Phytochemical Studies on some Species of Compositae Family

M.Phil. Thesis

by

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December 2008

Phytochemical Studies on some Species of Compositae Family

A thesis submitted to the Department of Chemistry, University of Dhaka, for the Degree of Master of Philosophy in Chemistry

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December 2008

Dedicated
To
My Parents and My Teachers

Certificate

This is to certify that **Roksana Akter Lubna**, an M.Phil. student bearing registration No.: 210 (session: 2002-2003), of the Department of Chemistry, University of Dhaka, has carried out her research work leading to M.Phil degree under our joint supervision at the Organic Research Laboratory of our department. It is to mention that she has done all her research work by herself and she has prepared the thesis entitled "**Phytochemical Studies on some Species of Compositae Family**". The results incorporated in this thesis has not been used or submitted elsewhere for award of any other degree.

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I do hereby declare that this dissertation entitled "**Phytochemical Studies on some Species of Compositae Family**". Department of Chemistry (Organic Chemistry) University of Dhaka, Dhaka, Bangladesh, in fulfillment of the requirement for the Degree of Master of Philosophy (M. Phil.) is the result of my own investigation.

I further declare that the work embodied in this thesis has not already been submitted in substance for the award of any degree or diploma.

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Abstract

Bangladesh is rich in different types of herbs having medicinal values and nutritional status. *Enhydra fluctuans* Lour. and *Spilanthes acmella* Murr. are two herbs of Compositae family widely distributed and easily available in our country. Both these plants are used as medicine by local Kabiraj and *Enhydra fluctuans* Lour. is also used as a leafy vegetable by the local people. So, investigations were carried out on these plants and these findings are presented in this dissertation. The total work is divided into five parts such as –

1. Phytochemical investigation:
   (i) Screening and estimation of phytochemicals,
   (ii) Isolation and characterization of some compounds.
2. Distribution of fatty acid composition.
3. Ascorbic acid analysis.
4. Iron content and ash content determination.
5. Antimicrobial activity.

1. Phytochemical investigation:

(i) The plants were collected locally, dried and powdered. Phytochemical screening of the samples indicated the presence of alkaloids, tanins, saponins, flavanoids, steroids and terpenoids in both the plants *Enhydra fluctuans* Lour. and *Spilanthes acmella* Murr. The amount of alkaloids, flavonoids and saponins in both the plants were estimated and the results are expressed in mg per 100 g of dry powder basis.

(ii) Plant powder of *Enhydra fluctuans* Lour. was extracted with petroleum ether (b.p. 40–60°C) followed by ethyl acetate in Soxhlet apparatus. Both the extracts were evaporated to dryness and subjected to VLC using different solvents with increasing polarity when several fractions were obtained. The fractions were monitored and similar fractions were combined. The two fractions (F_2 and F_7) from petroleum ether extract and one fraction from ethyl

acetate extract were found to be single by TLC study. These fractions yielded crystalline compounds. The crystals obtained from petroleum ether extract were marked as EF_3 and EF_4 and that obtained from ethyl acetate extract was marked as EFA. These three compounds were characterized by m.p., IR, ^{1}H-NMR and ^{13}C-NMR spectral data and in the case of compound EF_3 mass spectral data, was also included. From these physical constants and spectral data, the compounds EF_3, EF_4 and EFA were identified and their structures were established as *ent*-kaur-16-en-19-oic acid, stigmasta-5,22,25-trien-3β-ol and stigmasta-5,22,25-trien-3β-yl acetate.

Similarly, powdered leaves of *Spilanthes acmella* Murr. were extracted with petroleum ether followed by dichloromethane. The extracts were dried and separately subjected to VLC to isolate different compounds. Chromatographic fractionation of the petroleum ether extract yielded a single compound marked as SA-1, similarly two compounds SA-2 and SA-3 were obtained from dichloromethane extract. Characterization of the compounds using spectroscopic techniques revealed that compounds SA-1 and SA-2 were same and it was established as stigmasterol. On the other hand, structure of SA-3 was tentatively established as sitosterone.

2. Distribution of fatty acid composition:

Free fatty acid (FFA) and bound fatty acid (BFA) were extracted from both the plant powders and the relative percentages of different fatty acids both in the form of FFA and BFA were determined in gas chromatography. From this it was found that palmitic acid is the highest in both the plants. The fatty acids present in *Enhydra fluctuans* Lour. as FFA and BFA were found to be similar and these were caprylic acid, palmitic acid, stearic acid, arachidic acid, behinic acid and lignoceric acid. Free fatty acids found in *Spilanthes acmella* Murr. were lauric acid, palmitic acid, oleic acid, stearic acid, arachidic acid, behinic acid .The BFA of this plant were found to be similar as in FFA including the lauric acid.

3. Ascorbic acid analysis:

Ascorbic acid of *Enhydra fluctuans* Lour. was determined using 2,4-dinitrophenyl hydrazine method spectrophotometrically and it was found to be 31.64 mg/ 100 g of fresh leaves.

4. Iron content and ash content determination:

Iron content of both the plants were determined spectrophotometrically using 1,10-phenanthroline and the results showed that the iron content in *Enhydra fluctuans* Lour. and *Spilanthes acmella* Murr. were 0.6976 and 1.1816 mg/ 100 g dry powder, respectively. The ash content of *Enhydra fluctuans* Lour. and *Spilanthes acmella* Murr. were determined and these were found to be 14.93 and 16.55 %, respectively.

