Fig.17: IR spectrum of compound PC₂


```

#cc1oud/mos1hu222am
PC-2 (18ag)
18a223
CD300
Name : CD300.DMSO 1.1
Experiment : H1 2pu1
Temperature : 27 C
Date : Sep 24 96
Probe : Nalorac MD500-3mm

```

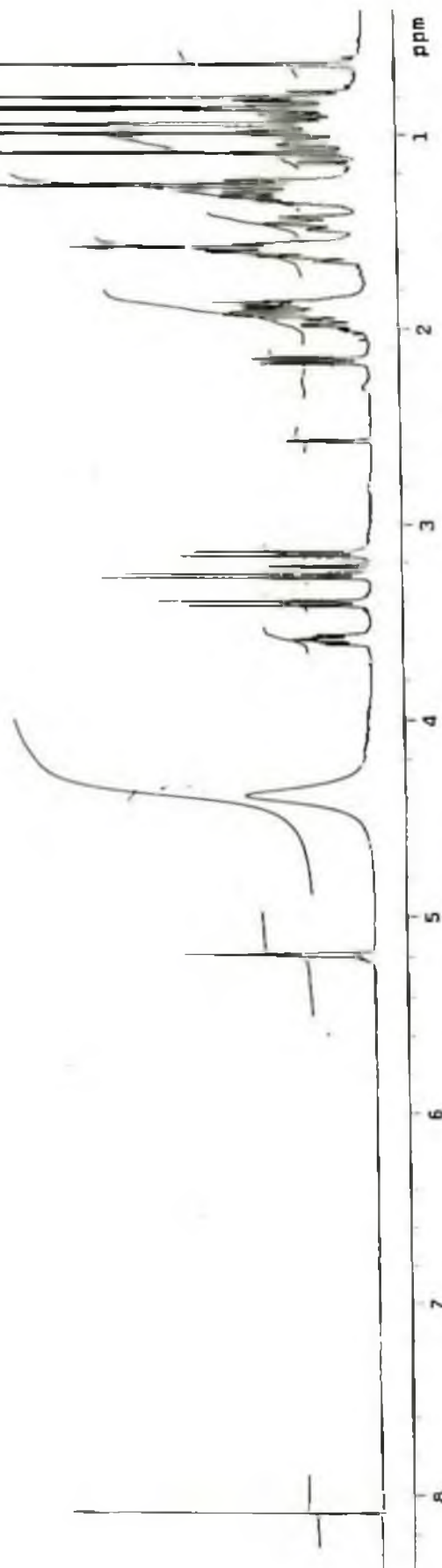


Fig.18a: Partial ¹H-NMR spectrum of compound PC₂ (8-0 ppm)

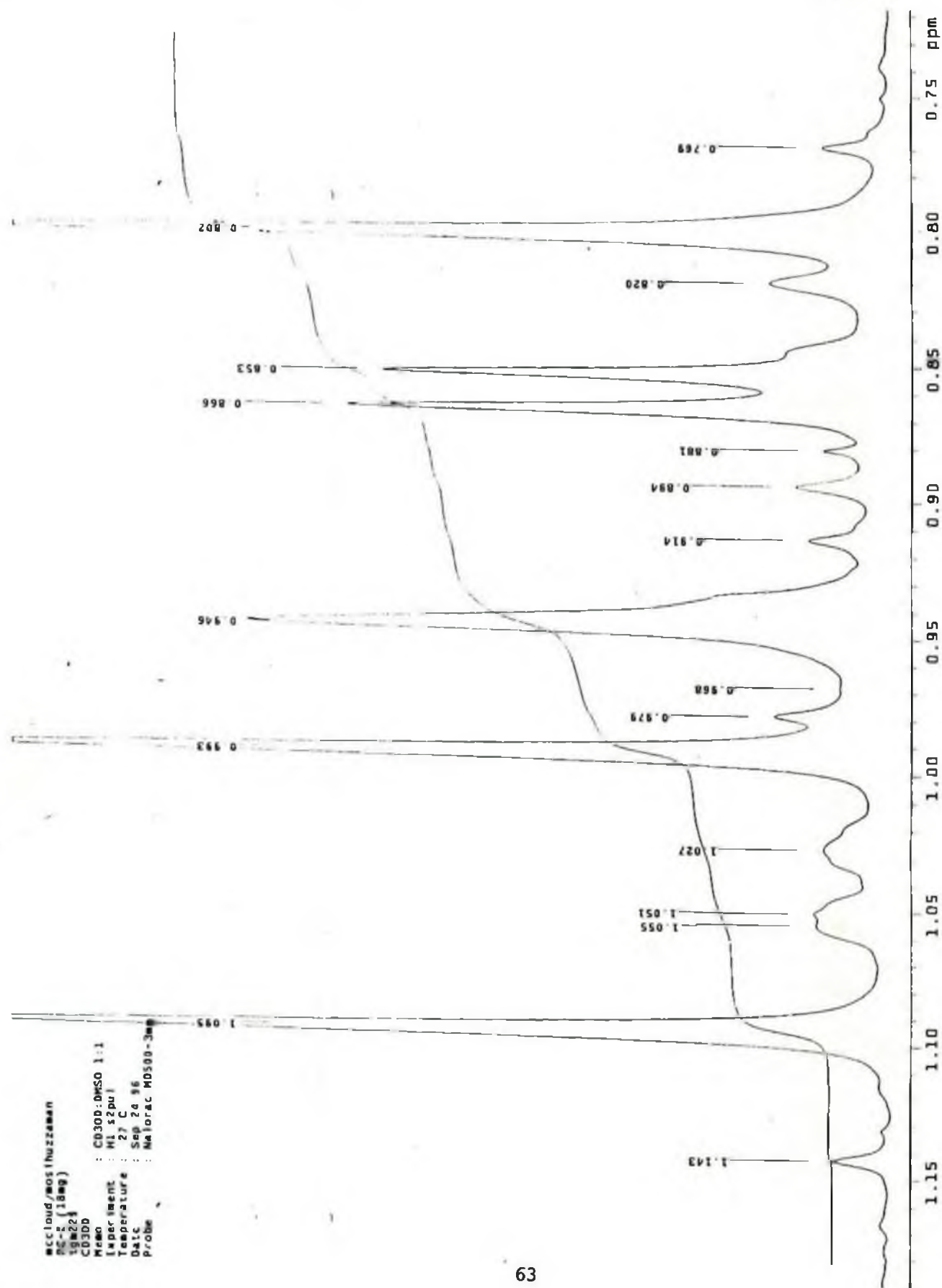


Fig.18b: Partial ^1H -NMR spectrum of compound PC_2 (1.15-0.75 ppm)

```

mccloud/mos1hu22aam
PC-2 (18mg)
tgm230
CD300
NMR
Experiment : C13 s2pu1
Temperature : 27 C
Date : Sep 24 96
Probe : NMRac MDS00-3mm