5. Antimicrobial activity:

The dried powder of *Enhydra fluctuans* Lour. and *Spilanthes acmella* Murr were extracted separately and successively with chloroform, ethyl acetate, methanol and water to obtain the crude extracts of both the plants in each solvents. Five bacteria (*Salmonella typhi* ATCC 9458, *Staphylococcus aureus* ATCC 25923, *Vibrio cholera* O1classical ATCC 9458, *Shigella dysenteriae* type-1 ATCC 25226, *Escherichia coli* ATCC 25922) were used to observe the antibacterial activity of these herb extracts. The chloroform extract of *Enhydra fluctuans* Lour. showed antibacterial activity against only *Staphylococcus aureus*.. The ethyl acetate extract of both *Enhydra fluctuans* Lour. and *Spilanthes acmella* Murr. were found to be positive against *Staphylococcus aureus*. Negative tests were observed for all the bacteria with methanol extract of both the plants. Water extract of *Enhydra fluctuans* Lour. showed activity against two organism *Vibrio cholera* and *Staphylococcus aureus* but antibacterial tests with water extract of *Spilanthes acmella* Murr. were found to be negative for all the bacteria. This study indicates that both plants contain biologically active ingredients.

Objective

Tropical Bangladesh is a store house of different types of plants, herbs, creepers and vegetables. All these have contribution towards mankind in various ways specially as a source of food and nutrition as well as medicinal use.

Among these plants and herbs, there are some herbs which belong to the family Compositae. This family is comprised of more than 2500 genera and thousands of species and most of them are available all over our Bangladesh. All the species of this family have medicinal uses and some of them are used as leafy vegetables. The species *Enhydra fluctuans* Lour. and *Spilanthes acmella* Murr. of this family are two important widely distributed herbs. These two species are used by local people and Kabiraj for remedy of various diseases like bowel complaints, eye imflammation, dirrheas, bronchites, leucoderma and other nervous diseases. This medicinal importance of these species of Compositae family inspried to investigate these plants.

Although attempts have been taken to evaluate their phytochemical studies to explore their medicinal value but enough work has not been done on these species.

Therefore, the present study has been undertaken to-

(a) isolate and characterize different phytochemicals,

(b) evaluate its nutritional status and food values (dertermination of iron content, ascorbic acid, and fatty acid composition), and

(c) study the antimicrobial activity of different extract of the plants and hence to explore its potentiality (if any) in the field of medicine.

CHAPTER ONE

Phytochemical analysis

A. Introduction

A. Introduction

A.1.1. Background :

Plants play an important role[1] in our life. Plants[2] have been providing the human beings with the basic necessities of life, that is, food, fuel and shelter, from the very beginning of their existence, and for their continued living and sustenance on this earth.

Plant and man[2] are inseparable. Because plants not only provide man the basic needs of life, but also the life sustaining oxygen gas. The simple compound carbon dioxide (CO_2) and water (H_2O) combine in the leaves of plant in the presence of chlorophyll and sunlight to produce glucose and by product oxygen which are essential for all forms of life. Plants play a vital role in consuming CO_2 (carbon dioxide) from atmosphere and save it from the negative impression of the green house effect. Since man encountered diseases, got injured and wounded, faced decay of health and death as soon as he started inhabiting this earth, the early man had to think about disease and its treatment at the very dawn of human intellect. Plants were the first natural substances that man could think of using as therapeutic agents for management of his health. Thus the human race started using plants as a means of treatment of diseases and injuries from very early days of his conscious living on earth. From ancient time to modern age the human race has successfully used plants and plant products as effective therapeutic tools for fighting against diseases and various other health hazards.

Again plants produce other substances by metabolic processes, such as glycosides, steroids, terpenoids, alkaloids, resins, essential oils, tannins, flavonoids, pigments etc. Which are usually called secondary metabolites. Some of the compounds are harmful to animal life but some of them have proved to be tremendously effective in therapeutic doses for successful remedies for a number of diseases. Many chemical compounds of diversified

nature from plants often played an important role to give a new direction for laboratory synthesis of many new classes of drug molecules.

The natural product researchers have been trying to find out the active constituents of toxic and nontoxic plants for treatment of various diseases. In modern medicine, with the advent of rapid isolation techniques, bioassay screening procedures and spectroscopic methods for structure determination, the phytochemistry has become an extremely exciting and productive field in mainstream of science. Hence is the importance of plant and its derivatives.

Chemistry[1] utilizing medicinal plants started to develop. In the recent decades, natural pesticides, antibiotics, vitamins and hormones are the follow up results of the researchers of chemistry of natural products. Scientists are now working together to find out new drugs from plants for incurable diseases likes diabetes, cancer and AIDS. So, it will not be at all surprising that the chemistry of natural products will lead to new contributions to therapy of the controlled diseases.

Ordinary people do not know the harmful and useful effects of the compounds stored in the plants; they also do not know what the side effects of such plant materials are. So the chemists have a great responsibility to find out the active ingredients, which are effective against various diseases and also to identify the toxic ingredients (if any).

A.1.2. Medicinal importance of plant materials:

A medicinal plant[3] is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes or which are precursors for synthesis of useful drugs. This definition has been given by the consultative group on medicinal plants of World Health Organization (WHO). Unfortunately this definition of the WHO group includes only the medicinal

plants whose therapeutic properties and chemical constituents have been established scientifically. But it does not take into consideration the vast majority of the medicinal plants, which have not yet been subjected to thorough scientific studies. These medicinal plants have been used in traditional medicine for hundreds of years with reputation as efficacious remedies although there may not be sufficient scientific data to substantiate their efficacy.

Thus plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are designated as medicinal plants. Although there are no apparent morphological characteristics in the medicinal plants that make them distinct from other plants growing with them, yet they possess some special qualities or virtues that make them medicinally important. It has now been established that the plants which naturally synthesize and accumulate some secondary metabolites, like alkaloids, sterols, terpenes, flavonoids, saponins, glycosides, cyanogenics, tannins, resins, lactones, quinines, volatile oils, etc and contain minerals and vitamins, possess medicinal properties.

Today there are[4] at least 120 distinct chemical substances derived from plants that are considered as important drugs currently in use in one or more countries in the world. Several of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances. The original plants substance/chemical name is shown under the "Drug" column rather than the finished patented drug name. For example, many years ago a plant chemical was discovered in a tropical plant, *Cephaelis ipecacuanha,* and the chemical was named emetine. A drug was developed from this plant chemical called Ipecac which was used for many years to induce vomiting mostly if someone accidentally swallowed a poisonous or harmful substance. Ipecac can still be found in pharmacies in many third world countries but has been mostly replaced by other drugs in the United States. Another example of this is the

plant chemical named taxol shown in the drug column below. The name taxol is the name of the plant chemical originally discovered in the plant. A pharmaceutical company copied this chemical and patented a drug named Paclitaxel which is used in various types of tumors today in the U.S. and many other countries.

The chemical substances derived from plants are sold as drugs worldwide but not in all countries. Some European countries regulate herbal substances and products differently than in the United States. Many European countries, including Germany, regulate herbal products as drugs and pharmaceutical companies prepare plant based drugs simply by extracting out the active chemicals from the plants. A good example is the plant substance/drug shown below, cynarin. Cynarin is a plant chemical found in the common artichoke (*Cynara scolymus*). In Germany, a cynarin drug is sold for liver problems and hypertension which is simply this one chemical extracted from the artichoke plant or a plant extract which has been standardized to contain a specific milligram amount of this one chemical. These products are manufactured by pharmaceutical companies, sold in pharmacies in Germany and a doctor's prescription is required to purchase them. In the United States artichoke extracts are available as natural products and sold in health food stores. Some products are even standardized to contain a specific amount of the cynarin chemical. Anybody can purchase these natural and standardized extracts over the counter without a prescription and he does not need to go to a pharmacy in the U.S. and obtain a cynarin drug with a prescription. Another similar example is the plant chemical, silymarin, shown in the drug column below. Silymarin is a chemical found in the milk thistle plant and natural milk thistle extracts and it is found in just about every health food store in the United States. However in Germany, silymarin drugs and milk thistle standardized extracts are sold only in pharmacies and require a doctor's prescription for liver problems.

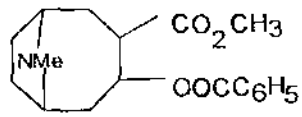
Some of the drug/chemicals shown below are still sold as plant based drugs requiring the processing of the actual plant material. Others have been

chemically copied or synthesized by laboratories and no plant materials are used in the manufacture of the drug. A good example of this is the plant chemical quinine, which was discovered in a rainforest tree (*Cinchona ledgeriana*) over 100 years ago. For many years the quinine chemical was extracted from the bark of this tree and processed into pills to treat malaria. Then a scientist was able to synthesize or copy this plant alkaloid into a chemical drug without using the original tree bark for manufacturing the drug. Today, all quinine drugs sold are manufactured chemically without the use of any tree bark. However, another chemical in the tree called quinidine which was found to be useful for various heart conditions couldn't be completely synthezised in the laboratory and the tree bark is still harvested and used to extract this plant chemical from it. Quinidine extracted from the bark is still used today to produce quinidine-based drugs. In the U.S. there are four patented brand-name heart drugs sold in pharmacies containing bark-extracted quinidine: Cardioquin, Quinaglute Dura-tabs, Quinidex Extentabs and Quin-Release.

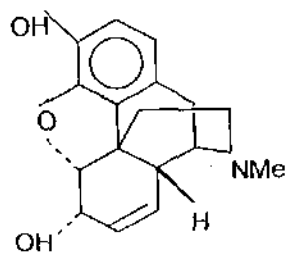
Table A.1: Some important drugs/chemicals from plant source and their actions/ chemical uses[4]