```

```
exp7 s2pu1
```

```

SAMPL F DEC. 6 VT
date Sep 24 96 dfrq 499.883
solvent CD300 dn H1
file ACQUISITION exp dper 26
sfrq 125.708 do -449.5
tn C13 de YVY
ai 1.000 de 10520
np 56000 dseq 1.0
bw 27881.6 drec 1.0
fs 15400 hmo 29.0
bs 128 texp PROCESSING
tpr 52
pw 5.5 lb 1.00
d1 0 wfile
tof 1250.0 proc f1
nt 61440 fn 131072
ct 5728 math f
a lock
gain 30 werr
f1 n exp svf(*fmo~
ln n c/vnar1/archive/mc~
dp n n cloud/tgm230*)
hs y lbs
nt nn ent
DISPLAY
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wp 27491.6
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ne ph

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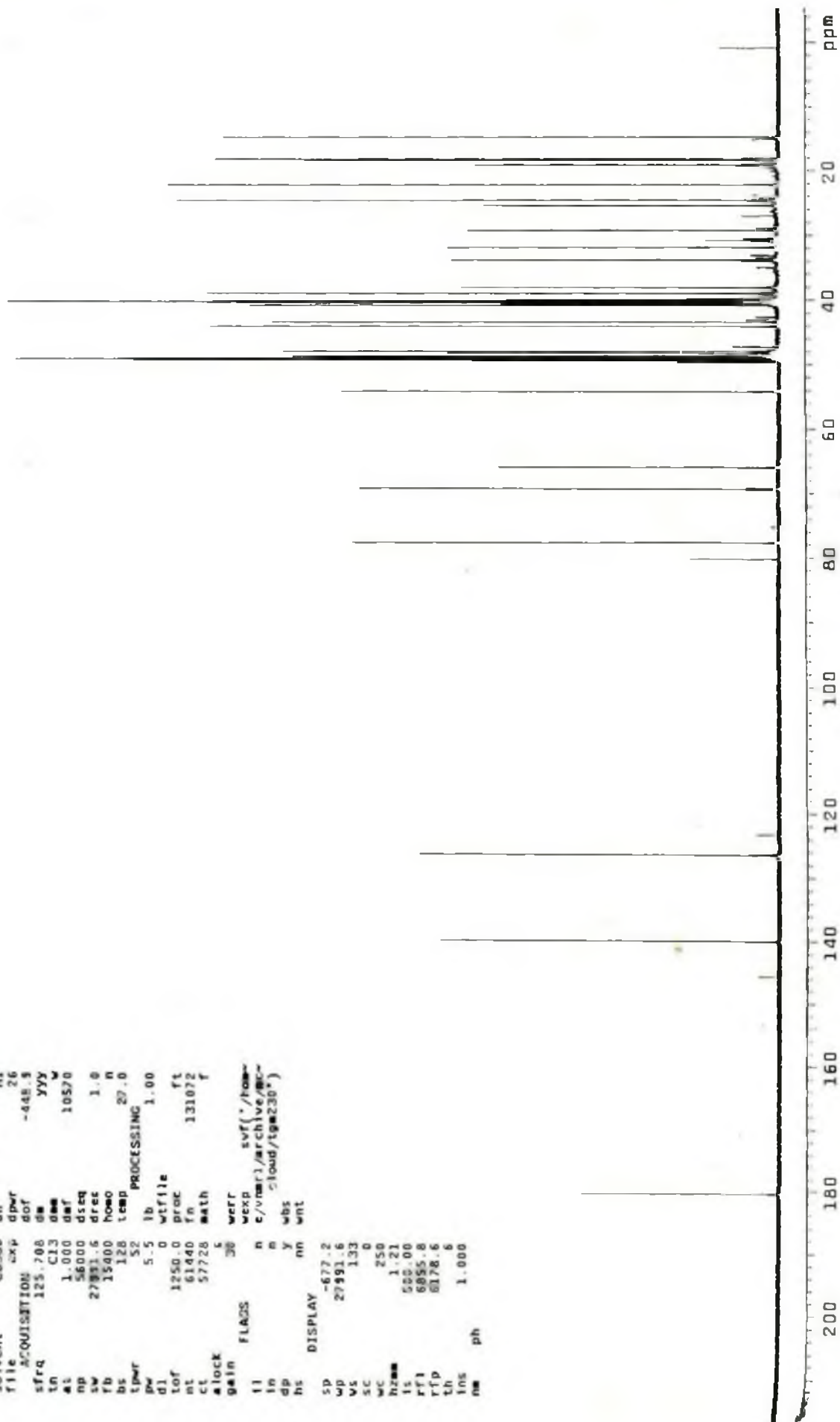


Fig 19: ¹³C-NMR spectrum of compound PC₂

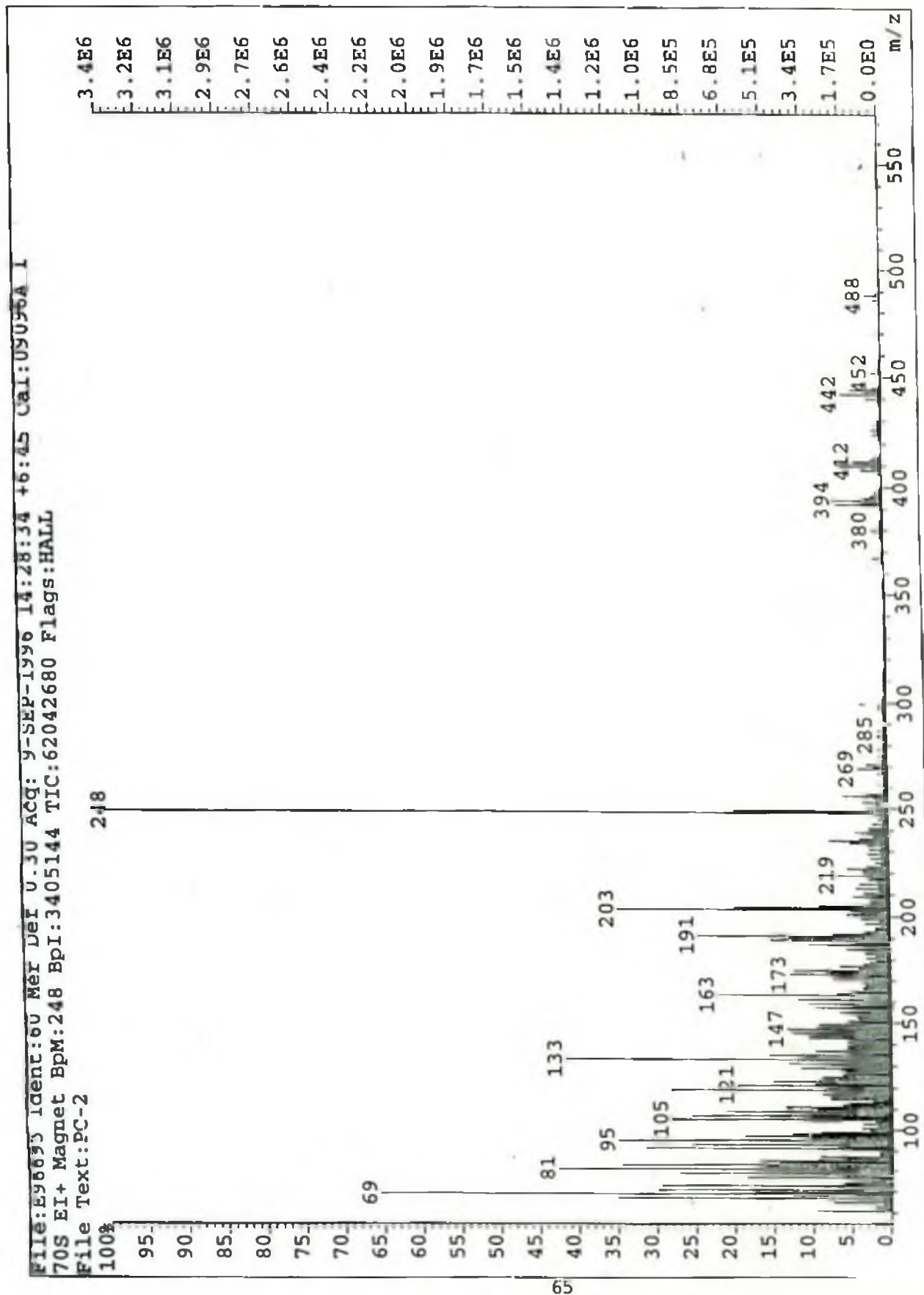


Fig. 20: Mass spectrum of compound PC-2

The ^{13}C -NMR spectrum (Fig. 24) of PC_3 had 51 signals. The ^{13}C spectrum of this compound had peaks very similar to stigmasterol (compound C_4) and stigmasterol glucoside. But some other signals corresponded to those β -sitosterol glucoside. The ^{13}C -NMR data of the compound PC_3 was compared with the ^{13}C -NMR data of known steroidal compounds β -sitosterol³⁹, β -sitosterol glucoside, stigmasterol⁵⁹ and stigmasterol glucoside. It was found that the major portion of the data fitted with stigmasterol glucoside and other minor data fitted with β -sitosterol glucoside. The comparison of the ^{13}C -NMR signals are given in Table-10. As may be seen from the Table some signals overlapped so that the total number of signals (51) were less than the number (70) of signals expected from the mixture of the two steroidal glucosides.

TABLE-10: COMPARISON BETWEEN ¹³C-NMR SPECTRAL DATA OF B-SITOSTEROL, STIGMASTEROL, B-SITOSTEROL GLUCOSIDE AND STIGMASTEROL GLUCOSIDE.

Serial No.	Chemical shift in ppm				
	B-sitosterol	Stigmasterol	B-sitosterol glucoside	Stigmasterol glucoside	Compound PC ₃
1	140.78	140.80	140.12	140.80	140.43
2	121.59	138.37	121.79	138.10	138.01
3	71.69	129.32	100.90	129.13	128.79
4	56.79	121.69	78.85	121.84	121.17
5	56.11	71.81	76.30	100.95	121.13
6	50.17	56.91	75.67	78.94	100.77
7	46.07	56.06	73.36	76.35	76.89
8	42.33	51.29	70.06	75.70	76.74
9	42.25	50.20	61.60	73.43	76.72
10	39.81	42.35	56.54	70.12	73.43
11	37.31	42.35	55.84	61.86	70.05
12	31.51	40.34	49.99	56.69	61.05
13	36.29	39.74	49.07	55.84	56.24
14	33.95	37.31	47.78	51.10	56.15
15	31.92	36.56	45.66	50.10	55.41
16	31.92	31.94	42.07	49.04	55.32
17	31.57	31.94	39.53	49.04	50.57
18	28.98	31.94	38.41	40.26	49.60
19	28.26	31.69	37.03	39.51	49.58
20	26.42	28.96	36.46	38.51	45.12
21	24.32	25.44	33.70	37.10	41.83
22	23.09	24.39	31.66	36.56	41.72
23	21.11	21.26	29.32	31.73	38.29
24	19.62	21.11	28.93	31.73	36.81
25	19.40	21.11	27.95	31.73	36.20
26	19.07	19.42	25.84	29.41	36.19
27	18.82	19.02	23.99	28.68	33.33
28	12.32	12.29	22.80	25.16	31.39
29	11.87	12.07	20.80	24.13	31.35
30			19.36	20.88	31.31
31			18.90	20.71	29.24
32			18.60	19.02	28.68
33			18.40	18.65	28.48
34			11.53	11.86	27.77
35			11.47	11.77	25.41
36					24.85
37					23.87
38					23.85
39					22.58
40					21.09
41					20.92
42					20.57
43					19.69
44					19.08
45					18.92
46					18.83
47					18.60
48					12.11
49					11.82
50					11.76
51					11.65

From Table-10 it may be observed that ^{13}C -NMR data of PC_3 had signals at 140.43, 138.01, 128.78, 121.17, 100.77, 56.24, 55.41, 50.57, 49.60, 41.83, 41.72, 38.29, 36.81, 36.20, 31.39, 31.35, 28.68, 25.41, 24.85, 21.09, 20.92, 19.69, 18.92, 12.11 and 11.82 ppm which were very similar to stigmasterol glucoside, stigmasterol and compound C_4 (see 3.3). The signals at 140.43, 121.13, 100.77, 56.15, 55.32, 49.58, 45.82, 41.72, 36.19, 33.33, 31.31, 29.24, 28.48, 27.77, 23.87, 22.58, 22.57, 19.08, 18.83, 18.60, 11.76 and 11.65 ppm were very similar to the compound B-sitosterol glucoside and B-sitosterol. The signals at 100.77, 76.89, 76.74, 76.72, 73.43, 70.05 and 61.05 were assigned to six carbons of glucose. Therefore, PC_3 was a mixture of major amount of stigmasterol glucoside and a minor portion of B-sitosterol glucosides. Stigmasterol glucoside and B-sitosterol glucoside were known compounds and their R_f values were very close so no attempt was made to separate them which could have been achieved by the use of preparative HPLC.

3.9 CHARACTERIZATION OF THE COMPOUND PC_3

The compound PC_3 was a colourless UV inactive white solid which melted at 80°C . The UV spectrum of PC_3 (Fig. 6) had absorption maximum at 320 nm indicating the presence of carbonyl group. The IR spectrum of compound PC_3 (Fig. 7) had absorption at 2900 and 2850 cm^{-1} due to aliphatic $-\text{C}-\text{H}$ stretching. The absorption band at 1615 cm^{-1} indicated $\text{C}=\text{C}$ stretching. Absorption bands at 1450 and 1370 cm^{-1} indicated $-\text{CH}_2$ and $-\text{CH}_3$ bending vibrations, respectively. The broad absorption band at 3400 cm^{-1} was characteristic of hydroxy group. The ^1H -NMR spectrum of PC_3 had a multiplet at 1.98-1.89, singlet at 1.29 and triplet at 0.87 ppm which were assigned to three methyl groups. A multiplet at 5.42 - 5.32, doublet at 4.60 ($J_{1,2}$ 24.0 Hz), and doublet of doublet at 4.29 ($J_{1,2}$ 4.76 Hz) ppm were due to the presence of olefinic protons. A multiplet at 7.44 indicated the presence of phenyl protons. Beside these, there were other signals which could not be directly assigned. Due to shortage of time HMQC, HMBC, COSE, and DEPT experimental NMR data could not be obtained. Therefore, with the available insufficient information it was not possible to assign any structure to the compound.

3.10 CHARACTERIZATION OF THE COMPOUND PC₁

The compound PC₁ was a colourless white solid which melted at 119°C. It was soluble in a mixture of chloroform methanol. The UV spectrum of PC₁ (Fig. 11) had absorption at 293 nm indicating the presence of double bond. The IR spectrum of compound PC₁ (Fig. 12) had absorption at 2900 and 2850 cm⁻¹ due to aliphatic stretching. The absorption band at 3300, 1615 and 1530 cm⁻¹ indicated the presence of benzene ring. The ¹H-NMR spectrum of PC₁ had multiplet at 1.74 - 1.22, singlet at 1.25 and triplet at 0.87 ppm which were assigned to three methyl groups. A pentet at 5.37 (J_{1,2} 5.36 Hz) multiplet at 5.33 - 5.32, triplet at 4.60, doublet of doublet at 4.27 and multiplet at 4.07 - 4.04 ppm were assigned to olefinic protons. In absence of further studies it was not possible to assign any structure to the compound.

3.11 CHARACTERIZATION OF THE COMPOUND L₁

The compound L₁ was a colourless white solid which melted 133 - 134°C. The UV spectrum of L₁ (Fig. 26) had absorption maximum at 321 nm indicating the presence of carbonyl group. The IR spectrum of L₁ (Fig. 27) had absorption at 2900 and 2850 cm⁻¹ due to aliphatic stretching. The absorption band at 3300 and 3200 cm⁻¹ were due to-OH stretching vibration. The ¹H-NMR spectrum had a triplet at 1.92, multiplet at 1.60 - 1.44 , singlet at 1.23 and triplet at 0.85 ppm which were assigned to four methyl groups. A multiplet at 5.40 - 5.31 and triplet at 4.03 ppm indicated the presence of olefinic protons. Two doublets of doublet at 3.89 (J_{1,2} 4.96 Hz) and 3.84 (J_{1,2} 4.97 Hz) ppm indicated the presence of a methine proton attached to a carbon containing alcoholic group. Beside these there were other signals which could not be directly characterized. From these insufficient and complex data it was not possible to assign any structure to the compound.

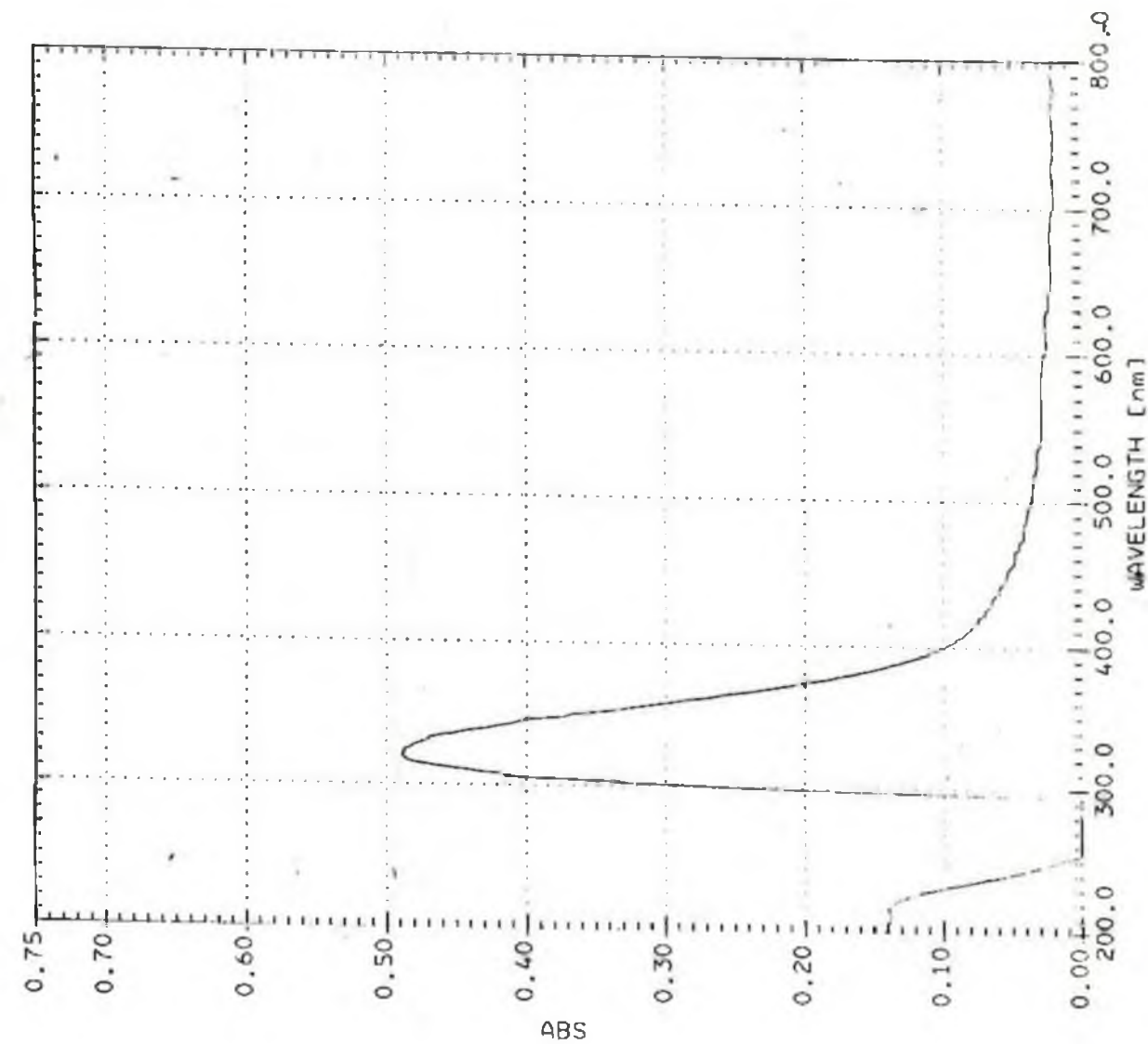


Fig.6: UV spectrum of compound PC₃

*** PEAK-PICK ***

-- PEAK --		-- VALLEY --	
No.	λ	ABS	λ
1	320.0	0.489	748.0
2			285.0

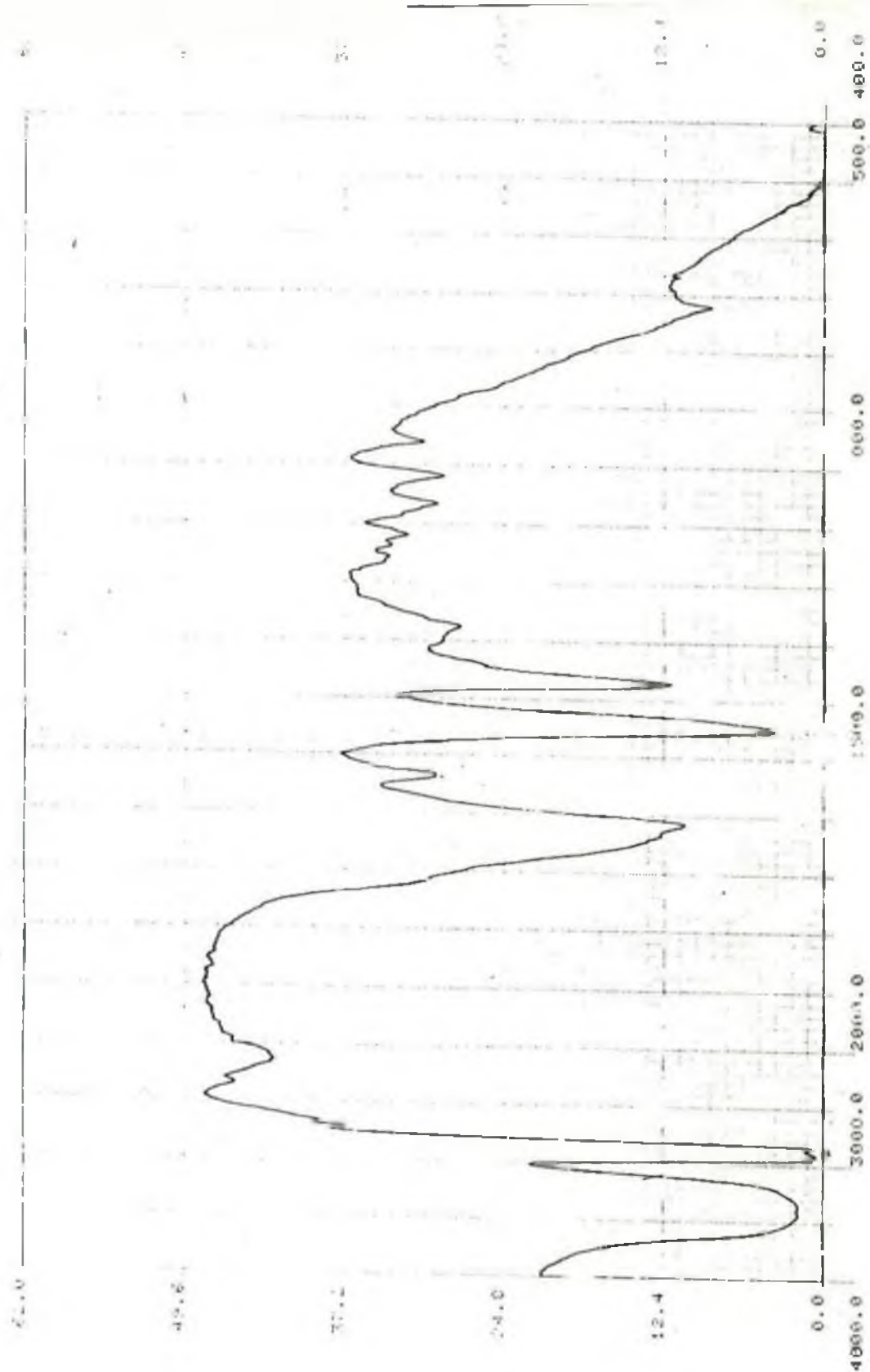


Fig.7: IR spectrum of compound PC₃

```

acc loud/mosibuzzaman
PC-3
19m231
DMSO
Memo      : 20mg in CDCl3:DMSO 1:1
Experiment : H1 12pu1
Temperature : 30 C
Date      : Sep 30 96
Probe    : Nalorac MD500-3mm
exp3 12pu1