Drug/Chemical	Action/Clinical use	Plant source
Atropine	Antispasmodic	*Atropa balladone*
Berberine, Brucine	Astrigent, Alcohol denaturant	*Strychnos nux-vomica*
Betulinic acid	Anticancerous	*Betula alba*
Caffeine	Stimulant, Analgesic	*Camellia sinesis*
Camphor	Antipyratic, Stimulant	*Conamomum camphori*
Camptothecin	Anticancerous	*Camptotheca acuminata*
Chymopapain	Proteolytic, mucolytic	*Carica papaya*
Cissampeline	Skeletal muscle relaxant	*Cissampelos pareira*
Colchiceine amide	Antitumor agent	*Colchicum autumnale*
Colchicine	Antitumor agent, anti-gout	*Colchicum autumnale*
Curcumin	Choleretic	*Curcuma longa*
Cynarin	Choleretic	*Cynara scolymus*
Codein	Anaesthetic, narcotic	*Erytroxylum coca*
Danthron	Laxative	*Cassia species*
L-Dopa	Anti-parkinsonism	*Mucuna sp*
Etoposide	Antitumor agent	*Podophyllum peltatum*
Ephedrine	Sympathomimatic bronchodilator	*Ephedra* spp.
Glaucarubin	Amoebicide	*Simarouba glauca*
Glycyrrhizin	Sweetener, Addison's disease	*Glycyrrhiza glabra*
Hesperidin	Capillary fragility	*Citrus species*
Irinotecan	Anticancer, antitumor agent	*Camptotheca acuminata*
Lapachol	Anticancer, antitumor	*Tabebuia sp.*
Menthol	Rubefacient	*Mentha species*
Papain	Proteolytic, mucolytic	*Carica papaya*
Pilocarpine	Parasympathomimetic	*Pilocarpus jaborandi*
Podophyllotoxin	Antitumor anticancer agent	*Podophyllum peltatum*
Quinidine	Antiarrhythmic	*Cinchona ledgeriana*
Quinine	Antimalarial, antipyretic	*Cinchona ledgeriana*
Rutin	Capillary fragility	*Citrus species*
Sennosides A, B	Laxative	*Cassia species*
Stevioside	Sweetner	*Stevia rebaudiana*
Taxol	Antitumor agent	*Taxus brevifolia*
Teniposide	Antitumor agent	*Podophyllum peltatum*
α-Tetrahydro-cannabinol (THC)	Antiemetic, decrease tension	*Cannabis sativa*
Theobromine	Diuretic, vasodilator	*Theobroma cacao*
Theophylline	Diuretic, brochodilator	*Theobroma cacao*
Topotecan	Antitumor, anticancer agent	*Camptotheca acuminata*
Trichosanthin	Abortifacient	*Trichosanthes kirilowii*
Tubocurarine	Skeletal muscle relaxant	*Chondodendron tomentosum*
Vasicine	Cerebral stimulant	*Vinca minor*
Vinblastine	Antitumor, Antileukemic agent	*Catharanthus roseus*
Vincristine	Antitumor, Antileukemic agent	*Catharanthus roseus*

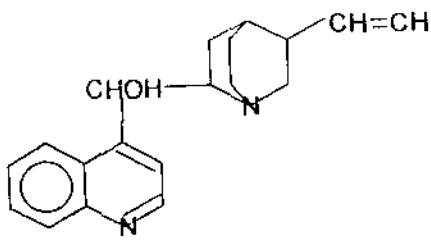
A.1.3. Structures of some of the modern drugs originated from natural products are given below:



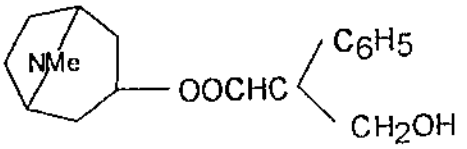
Cocaine



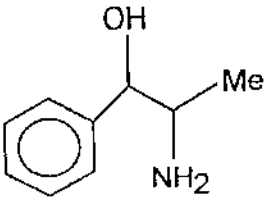
Morphine



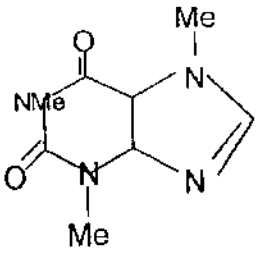
Quinine



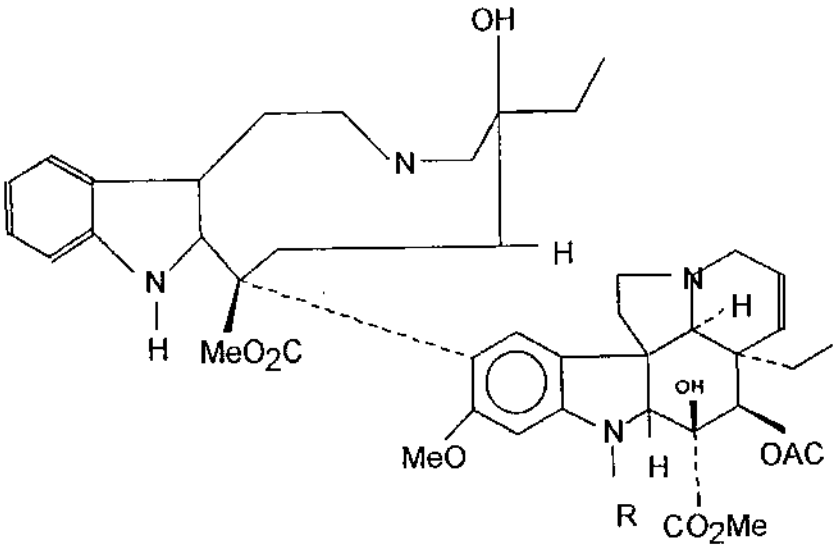
Atropine



Ephedrine



Caffeine



Vincristine

A.1.4. Economic importance of medicinal plants (An over-view with special reference to Bangladesh)[5] :

Medicinal plants are rich sources of bioactive compounds and thus serve as important raw materials for drug production. They constitute a precious natural wealth of a country and contribute a great deal to its health care programmes. Judicious and scientific exploitation of this wealth can significantly improve the general health of the people. Being a valuable item of commerce, a country can also earn a good amount of foreign exchange by exporting this natural wealth to other countries. There are many countries in the world which earn a substantial amount of foreign currency by exporting medicinal plants and crude plant drugs. India and Thailand are two glaring examples of such countries in this subcontinent which earn crores of rupees by exporting medicinal plants and their semi-processed products to other countries including Bangladesh.

Being naturally gifted by a suitable tropical climate and fertile soil, Bangladesh possesses a rich flora of tropical plants. About 5000 species of phanerogams and pteridophytes grow in its forests, jungles, wastelands and road sides as indigenous, naturalised and cultivated plants. Out of them more than a thousand have been claimed to possess medicinal and/or poisonous properties, of which 546 have recently been enumerated with their medicinal properties and therapeutic uses. In addition to possessing various other medicinal properties, 257 of these medicinal plants have been identified as efficious remedies for diarrhoeal diseases and 47 for diabetes.

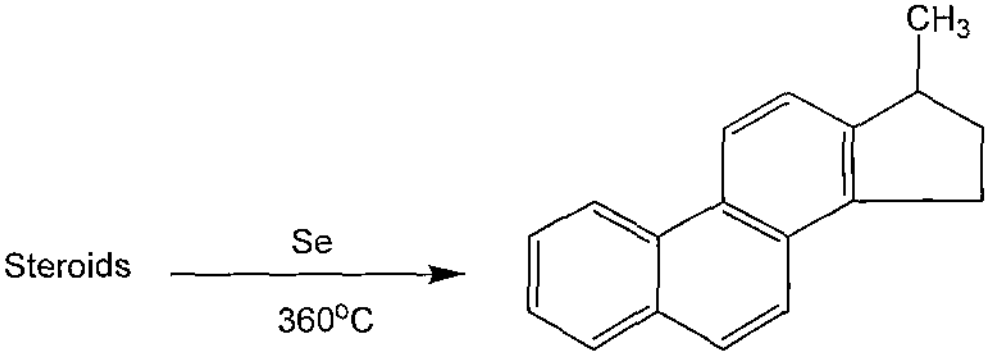
Every year Bangladesh transports a large quantity of pharmaceuticals raw materials including medicinal plants and semi-processed plant products to feed its various drug manufacturing industries. These expenditures seriously aggravate the already bad situation with regard to our dwindling foreign exchange position, and something has to be done to reduce this spending.

Bangladesh possessing a rich flora of medicinal plants should make serious efforts to derive maximum economic benefit from these plants by using them as raw materials for its indigenous drug manufacturing industries.

In order to achieve these goals of export of medicinal plants and local manufacture of drugs and medicines from indigenous plant materials, it is necessary to have a correct inventory of the total volume of this natural wealth in the country. This is possible only by carrying out scientific surveys throughout the country in a planned manner to determine the types and number of medicinal plants available in the country and the feasibility of growing them in commercial scale.

A.1.5. Steroids :

Compounds which produce Diel's hydrocarbon on selinium dehydrogenition (3'-methyl-1,2 cyclopenteno-phenanthrene) are known as steroids.



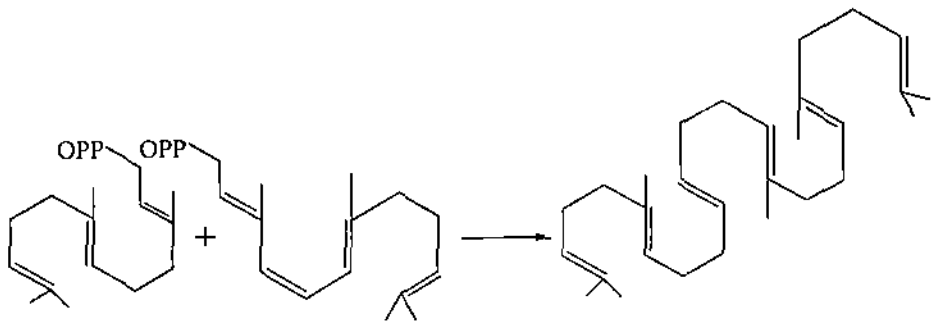
Steroids contain four rings fused together. Among the four rings three are six membered and other one is five membered.

The precursor of steroids in biological system is squalene, a triterpene which was originally produced by joining of six isopentenyl pyrophosphate.

Many hormones, bile acids and vitamins have this steroidal structure. Usual birth control pill for women contains steroidal hormones. Steroids are extracted by lipophilic solvents like chloroform, petroleum ether etc.