=====
date Sep 30 96 dfrq 499.883
solvent DMSO dn H1
file ACQUISITION exp dpr 30
sfrq 499.884 dm -579.7
tn H1 dm nm
at 5.082 dmf 200 C
np 6582.4 dseq
sv 6498.8 dres 1.0
fb 3600 homo
bs 16 temp 30.0
ss 1 PROCESSING
tpr 60 lb 8.30
pw 3.0 wflke
dl 5.000 proc FL
tof 300.0 fn 131072
nt 32 math f
ct 32 verr
gain n werr svf(../hom-
e/vmar1/archive/ac-
cloud/19m231)
ll in n wbs vtl
dp n y wnt
hs DISPLAY nm
sp -426.3
wp 6493.8
vs 3781
sc 35
wc 215
hznm 0.14
ls 3780.43
rf1 426.3
zfp 6
th 3
ins 47.002
nm ph

```

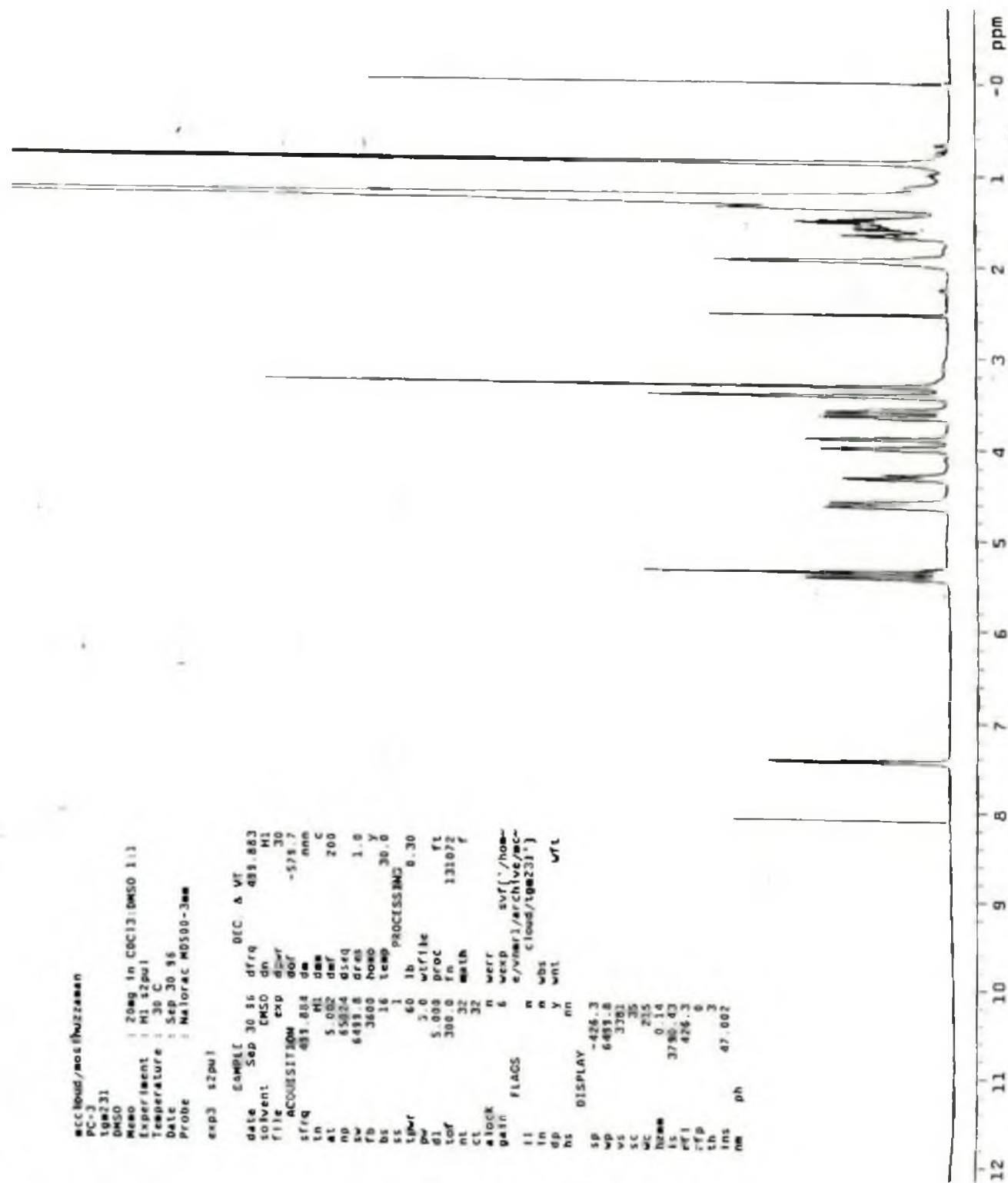


Fig. 8: ¹H-NMR spectrum of compound PC₃

```

#cloud/ios1hu22aah
PC-3
20m232
CDC13
200g in CDC13:DMSO 1:1
Experiment : C13 s2pu1
Temperature : 27 C
Date : Sep 30 96
Probe : Nalorac MDS00-3mm
exp6 s2pu1

SAMPLE
date Sep 30 96 dfrq DIC & VT
solvent CDC13 dn 499.881
file exp 26
ACQUISITION
exp -43d.3
sfrq 125.708 da yvy
in C13 da 10570
at 1.001 arf 10570
np 5.332 dseq n
sw 27981.6 dres 90.0
fb 15400 hmo n
bs 128 temp PROCESSING 27.0
tpur 52
pw 5.5 tb 1.00
d1 0 wfile
toF 1250.0 proc ft
nt 61440 fr 131072
ct 57854 mull f
clock n
gain 30 wery
flags m wexp svf( "/noe-
e:/user1/archive/sc-
cloud/igm232")
in n
dp n y wbi
hs mm woi
DISPLAY
sp -1685.4
wp 27991.6
vc 171
sc 0
wc 250
hzmm 0.54
ls 28867.15
fft 11364.3
rfp 9678.5
th 7.7
ins 7.763
no cdc ph

```

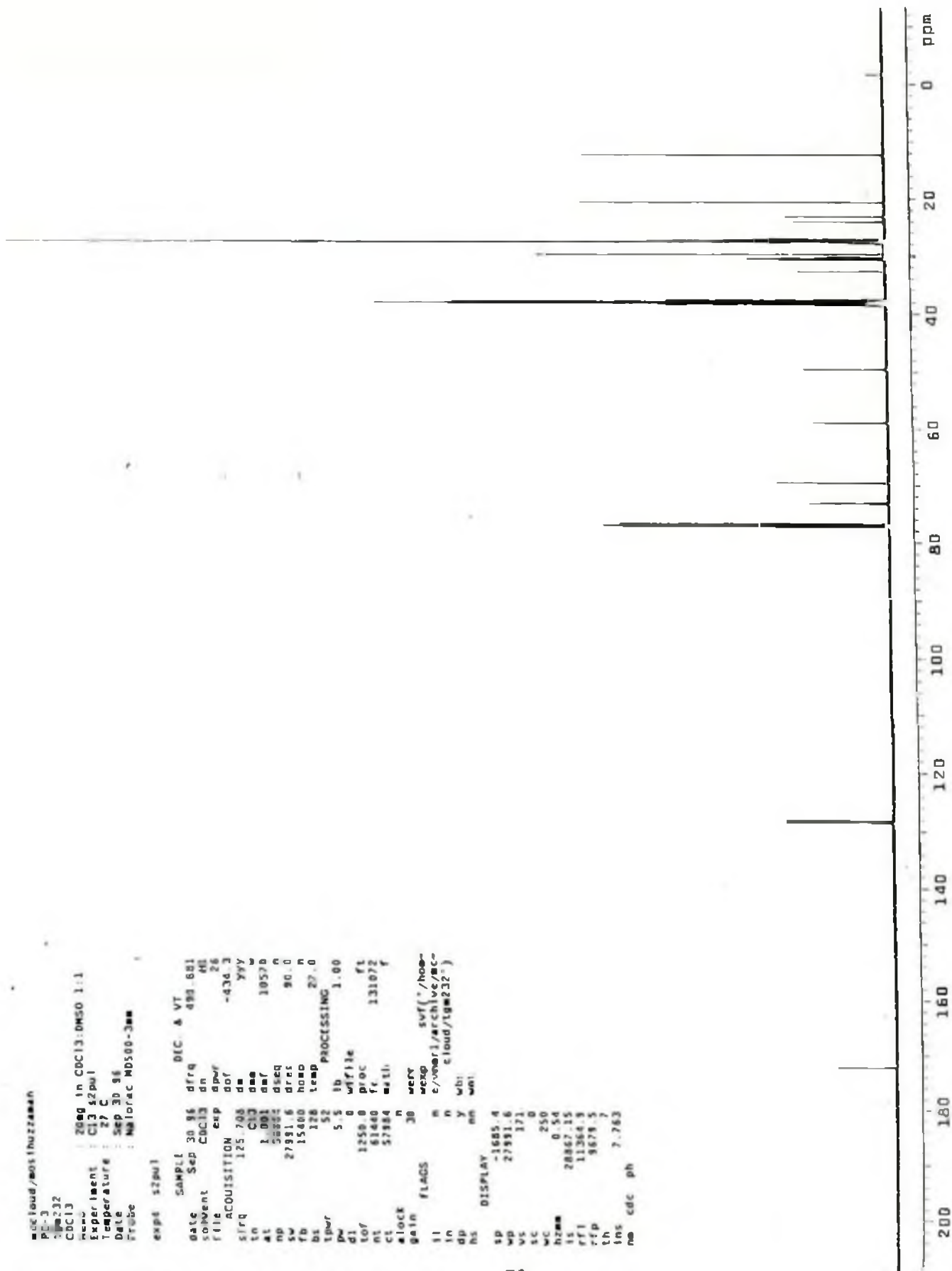


Fig.9: ^{13}C -NMR spectrum of compound PC₃

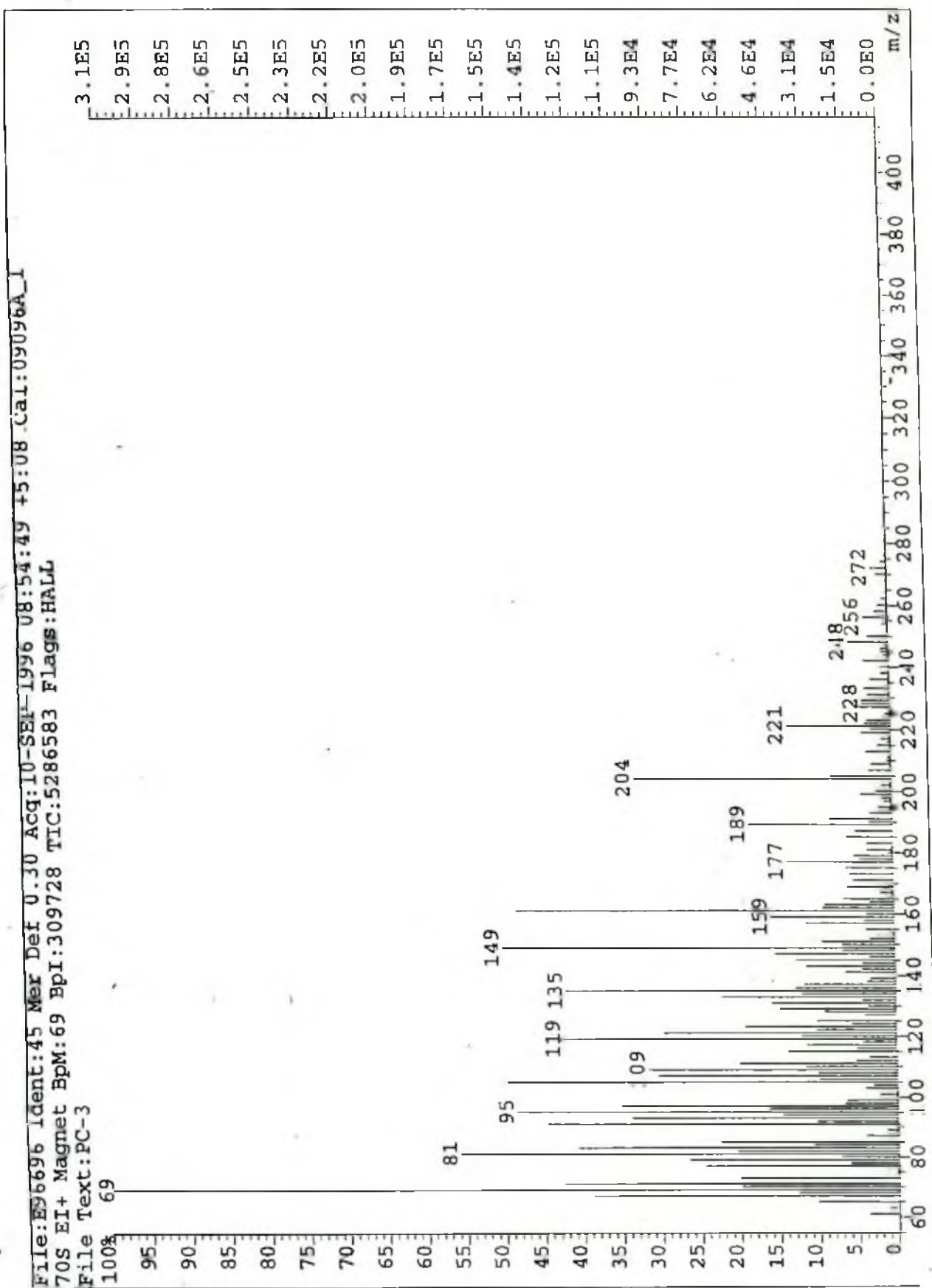
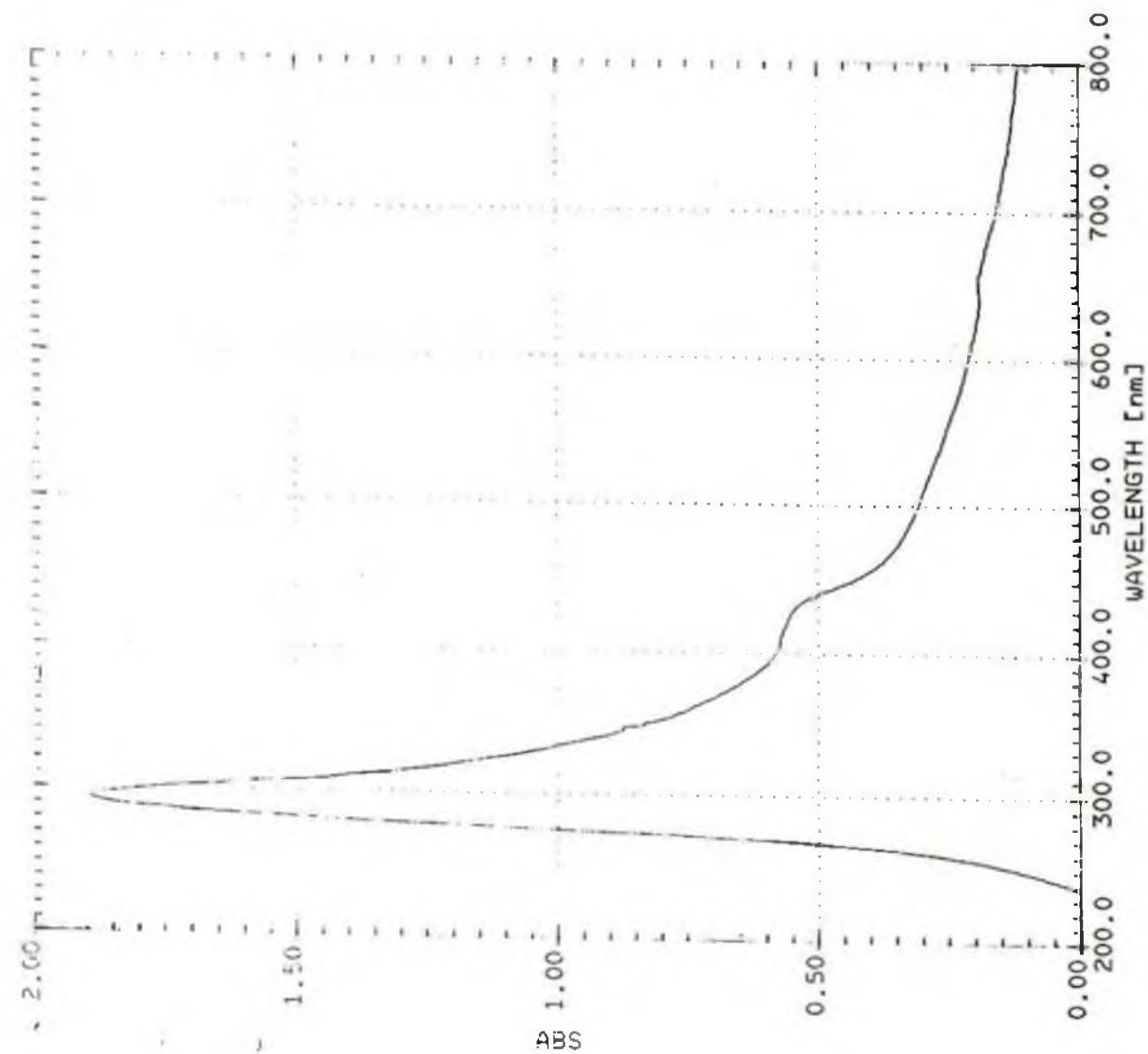


Fig.10: Mass spectrum of compound PC₃



*** PEAK-PICK ***
 -- PEAK -- -- VALLEY --
 No. 1 ABS 4 ABS
 1 293.0 1.84 413.0 -0.082

Fig.11: UV spectrum of compound PC₁

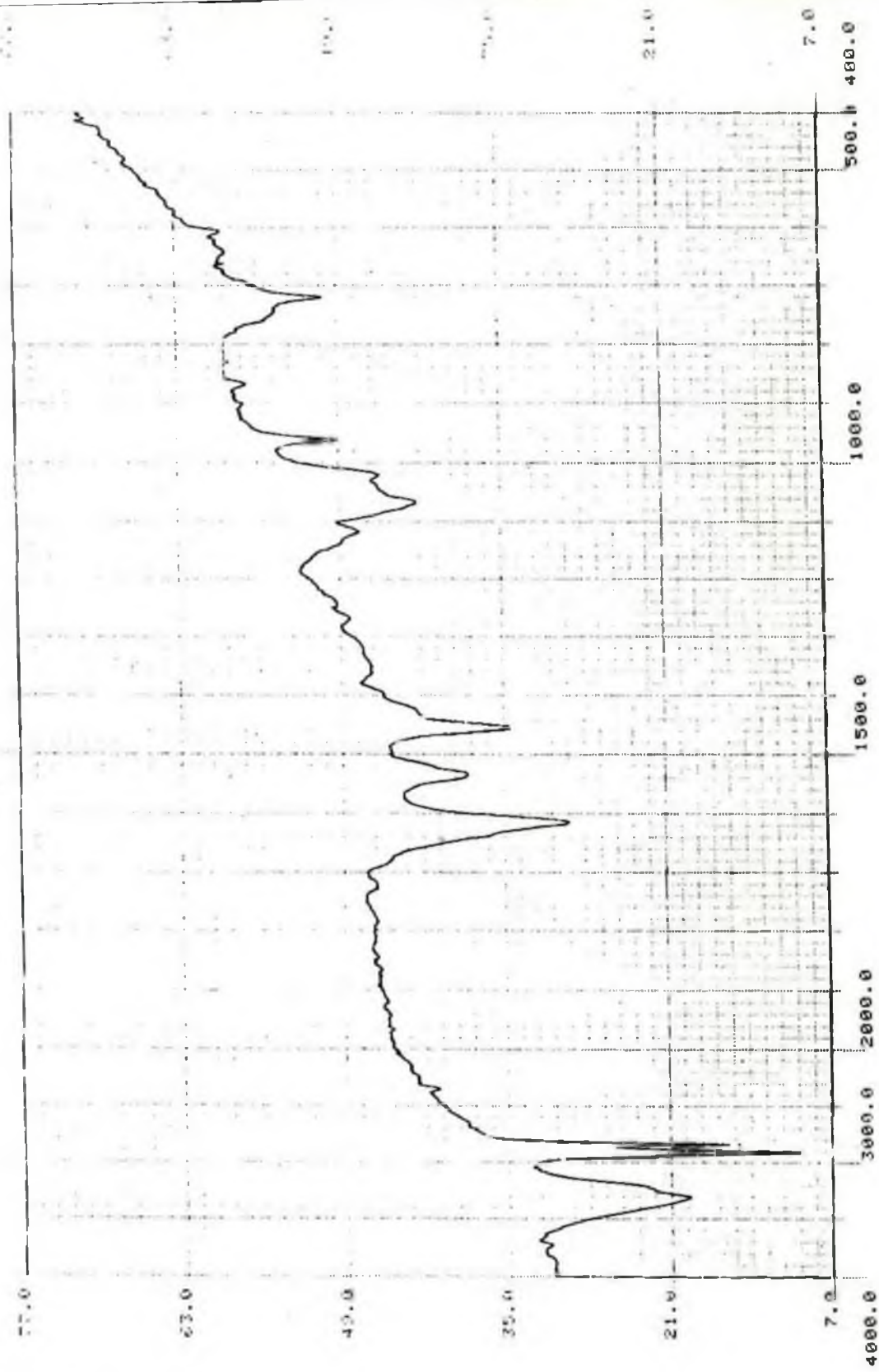


Fig 12: IR spectrum of compound PC1