A.1.6. Biosynthesis of steroids:

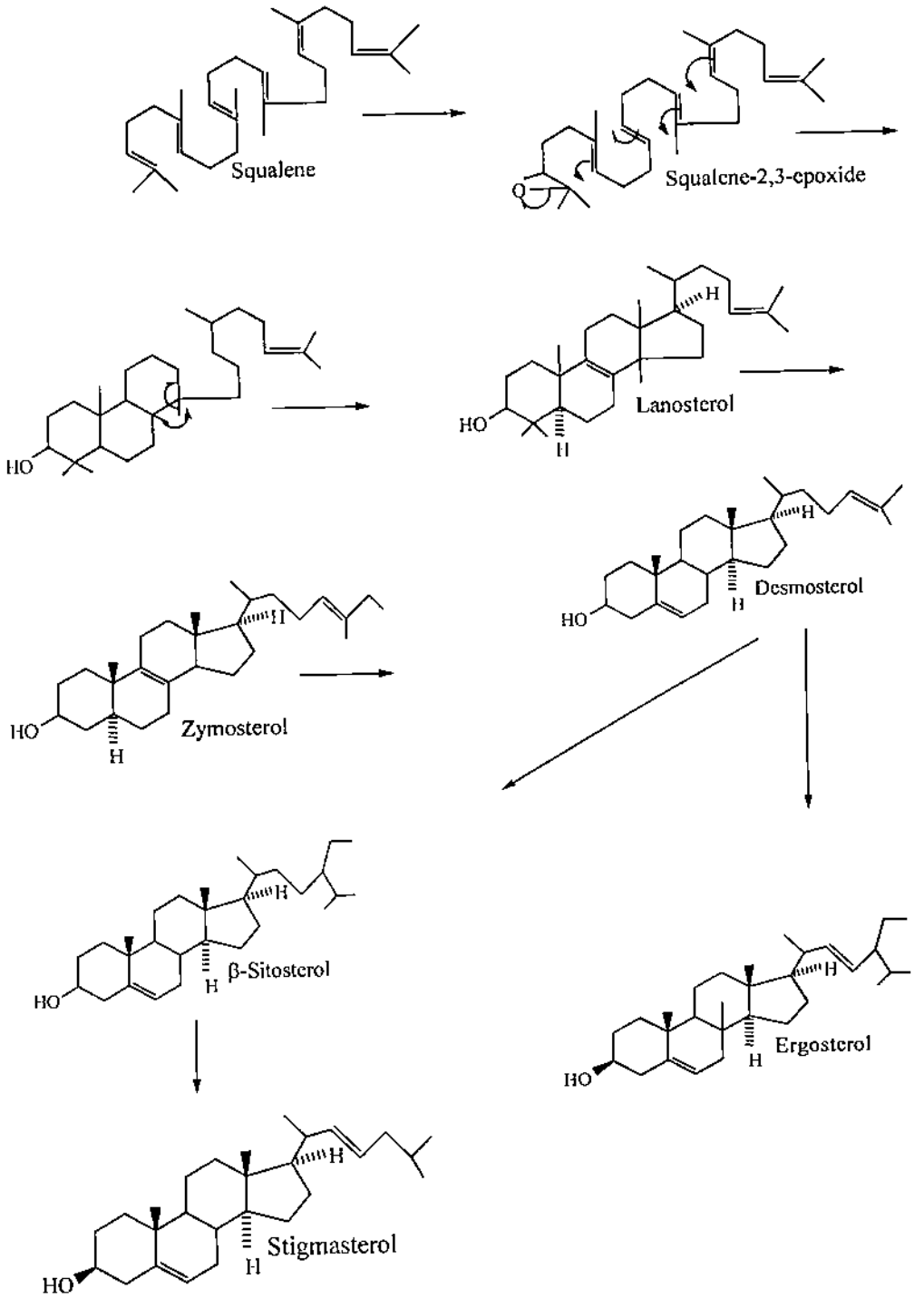
Steroids[6,7] are not triterpenes as they possess C_{27}-C_{29} skeletons; rather than a C_{30} skeleton, although both of them are derived from the same C_{30} precursor squalene. This squalene is derived from two farnesyl-pyrophosphate units, upon joining each other in the unusual head to head' fashion.



Squalene

Cyclisation of squalene-2,3-epoxide in a chair-boat-chair-boat conformation and by a subsequent sequence of rearrangements leads to lanosterol, cycloartenol etc.

Desmosterol is formed from lanosterol by a sequence of modification reactions. β-sitosterol and stigmasterol are fomed by the addition of extra carbon atoms to the side chain of desmosterol in plants.



Scheme A.1: Biosynthesis of phytosterols.

B. The family Compositae

B. The family Compositae

B.1.1. Introduction :

The family Asteraceae or Compositae (known as the aster, daisy, or sunflower family) is the largest family of flowering plants, in terms of number of species.

The name 'Asteraceae' is derived from the type genus Aster, while 'Compositae', an older but still valid[8] name, means composite and refers to the characteristic inflorescence, a special type of pseudanthium found in only a few other angiosperm families. The study of this family is known as synantherology.

According to the Royal Botanical Gardens of Kew, the family comprises more than 1,600 genera and 23,000 species. The largest genera are *Senecio* (1,500 species), *Vernonia* (1,000 species), *Cousinia* (600 species), *Centaurea* (600 species). The circumscription of the genera is often problematic and some of these have been frequently divided into minor subgroups[9].

Compositae are cosmopolitan, but most common in the temperate regions and tropical mountains.

B.1.2. Taxonomy:

The family has been universally recognized and placed in the order Asterales.

Traditionally two subfamilies were recognised: Asteroideae (or 'Tubuliflorae') and Cichorioideae (or 'Liguliflorae'). The latter is paraphyletic and has been divided into many minor groups in most newer systems[10]. A list of subfamilies is shown below[11]:

Barnadesioideae: 9 genera, 93 species. South America, mainly the Andes.