```

mccloud/moithuzzaman
PC-1 (12mg)
tgm227
DMSO
Name : 150ul C0C13 : 150ul DMSO d6
Experiment : H1 s2pu1
Temperature : 30 C
Date : Sep 18 96
Probe : Nalorac MDS80-3mm
exp6 s2pu1

SAMPLE DEC. & VT
date Sep 18 96 dfrq 499.884
solvent DMSO dn H1
file /home/vme-sper 30
r1/archive/mccloud-sof 16.5
nnn
nnn
ACQUISITION
sfrq 499.884 daf 200
tn H1 dseq
at 5.002 dres 1.0
np 65024 homo Y
sw 6488.8 temp 30.0
fb 3800 lb 0.30
bi 16 wfile
ss 1 wfile
tpr 60 proc ft
pu 5.0 fn 131072
d1 5.000 math f
tof 300.0
nt 32 verr
ct 32 wep svf(*/home-
alock n e/vme1/archive/mc-
gain 5 cloud/tgm227*)
flags wbs wnt wft
ll n
ln n
dp y
hs nn
DISPLAY
sp -414.0
wp 6499.8
vs 1918
sc 35
wc 215
hzam 0.12
ls 9358.70
rf1 414.0
rfp 0
th 13
ins 1.000
na ph

```

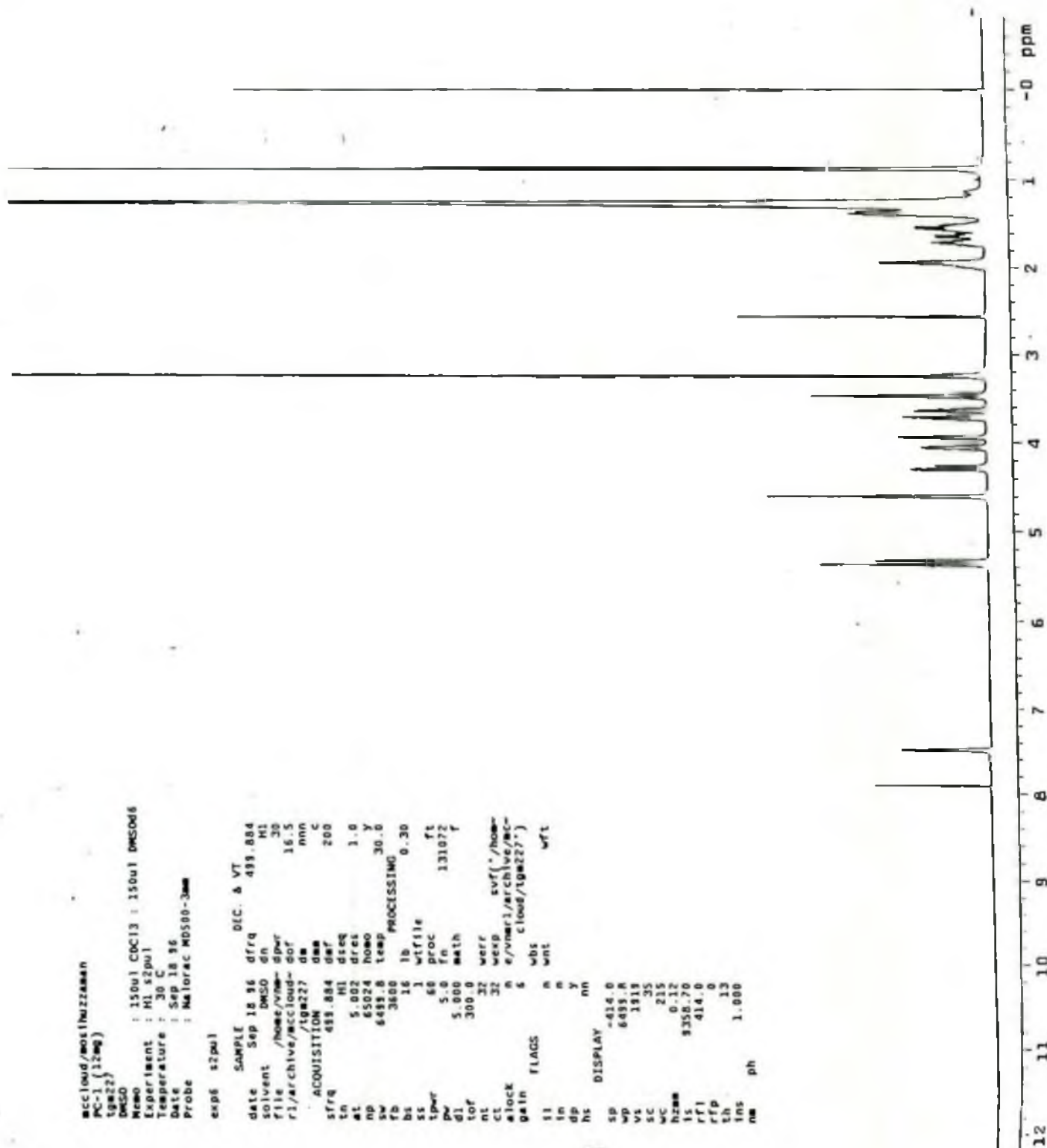


Fig.13: ¹H-NMR spectrum of compound PC₁

```

mccloud/bos/nuzzaman
PC-1 (12mg)
tgm228
DMSO
Miso : 150u1 DMSO : 150u1 CDC13
Experiment : C13 s2pu1
Temperature : 27 C
Date : Sep 18 96
Probe : Mafiac NDS00-3mm
exp7 s2pu1

SAMPLE DEC. & VT
date Sep 18 96 dfrq 499.883
solvent DMSO dn H1
file /home/vme-dwr 26
r1/archive/mccloud-dof -318.0
r1/tgm228 da yyy
ACQUISITION dms 10570
sfrq 125.708 daf
tn C13 dseq 1.0
at 1.000 dres 1.0
np 56000 homo 27.0
sw 27881.6 teap
fb 15400 PROCESSING
bs 128 lb 1.00
tpwr 52 wtfile
pw 5.5 proc ft
d1 0 fn 131072
tof 1250.0 math f
nt 61440
ct 57884 werr
aLOCK 4 exp svf('/home-
gain 30 e/vme1/archive/mc-
cloud/tgm228)
FLAGS n wbt halt
ll in n wnt
dp y
hs mn
DISPLAY
sp -908.3
wp 27981.6
vs 173
sc 0
wc 250
bzmm 4.11
ls 500.00
rf1 5872.8
rfp 4965.5
th 16
ns 1.000
ne ph

```

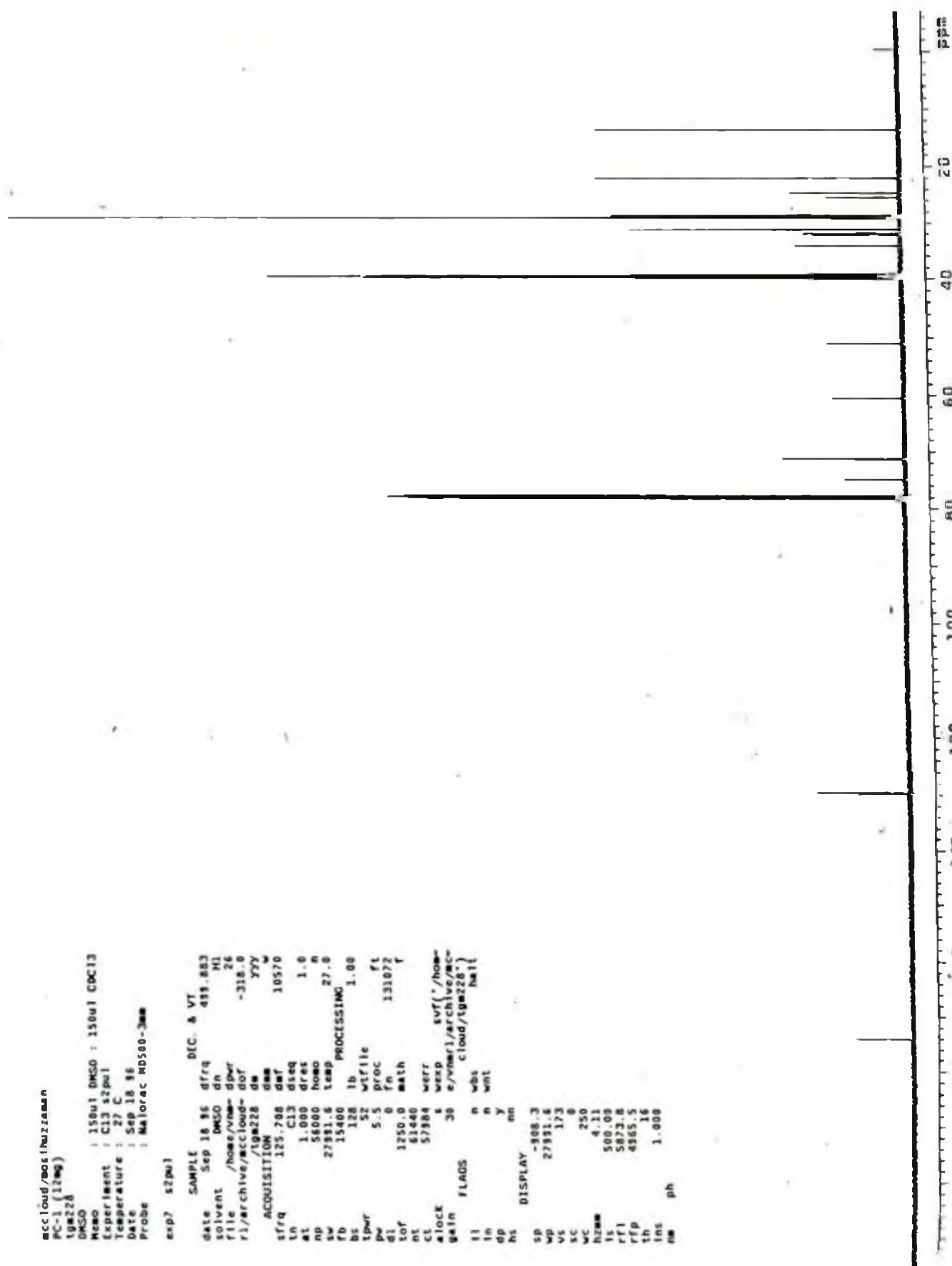
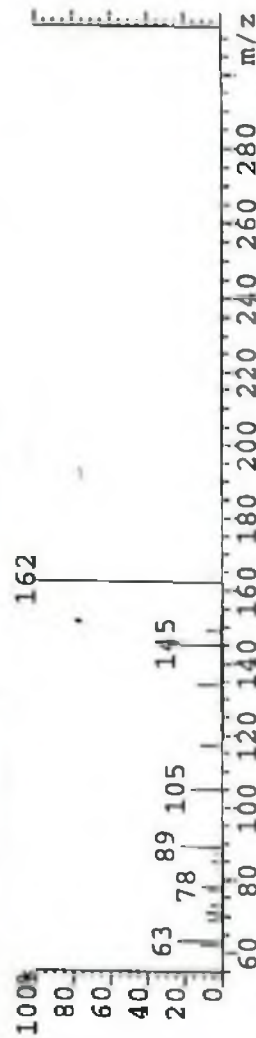


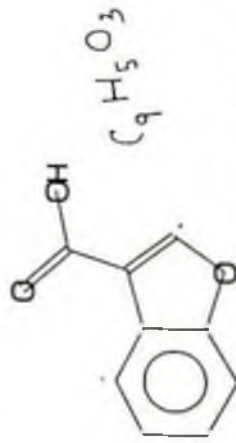
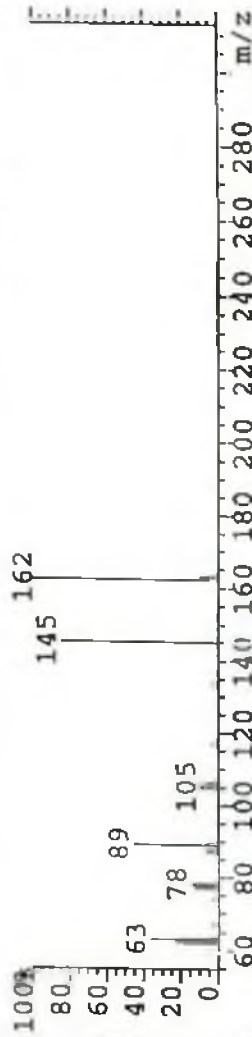
Fig.14: ¹³C-NMR spectrum of compound PC₁

Libraries 1: NIST (62235) 2: NISTREP (12593)

Peaks: 8 5 6 7 3 4 1 0
 Matches: 0 0 3 0 26 175 18712 49800
 Total: 0 0 3 29 204 6316 25028 74828
 Normal Search: 11 of 29 Rank: Purity Mass Weighting: On Presearch Minimum: 20
 File Text: PC-1 File: E96694 Ident: 21 Mer Def 0.30 Acquired: 9-SEP-1996 13:34:54 +2:25
 70S EI+ Function: Magnet TIC: 1152426



Rank: 1 NIST #12870 Purity: 777 Mixture: 785 Matched: 6 MF: C9H6O3 MW: 162 CAS: 26537-68-8 NIST: 65064
 Name: 3-Benzofuran-2-carboxylic acid SMILES: OC(=O)c1ccccc1c2ccccc12



Rank: 2 NISTREP #5411 Purity: 762 Mixture: 835 Matched: 5 MF: C8H6N2O2 MW: 162 CAS: 15804-19-0 NIST: 29
 Name: 2,3-Quinoxalinedione, 1,4-dihydro- SMILES: Oc2nc1cccc1nc2O

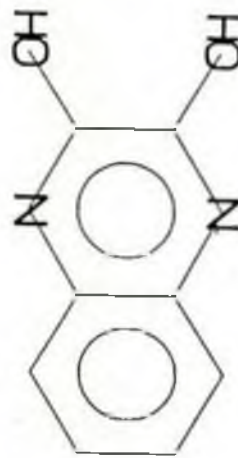
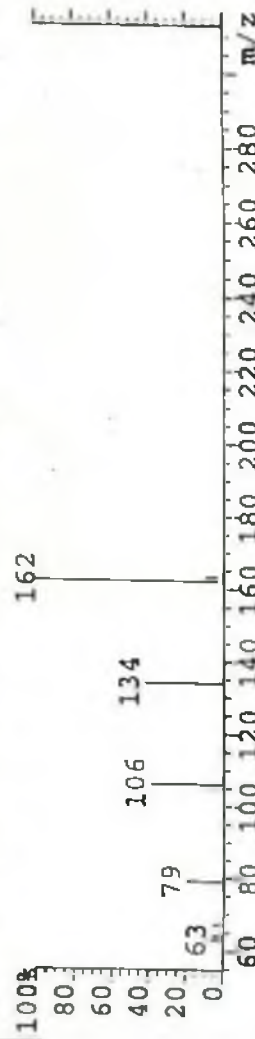


Fig. 15: Mass spectrum of compound PC1

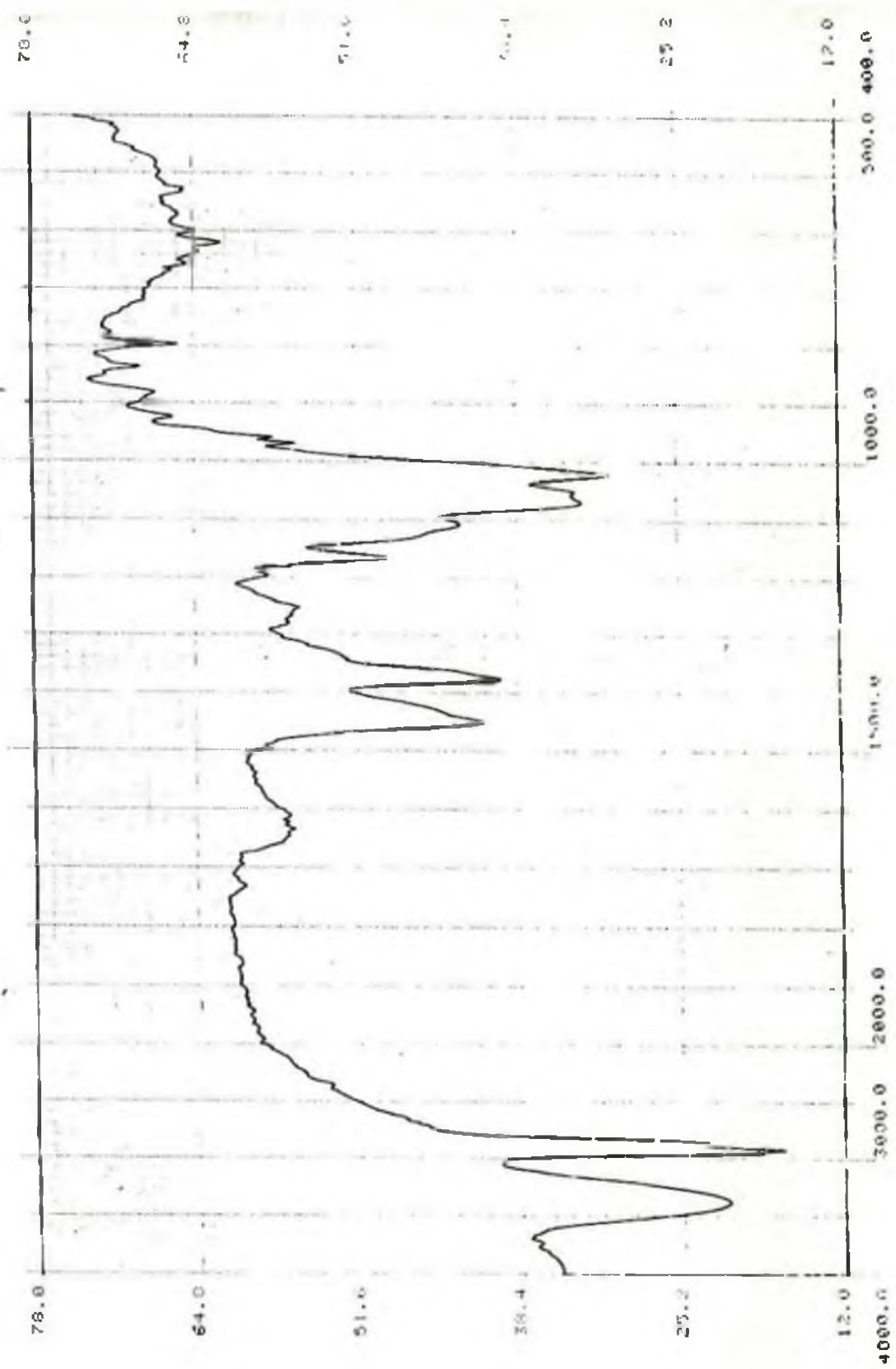


Fig 22: IR spectrum of compound PC₄

```

#cc1oud/mos1hu2zaman
PC-5
tgm235
DMSO
Meso : 9mg/300ul (filtered)
Experiment : H1 s2pu1
Temperature : 27 C
Date : Oct 2 95
Probe : Natorac MD500-3mm

exp3 s2pu1

SAMPLE 2 95 dfrq DEC. & VT
date Oct DMSO dn 439.883
solvent H1
file exp dper 30
ACQUISITION exp dor -434.3
sfrq 439.884 dm nnn
tn H1 dm c
at 5.002 daf 200
np 65024 dseq
sw 6499.8 dres 1.0
fb 3600 homo Y
bs 16 temp 27.0
ss 1 PROCESSING
tpr 60 lb 0.30
pw 5.0 wfile
dl 5.000 proc ft
tof 300.0 fn 131072
nt 32 math f
ct 32
alock n werr
gain 6 wexp svf('homo-
e/vmar1/archive/ac-
cloud/tgm235')
ll n n wbs
in n y wnt wft
ds DISPLAY -442.5
sp 6499.8
wp 374
vs 35
sc 215
wc hzmm 1.01
ls 18036.01
rfi 442.5
rfp 0
th 10
ins 83.971
na ph

```

82

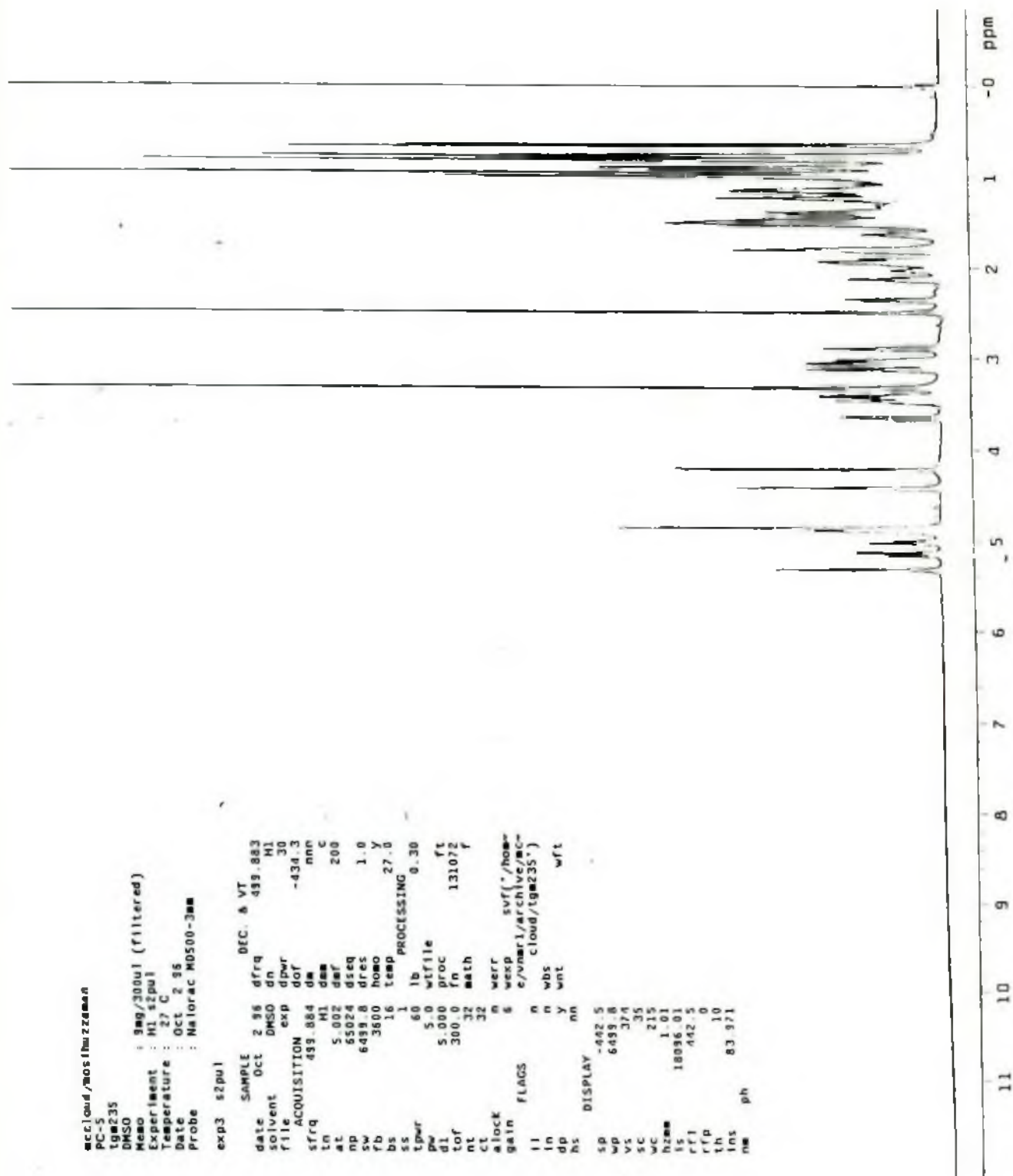


Fig.23: ¹H-NMR spectrum of compound PC₅

```

mccloud/mos(huzzaman
1-3
tga240
DMSO
Memo      : 17mg extracted from a wad
of cotton
Experiment : C13 s2pul
Temperature : 27 C
Date       : Oct 7 '96
Probe     : Nalorac MD500-3mm
exp4 s2pul

SAMPLE DEC. & VT
date Oct 7 '96 dfrq 419.883
solvent DMSO d1 M1
file ACQUISITION exp dpr 26
      -1139.5
      YYY
dfrq 125.708 dm
tn C13 dam 10570
at 1.000 def
np 58000 dseq 1.0
sw 27931.6 dret
rb 15400 homo
bs 128 temp 27.0
ss 4 PROCESSING 1.00
tpwr 52 lb
pw 5.5 wfile
d1 0 proc ft
tof 1250.0 fn 131072
mt 61440 math f
ct 58112
alock s
gain not used
flags m wbp svf"/hom-
      e/vmer1/archive/mc-
      cloud/tga240")
ls in m wbs
dp in y wnt
hs DISPLAY nn
sp -833.0
wp 27931.6
vs 557
sc 0
wc 250
hzmm 0.82
ls 500.00
rfi 5858.4
rfd 4165.5
th 1.4
lms cdc ph
nm 1.000

```

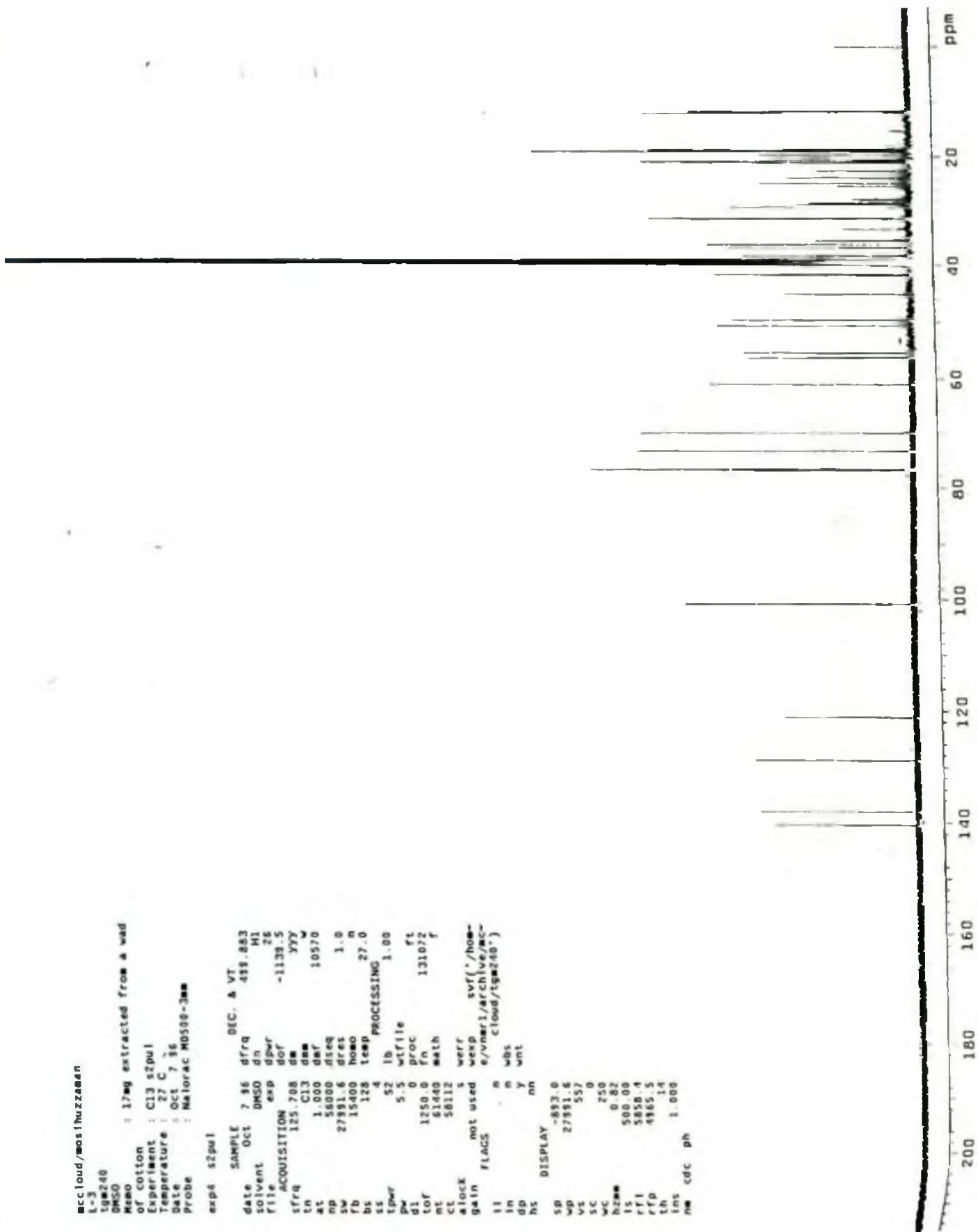


Fig.24: ^{13}C -NMR spectrum of compound PC₅

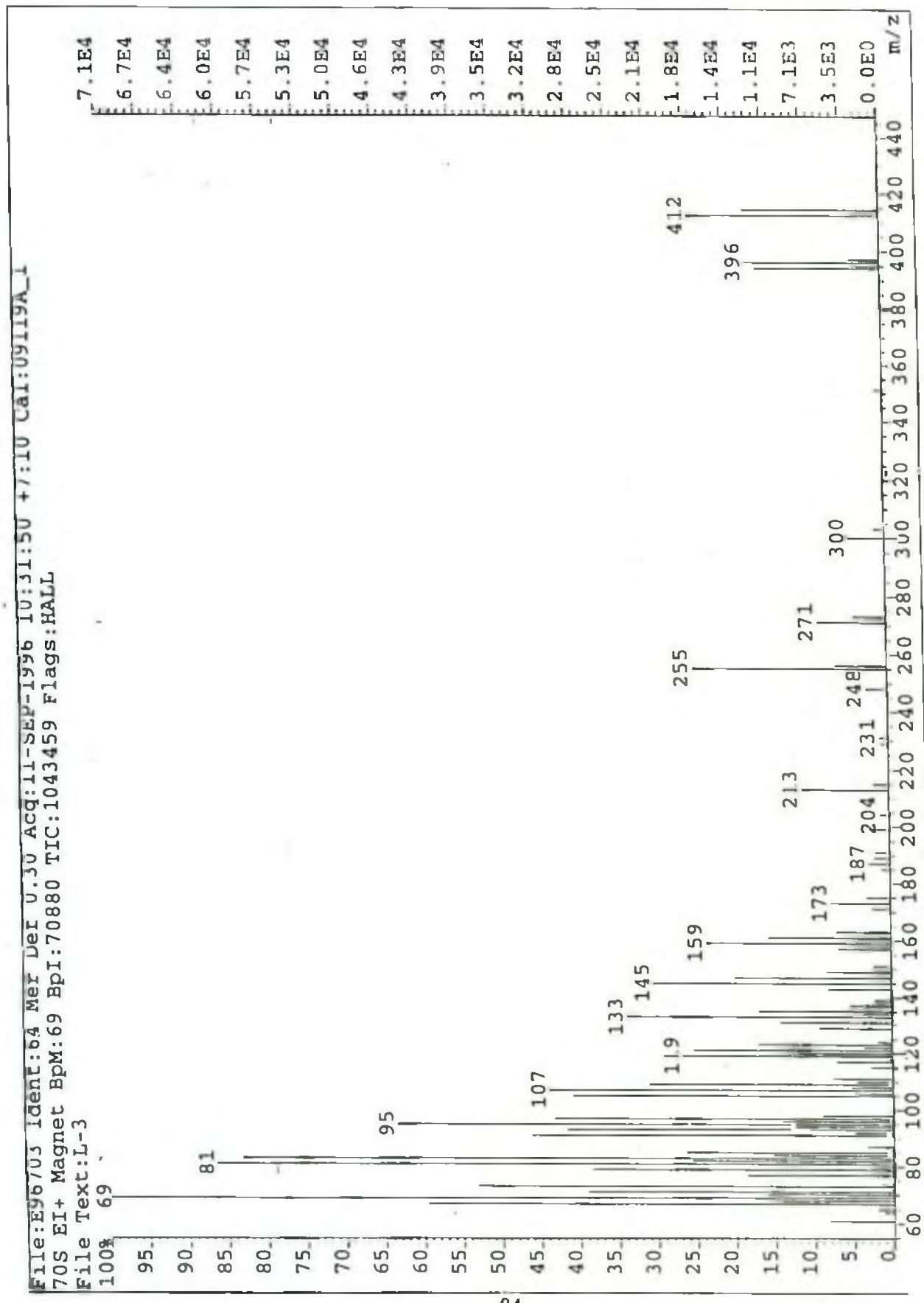


Fig.25: Mass spectrum of compound PC4