Stifftia and close relatives: South America and Asia.

Mutisioideae: 58 genera, 750 species. South America.

Gochnatioideae: 4 or 5 genera, 90 species

Hecastocleidoideae: Only *Hecastocleis shockleyi*. South-western U.S.A..

Carduoideae: 83 genera, 2,500 species. Worldwide.

Pertyoideae: 5 or 6 genera, 70 species.

Gymnarrhenoideae: Only *Gymnarrhena micrantha*. Northern Africa.

Cichorioideae: 224 genera, 3,200 species. Worldwide.

Corymbioideae: Only the genus *Corymbium*, with 7 species.

Asteroideae: 1,130 genera and 16,200 species. Worldwide.

It is noteworthy that the four subfamilies Asteroideae, Cichorioideae, Carduoideae and Mutisioideae comprise 99% of the specific diversity of the whole family (appr. 70%, 14%, 11% and 3% respectively).

B.1.3. Description:

Compositae are usually herbs, but some shrubs, trees and climbers do exist. They are generally easy to distinguish, mainly because of their characteristic inflorescence and many shared apomorphies[9].

B.1.4 . Leaves and stems:

The leaves and the stems very often contain secretory canals with resin or latex (particularly common among the Cichorioideae). The leaves can be alternate, opposite, or whorled. They may be simple, but are often deeply lobed or otherwise incised, often conduplicate or revolute. The margins can be entire or dentate.

B.1.5. Flowers:

A typical Asteraceae or Compositae flower head (here *Bidens torta*) showing the individual flowers.

The most evident characteristic of Asteraceae or Compositae is perhaps their inflorescence: a specialised *capitulum*, technically called a *calathid* or *calathidium*, but generally referred to as flower head or, alternatively, simply *capitulum*[12]. The *capitulum* is a contracted raceme composed of numerous individual sessile flowers, called the florets, all sharing the same receptacle.

The *capitulum* of the Asteraceae has evolved many characteristics that make it look superficially like a big single flower. This kind of flower-like inflorescences are quite widespread amongst plants and have been given the name of *pseudanthia*.

Figure B.1 : A typical Asteraceae flower head (here *Bidens torta*) showing the individual flowers.

Many bracts form an involucre under the basis of the capitulum; these are called "phyllaries", or "involucral bracts". They may simulate the sepals of the pseudanthium. These are mostly herbaceous but can also be brightly coloured (e.g. *Helichrysum*) or have a scarious texture. The bracts can be free or fused, and arranged in one to many rows, overlapping like the tiles of a roof

(imbricate) or not (this variation is important in identification of tribes and genera).

Each floret may itself be subtended by a bract, called a "palea" or "receptacular bract". These bracts as a group are often called "chaff". The presence or absence of these bracts, their distribution on the receptacle, and their size and shape are all important diagnostic characteristics for genera and tribes.

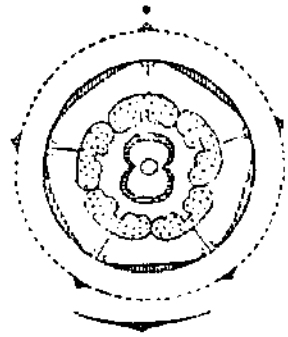


Figure B.2: Flower diagram of Carduus (Carduoideae)

The florets have five petals fused at the base to form a corolla tube and they may be either actinomorphic or zygomorphic. Disc florets are usually actinomorphic, with five petal lips on the rim of the corolla tube. The petal lips may be either very short, or long, in which case they form deeply lobed petals. The latter is the only kind of floret in the Carduoideae, while the first kind is more widespread. Ray florets are always highly zygomorphic and are characterised by the presence of a ligule, a strap-shaped structure on the edge of the corolla tube consisting of fused petals. In the Asteroideae and other minor subfamilies these are usually borne only on florets at the circumference of the capitulum and have a 3+2 scheme – above the fused corolla tube, three very long fused petals form the ligule, with the other two petals being inconspicuously small. The Cichorioidea has only ray florets, with a 5+0 scheme – all five petals form the ligule. A 4+1 scheme is found in the Barnadesioideae. The tip of the ligule is often divided into teeth, each one

representing a petal. Some marginal florets may have no petals at all (filiform floret).

The calyx of the florets may be absent, but when present it is always modified into a pappus of two or more teeth, scales or bristles and this is often involved in the dispersion of the seeds. As with the bracts, the nature of the pappus is an important diagnostic feature.

There are usually five stamens. The filaments are fused to the corolla, while the anthers are generally connate (*syngenesious* anthers), thus forming a sort of tube around the style (theca). They commonly have basal and/or apical appendages. Pollen is released inside the tube and is collected around the growing style, expelled with a sort of pump mechanism (*nüdelspritze*) or a brush.

The pistil is made of two connate carpels. The style has two lobes; stigmatic tissue may be located in the interior surface or form two lateral lines. The ovary is inferior and has only one ovule, with basal placentation.

B.1.6. Fruits and seeds:

The fruit of the Asteraceae or Compositae is a specialised type of achene, sometimes called *cypsela* (plural *cypselae*). One seed per fruit is formed. It may sometimes be flat, winged or spiny and it adheres to the persistent pappus. Its morphology is often used to help determine plant relationships at the genus and species level[13]. The seeds usually have little or lack endosperm[9].