```

*** PEAK-PICK ***
-- PEAK -- -- VALLEY --
No.  λ      ABS      λ      ABS
-----
1  321.0  0.545    787.0  0.046
2  285.0 -0.358

```

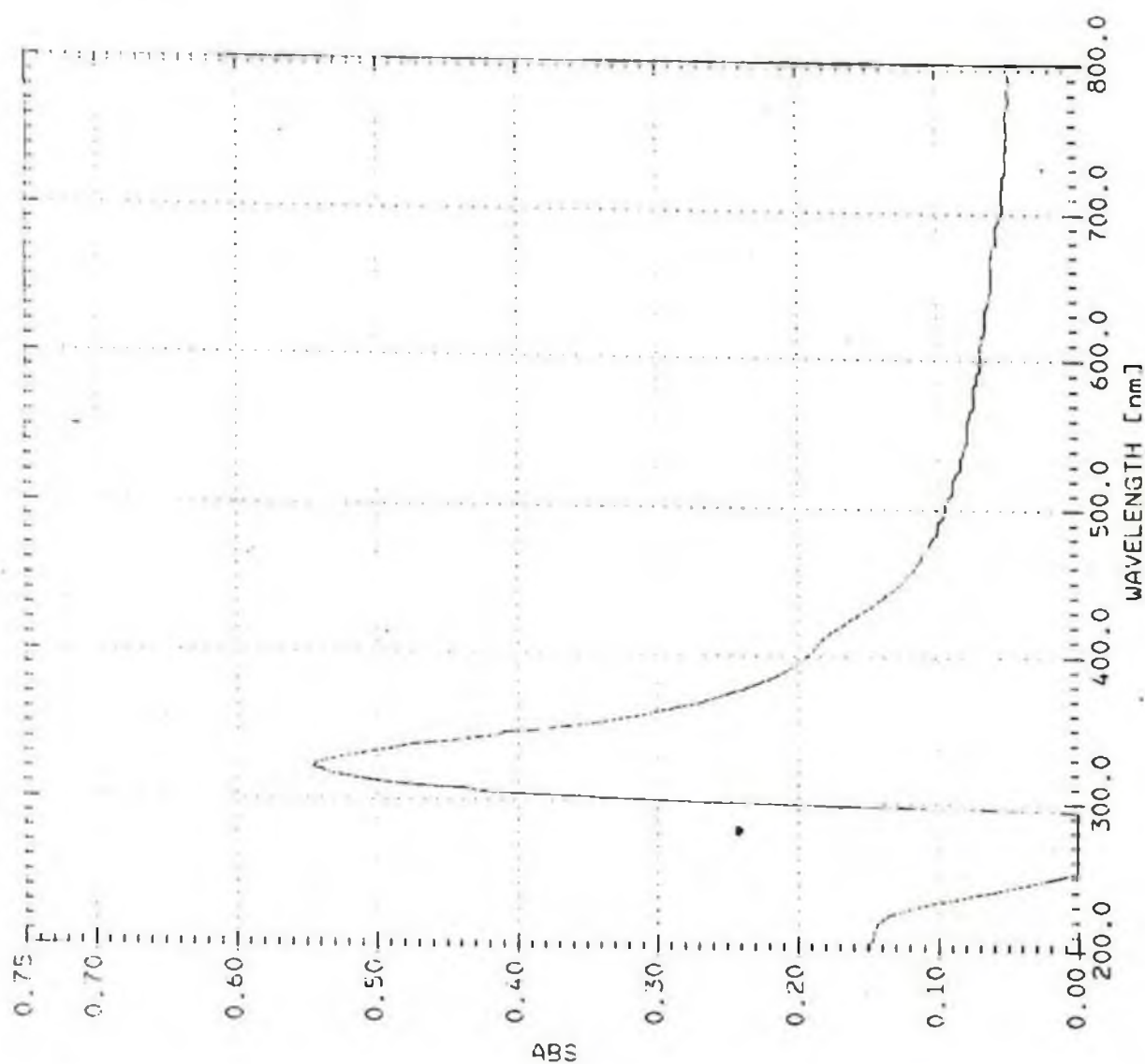


Fig.26: UV spectrum of compound L₁

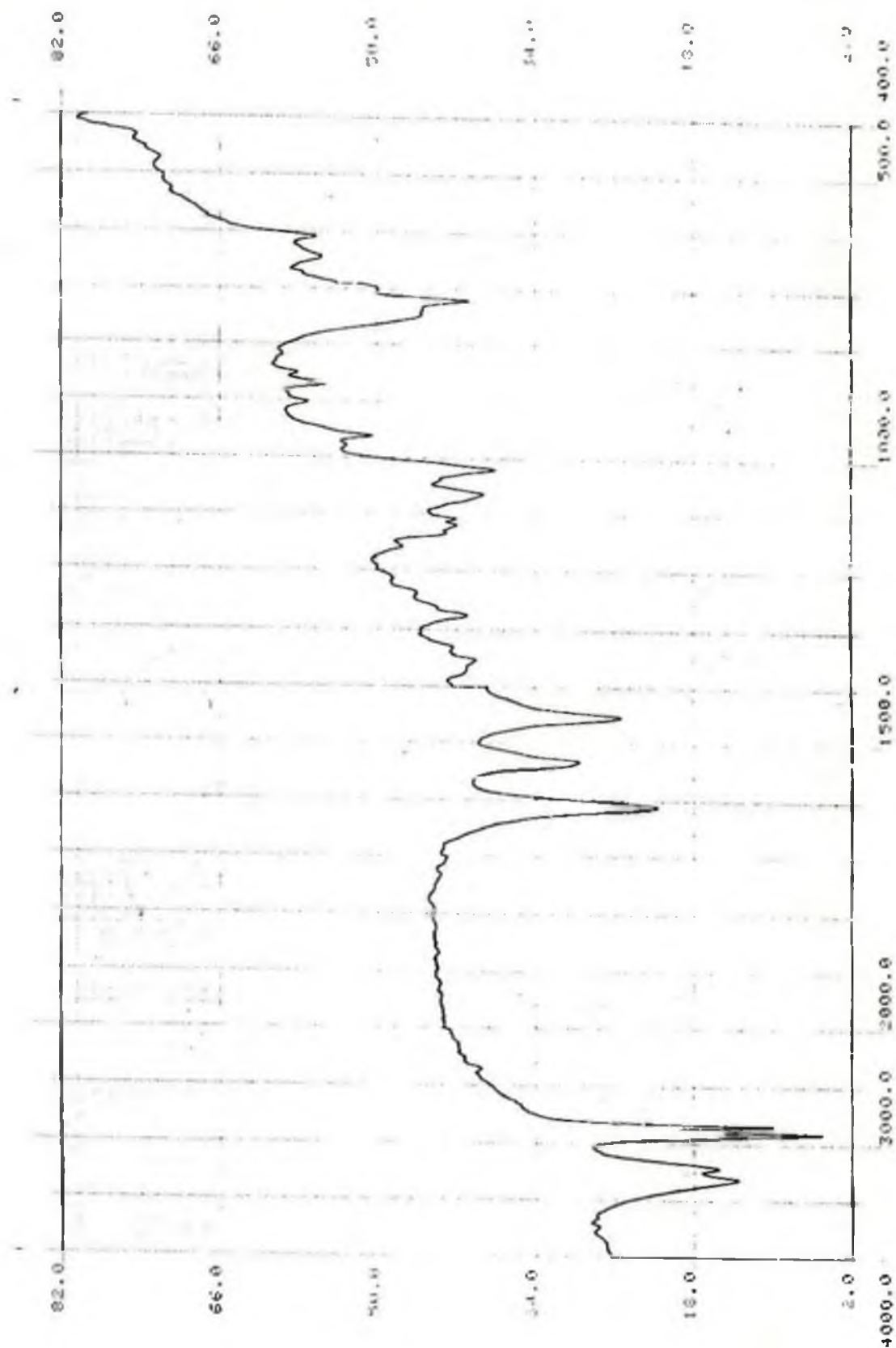
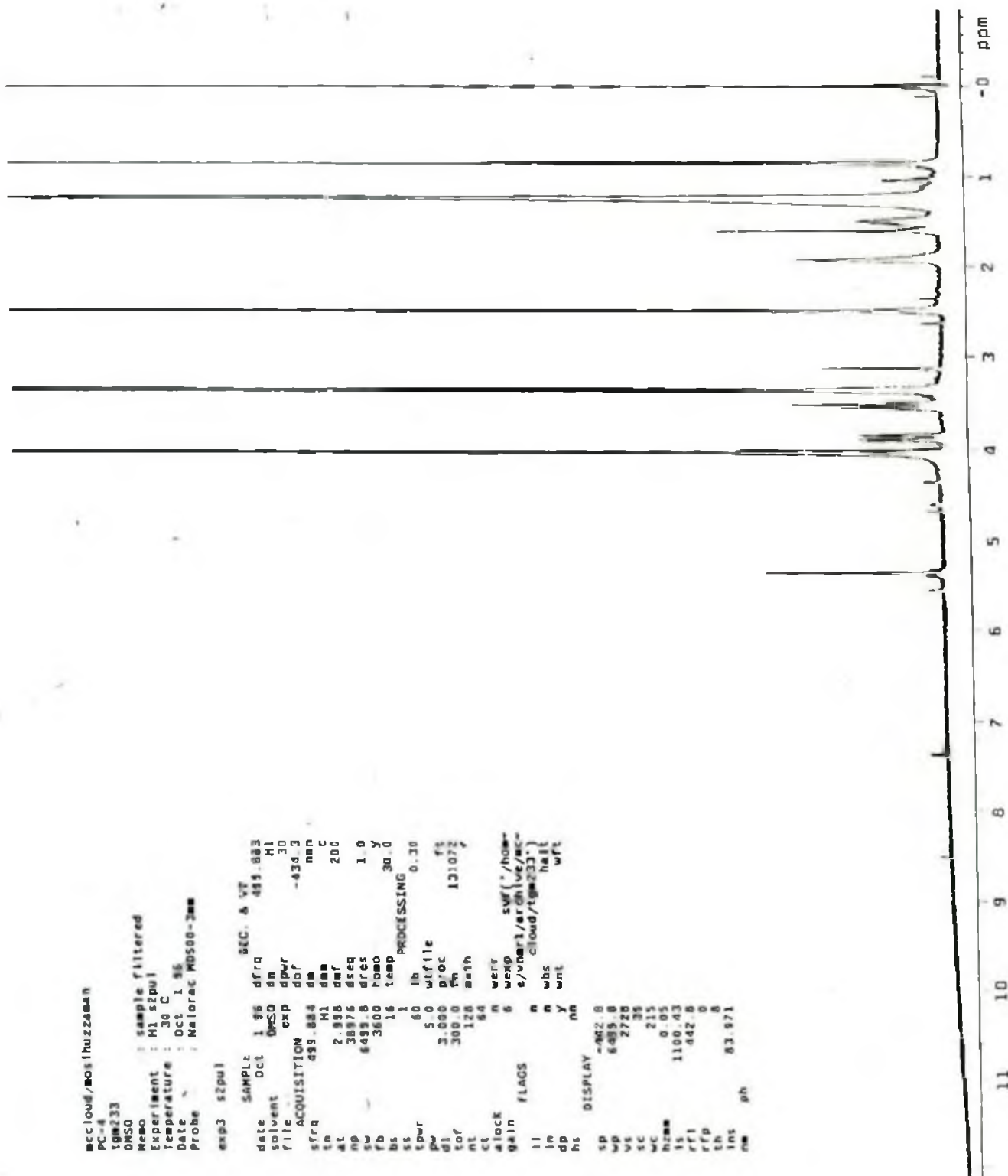


Fig.27: IR spectrum of compound L₁

```

cccloud/mos/huzzamh
PC-4
100233
DMSO
Memo : sample filtered
Experiment : M1 s2pul
Temperature : 30 C
Date : Oct 1 95
Probe : Nalorac MDS00-3mm
exp3 s2pul
SAMPLE SEC. & VT
date Oct 1 95 dfrq 459.883
solvent DMSO d1 H1
file exp dper 30
ACQUISITION ddr -43d.3
nnc
sfrq 459.884 dm
in M1 dm c
at 2.958 ddr 200
np 38976 dseq
pw 6499.8 dres 1.0
fb 3600 romo y
ss 16 temp PROCESSING 30.0
tpwr 60 lb 0.30
pu 5.0 wfile
d1 3.000 proc fs
tor 300.0 fm 131072
nt 128 math
ct 64
a1ock n
gain 6 wexp svt('hnm-
e/vnart/archive/cc-
cloud/100233')
ll n wbs halt
dp n y wnt
ns nm
DISPLAY -402.8
sp -402.8
wp 6499.8
vt 2720
sc 35
wc 215
hzmm 0.05
fs 1100.43
rfl 642.8
rfp 0
th 0
ins 83.971
ne ph

```

Fig.28: ¹H-NMR spectrum of compound L1

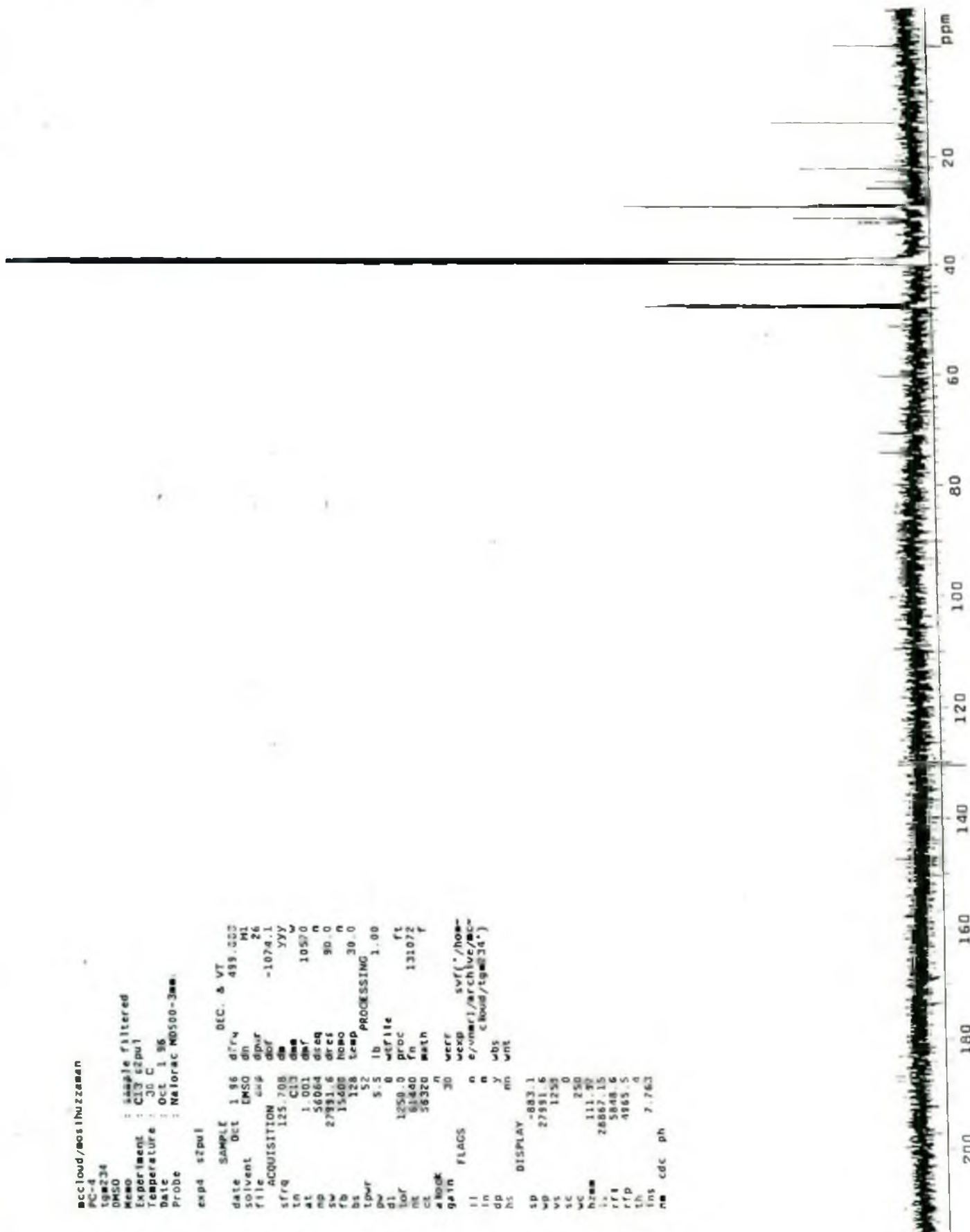


Fig. 29: ^{13}C -NMR spectrum of compound **Li**

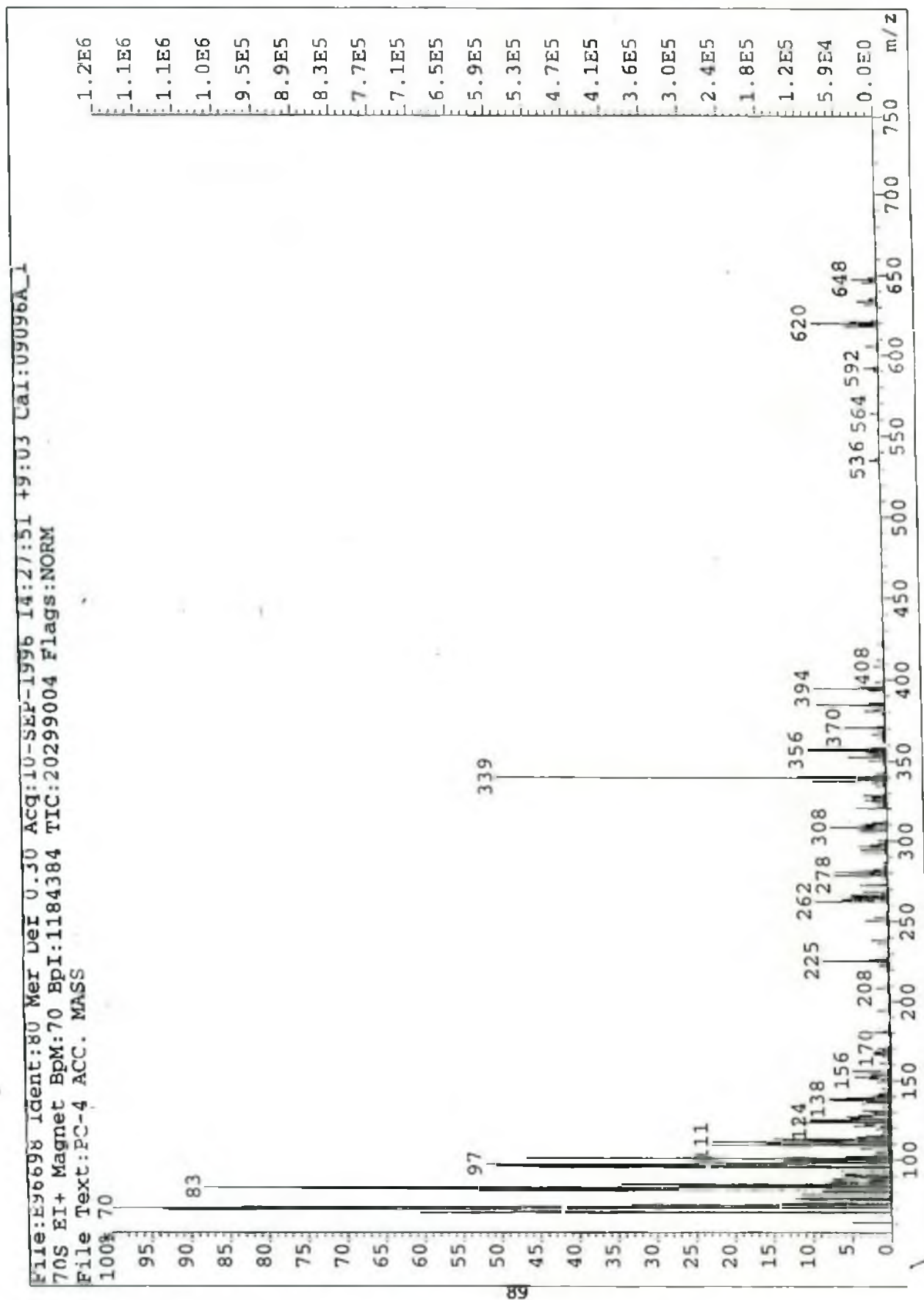


Fig.30: Mass spectrum of compound L1

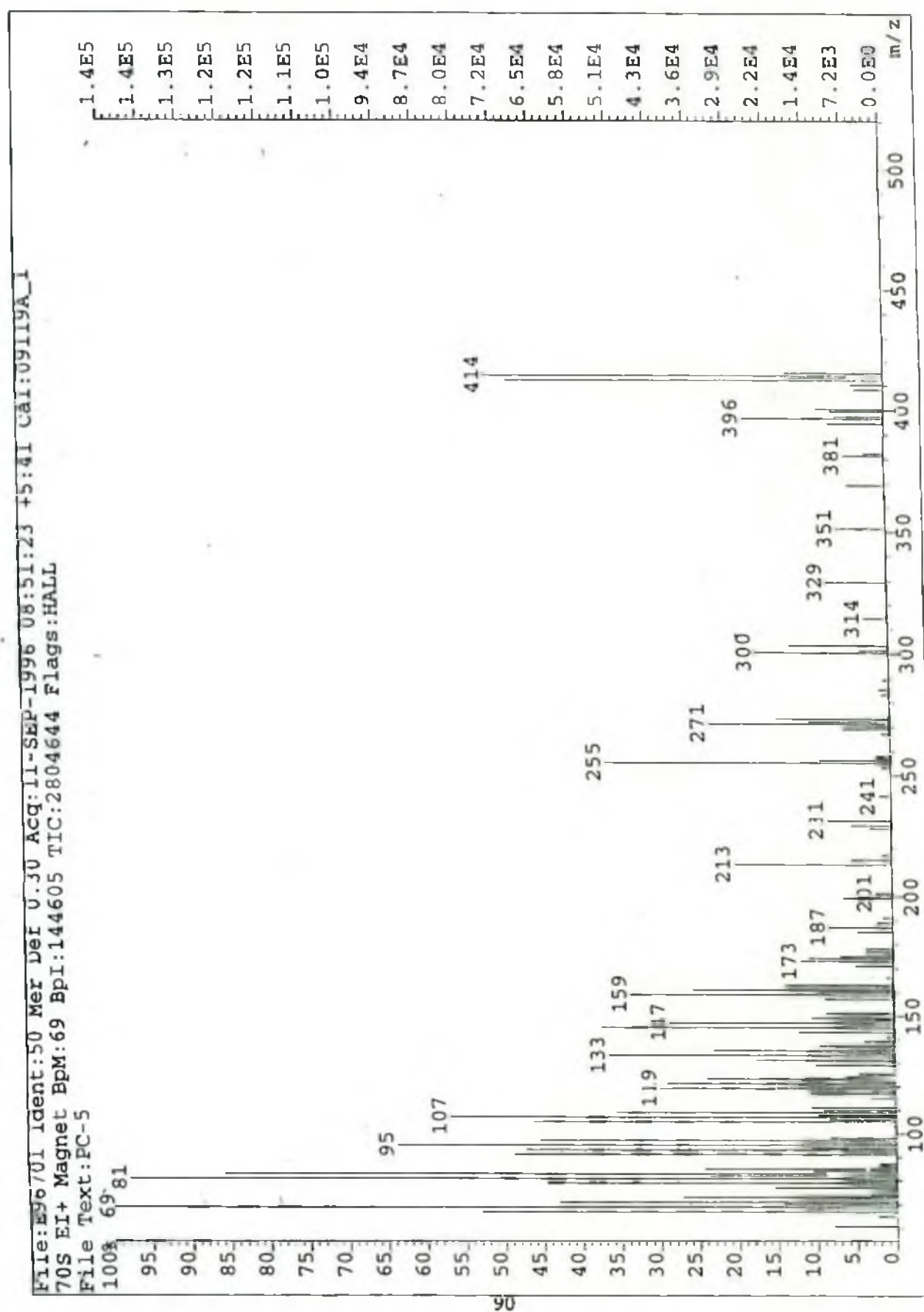


Fig.31: Mass spectrum of compound L3

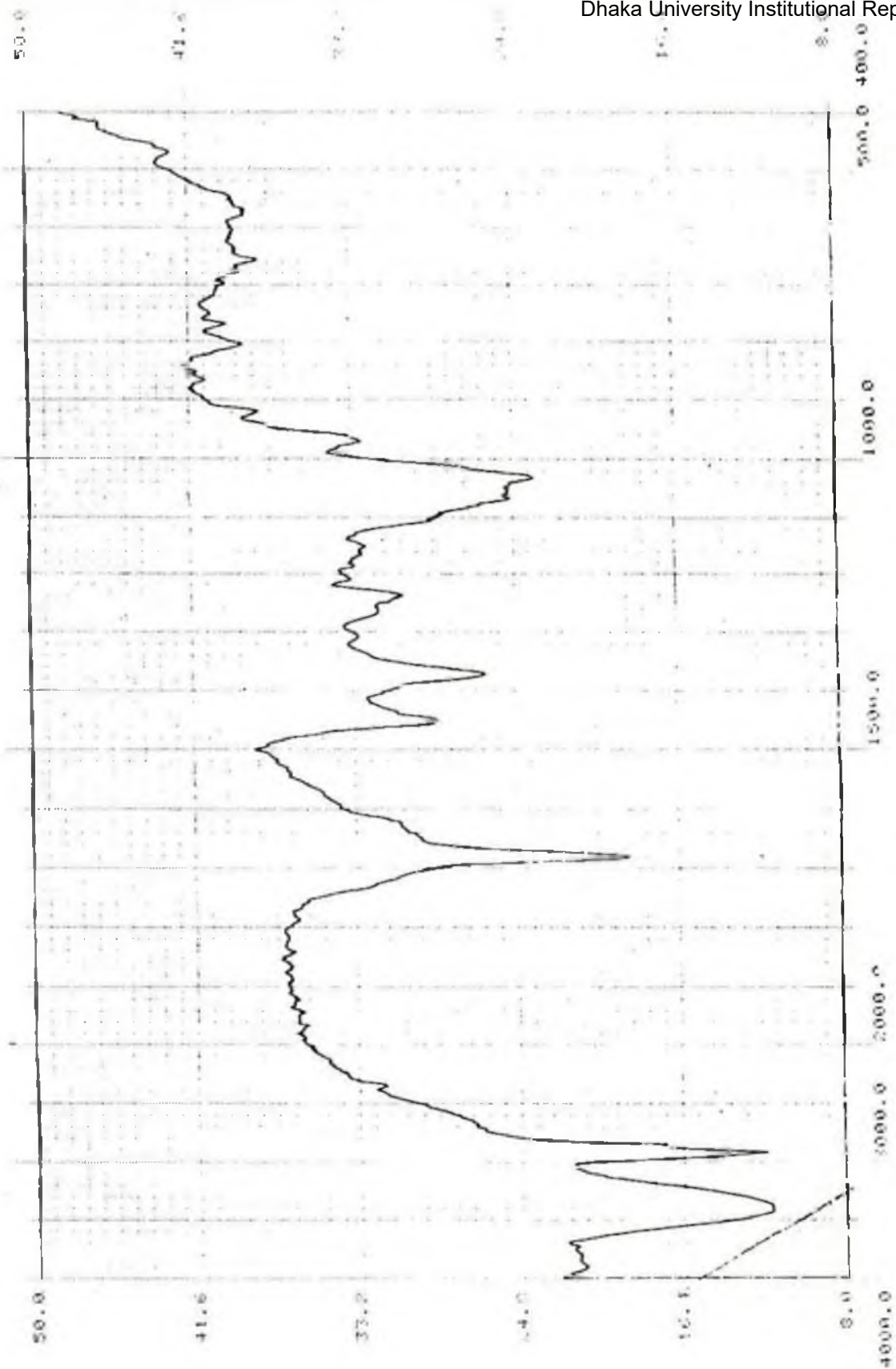
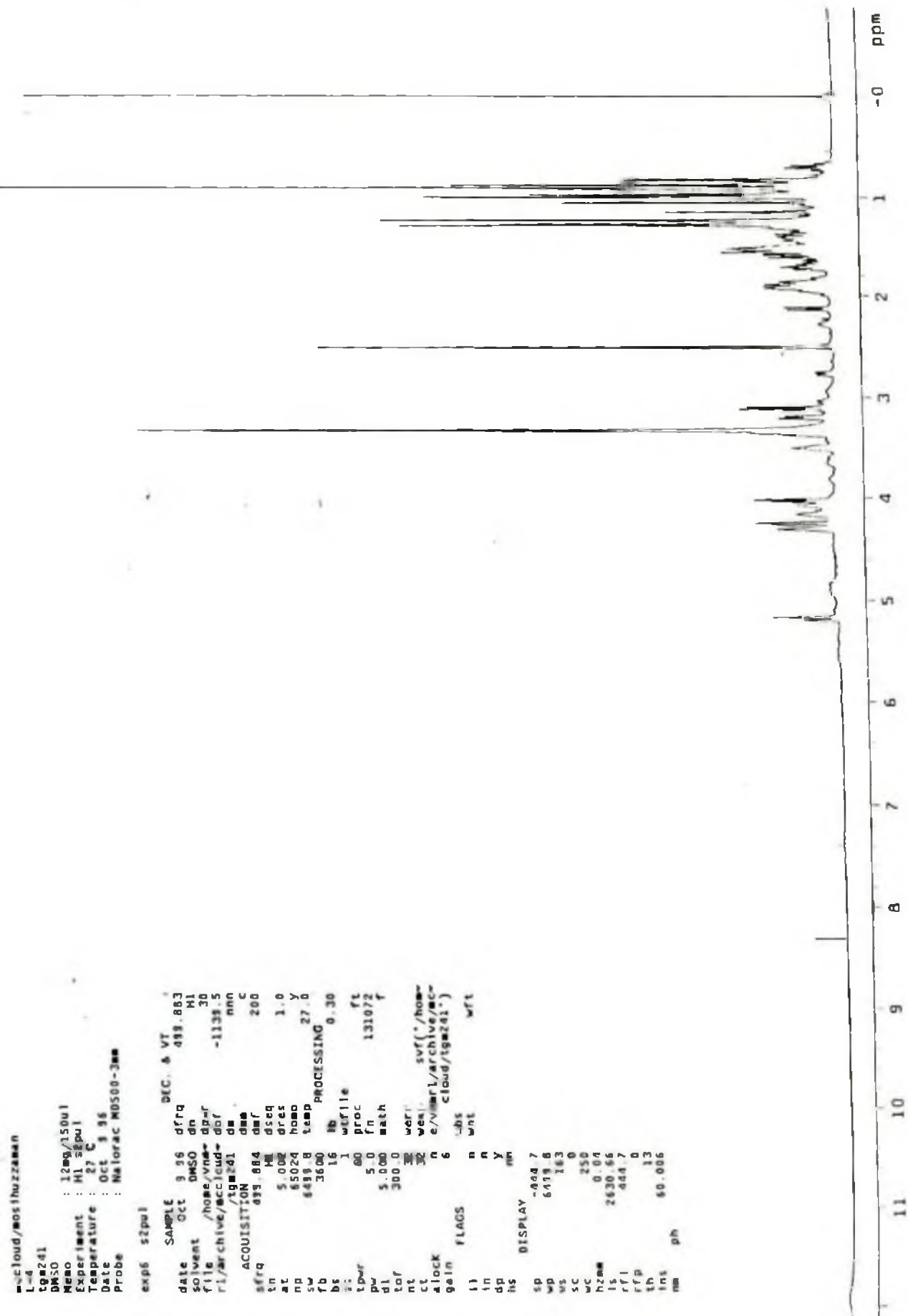


Fig.32: IR spectrum of compound L4

Fig.33: ¹H-NMR spectrum of compound L1

```

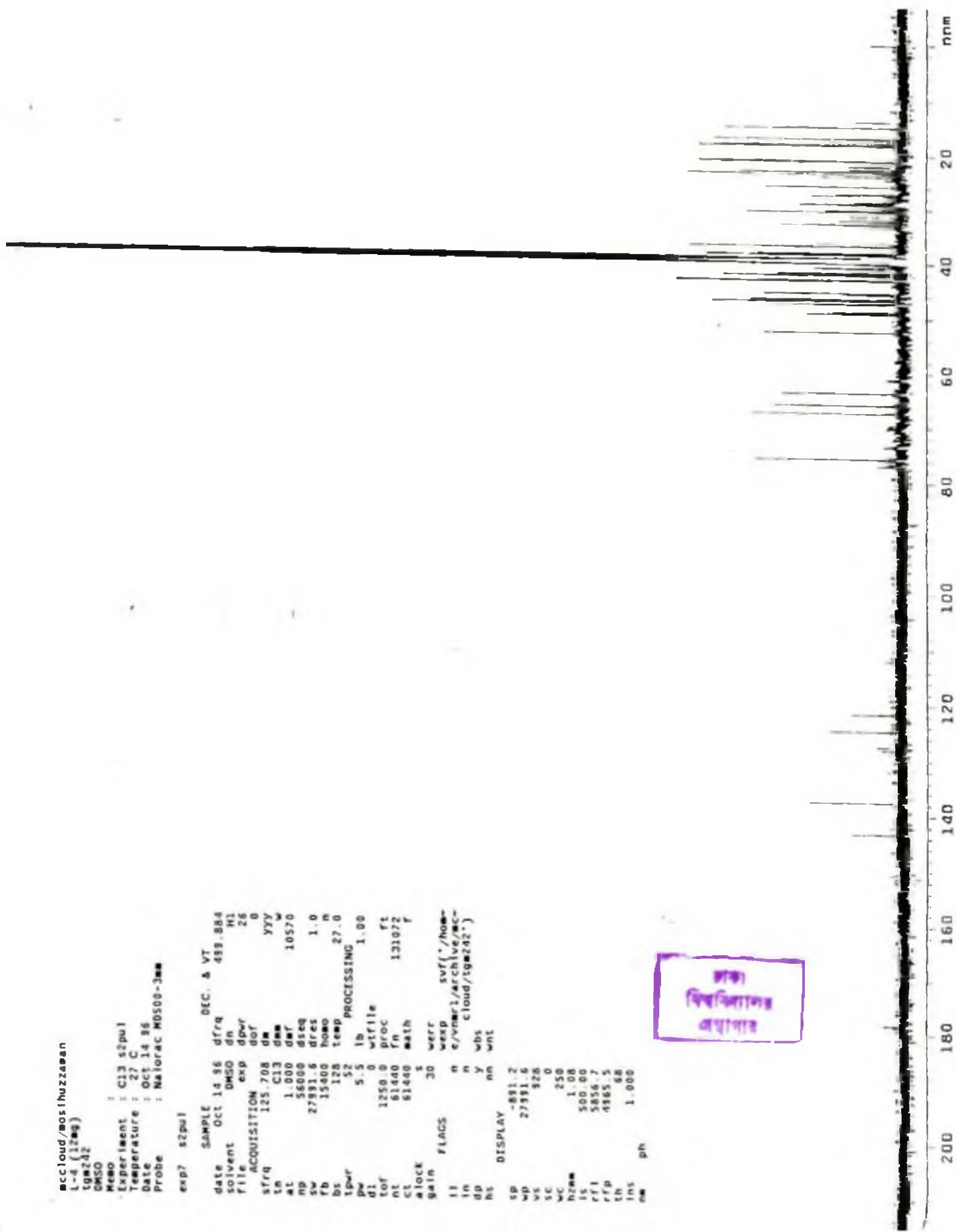
mccloud/mos1huzaan
1-4 (12eg)
tge242
DMSO
Miso
Experiment : C13 s2pul
Temperature : 27 C
Date : Oct 14 86
Probe : Nalorac MDS08-3mm
exp7 s2pul

SAMPLE DEC. & VT
date Oct 14 86 dfrq 489.884
solvent DMSO dn H1
file exp dpr 26
ACQUISITION exp dof 0
sfrq 125.708 dm yyy
tn 1.000 dm 10570
at 1.000 dmf
np 56000 dsq
sw 27991.6 dres 1.0
rb 15400 hmo
bs 128 temp 27.0
tpwr 52
PROCESSING
pw 5.5 lb 1.00
d1 0 wfile
tof 1250.0 proc ft
nt 61440 fn 131072
ct 61440 math f
clock s
gain 30 werr
f1 n wexp svf(*hmo-
f2 n e/vnart/archiv/mc-
f3 n cloud/tge242*)
f4 n
f5 n
f6 n
f7 n
f8 n
f9 n
f10 n
f11 n
f12 n
f13 n
f14 n
f15 n
f16 n
f17 n
f18 n
f19 n
f20 n
f21 n
f22 n
f23 n
f24 n
f25 n
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f27 n
f28 n
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f32 n
f33 n
f34 n
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f36 n
f37 n
f38 n
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f40 n
f41 n
f42 n
f43 n
f44 n
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f179 n
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f229 n
f230 n
f231 n
f232 n
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f250 n
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f252 n
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f256 n
f257 n
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f259 n
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f261 n
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f263 n
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f268 n
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f278 n
f279 n
f280 n
f281 n
f282 n
f283 n
f284 n
f285 n
f286 n
f287 n
f288 n
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f290 n
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f294 n
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f296 n
f297 n
f298 n
f299 n
f300 n
f301 n
f302 n
f303 n
f304 n
f305 n
f306 n
f307 n
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f316 n
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f318 n
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f325 n
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f329 n
f330 n
f331 n
f332 n
f333 n
f334 n
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f412 n
f413 n
f414 n
f415 n
f416 n
f417 n
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Fig 34 ^{13}C -NMR



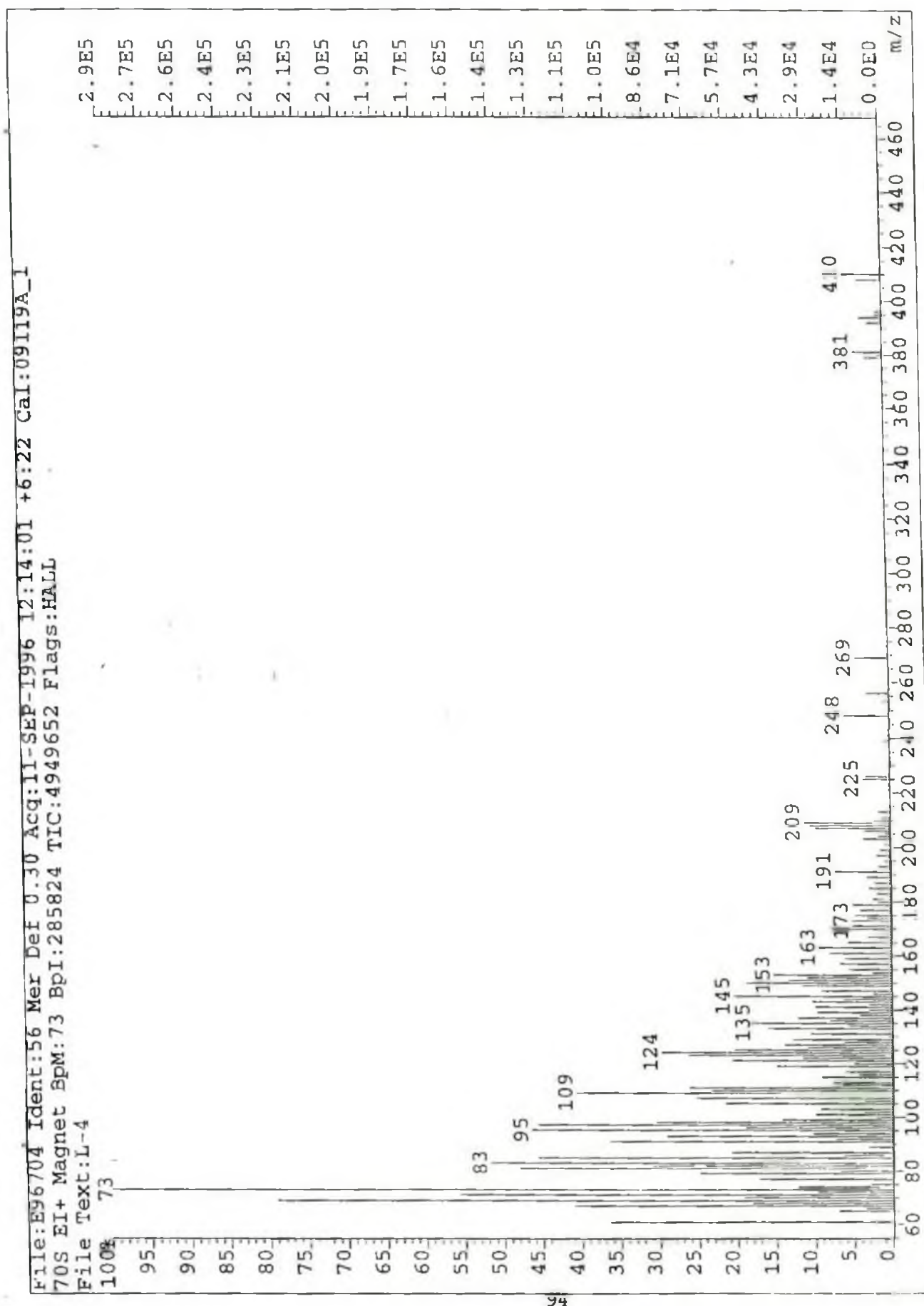


Fig.35: Mass spectrum of compound L4

3.12 CHARACTERIZATION OF THE COMPOUND L₄

The compound L₄ was white and had a R_f value of 0.45 in chloroform : methanol (8 : 2). It was soluble in chloroform and melted at 266°C. The IR spectrum of L₄ (Fig. 33) had absorption band at 3400 cm⁻¹ due to -OH stretching. Absorption band at 2900 and 2850 cm⁻¹ were due to aliphatic stretching. Absorption band at 1680 cm⁻¹ was assigned to C=O stretching. The ¹H-NMR spectrum had a multiplet at 1.99 - 1.82 and eight singlets at 1.28, 1.27, 1.23, 1.15, 1.05, 0.97, 0.91, 0.90 and 0.88 ppm which were assigned to methyl groups. The signals at 4.30, 4.25, 4.16, 4.07, 4.02, and 5.20 ppm indicated the presence of olefinic protons. The ¹³C-NMR spectrum (Fig. 35) had 59 signals indicating that the compound was impure. From these complex data it was not possible to assign any structure to the compound.

SUMMARY

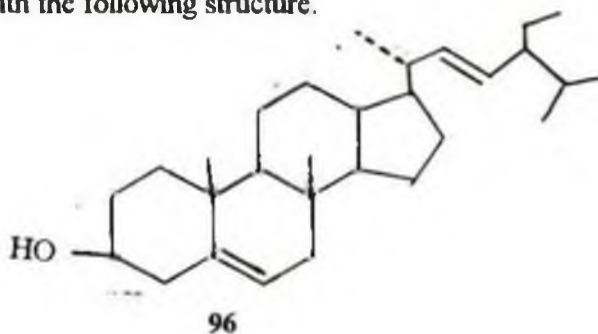
SUMMARY

Centella Asiatica is a very common plant of Umbelliferae family. It is available in different parts of Bangladesh and the Indian subcontinent. For the present work, *Centella asiatica* leaves (26 Kg) were collected from a village near Dhaka.

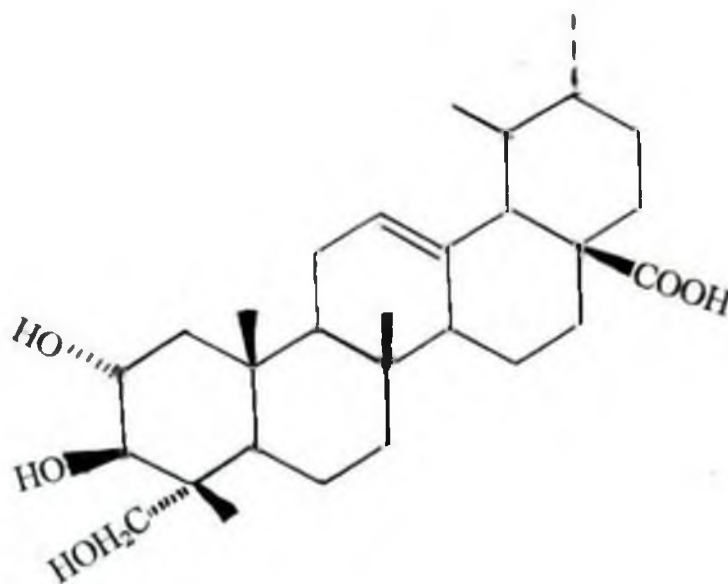
The dried and powdered leaves of *Centella asiatica* were extracted successively with chloroform followed by methanol. The percentage of extractives obtained were 3.6 for chloroform extract (CE) and 8.5 for methanol extract (ME). The methanol extract was further partitioned with ethyl acetate followed by butanol. It was found that the methanol extract contained nearly 3.23% of ethyl acetate soluble material and 18.23% of butanol soluble material.

The chloroform extract (CE) was fractionated by column chromatography and eighteen different fractions were obtained. Three of them CF₄, CF₆ and CF₇ were further fractionated. Fraction CF₄ gave six different sub-fractions out of which CF₄-3 gave a pure compound (C₄). Fraction CF₆ gave five different sub-fractions by column chromatography out of which CF₆-1, CF₆-3, CF₆-4 and CF₆-5 gave four pure compounds (PC₃, PC₁, PC₂ and PC₅ respectively). Fractions CF₇ gave seven different sub-fractions out of which CF₇-1, CF₇-4 and CF₇-6 gave three pure compounds (L₁, L₃ and L₄, respectively).

Compound C₄ gave positive colour with vanillin-sulphuric acid reagent, characteristic of terpenoids and steroids. Since compound C₄ gave positive test for steroids, it was suspected to be a steroid. It was characterised by various spectroscopic methods and was found to contain two olefinic double bonds, one hydroxy group, six methyl groups, nine methylene, eleven methyne groups and three quaternary carbons. Combining chemical and spectroscopic data the compound C₄ was characterised as stigmesterol with the following structure.



Compound PC₂ was characterized by different spectroscopic methods and was found to contain one olefinic double bond, one carboxylic group, three hydroxy groups, six methyl groups, ten methyne groups, five methylene groups and four quaternary carbons. Combining spectroscopic data the compound PC₂ was tentatively characterized as asiatic acid.



Compound PC₃ was a colourless solid with R_f value 0.43 in chloroform : methanol 9 : 1 . It was characterized by various spectroscopic methods and was found that it was a mixture of stigmasterol glucoside and β -sitosterol glucoside but the stigmasterol glucoside was the major compound.

The compound PC₃ was a colourless UV inactive white solid which melted at 80°C. TLC examination showed one distinct spot in chloroform : methanol (9.5 : 0.5) with a R_f value of 0.38. Attempts to characterize it by various spectroscopic methods but due to insufficient and complex data it was not possible to assign any structure to the compound.

Compound PC₁ was a UV inactive white solid which melted at 119°C . Due to shortage of time the compound could not be characterized.

The compound L₁ was a white compound. TLC examination showed one distinct spot but from the available insufficient and complex data it was not possible to characterized the compound.

Compound L₄ was an unsaturated compound and most probably contained a ketone group. However, the available mass, ¹H-NMR and ¹³C-NMR spectrum of the compound could not be thoroughly analysed due to shortage of time. Further work is necessary for the detrrmination of its structure.

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