Figure B.3: Seeds are dispersed by the wind in Carlina

B.1.7. Metabolites:

Asteraceae or Compositae generally store energy in the form of inulin.

They produce iso/chlorogenic acid, sesquiterpene lactones, pentacyclic triterpene alcohols, various alkaloids, acetylenes (cyclic, aromatic, with vinyl end groups), tannins. They have terpenoid essential oils which never contain iridoids[11].

B.1.8. Ecology:

Asteraceae or Compositae are especially common in open and dry environments[9].

Many members of the Asteraceae or Compositae are pollinated by insects, but anemophyly is also present (e.g. *Ambrosia*, *Artemisia*). There are many apomictic species in the family.

Figure B.4: Epizoochory in *Bidens tripartite*

Seeds are ordinarily dispersed intact with the fruiting body, the cypsela. Wind dispersal is common (*anemochory*) assisted by a hairy pappus. Another common variation is (*epizoochory*), in which the dispersal unit, a single cypsela (e.g. *Bidens*) or entire capitulum (e.g. *Arctium*) provided with hooks, spines or some equivalent structure, sticks to the fur or plumage of an animal (or even to clothes, like in the photo) just to fall off later far from its mother plant.

B.1.9. Evolution:

Diversification of Asteraceae or Compositae may have been within 42-36 million years, the stem group perhaps being up to 49 million years old[11].

It is still unknown whether the precise cause of their great success was the development of the calathid, their ability to store energy as fructans (mainly inulin), which is an advantage in relatively dry zones, or some combination of these and possibly other factors[10].

B.1.10. Uses:

Commercially important plants in the Asteraceae include the food crops *Lactuca sativa* (lettuce), *Cichorium* (chicory), *Cynara scolymus* (globe artichoke), *Helianthus annuus* (sunflower), *Smallanthus sonchifolius* (yacón), *Carthamus tinctorius* (safflower) and *Helianthus tuberosus* (Jerusalem artichoke).

Other commercially important species include Compositae used as herbs and in herbal teas and other beverages. Chamomile which comes from two different species, the annual *Matricaria recutita* or German chamomile, and the perennial *Chamaemelum nobile*, also called Roman chamomile. *Calendula*, also called the pot marigold is grown commercially for herbal teas and the potpourri industry. Echinacea (*Echinacea purpurea*), used as a medicinal tea. Winter tarragon, also called Mexican mint marigold, *Tagetes lucida* is commonly grown and used as a tarragon substitute in climates where tarragon will not survive. Finally, the wormwood genus *Artemisia* includes absinthe (*A. absinthium*) and tarragon (*A. dracunculus*).

Figure B.5 : Sunflowers are a commonly cultivated member of Asteraceae

Industrial use of Compositae is also known. Common in all commercial poultry feed, marigold (*Tagetes patula*) is grown primarily in Mexico. Marigold oil, extracted from *Tagetes minuta* is used by the metric ton in the cola and cigarette industry.

Plants in Asteraceae or Compositae are medically important in areas that don't have access to Western medicine. They are also commonly featured in medical and phytochemical journals because the sesquiterpene lactone compounds contained within them are an important cause of allergic contact dermatitis. Allergy to these compounds is the leading cause of allergic contact dermatitis

in florists in the US.[14] Pollen from Ragweed Ambrosia is among the main causes of so called hay fever in the United States[15].

Many members of the family are grown as ornamental plants for their flowers and some are important ornamental crops for the cut flower industry. Some examples are *Chrysanthemum*, *Gerbera*, *Calendula*, *Dendranthema*, *Argyranthemum*, *Dahlia*, *Tagetes*, *Zinnia* and many others.

Figure B.6: A Dahlia cultivar

Many members of Asteraceae or Compositae are copious nectar producers and are useful for evaluating pollinator populations during their bloom. *Centaurea* (knapweed), *Helianthus annuus* (domestic sunflower), and some species of *Solidago* (goldenrod) are major "honey plants" for beekeepers. Solidago produces relatively high protein pollen, which helps honey bees overwinter.

Some members of the Asteraceae or Compositae are economically important as weeds. Notably in the United States are the ragwort, *Senecio jacobaea*, groundsel *Senecio vulgaris* and *Taraxacum* (dandelion).

The genera *Tanacetum*, *Chrysanthemum* and *Pulicaria* contain species with insecticidal properties.

Parthenium argentatum (Guayule) is a source of *hypoallergenic latex.*

B.2.1. Species of Compositae available in Bangladesh:

The plants of this species grow abundantly in Bangladesh. Some of the species are listed Table B.2.1.

Table B.2.1: Species of Compositae available in Bangladesh[5] :

Genera	Botanical names	Native names
Ageratum	*Ageratum conyzides* (Linn)	Fulkuri, Uhanti
Bhumea	*Bhumea lacera* (Linn)	Kiekursunga, Shialmutra
Carthamus	*Carthamus tinctorius* (Linn)	Kajirah, Kushum
Eclipta	*Eclipta alba* (Linn)	Kalokeshi, Keshraj
Elephantopus	*Elephantopus scaber* (Linn)	Gojialata, Shamdalan
Enhydra	*Enhydra fluctuans* (Lour)	Helencha, Shak, Hincha
Eupaturium	*Eupaturium odoratum* (Linn)	Asanlata
Eupatorium	*Eupatorium triplinerve* (Vahl)	Ayapan, Ayapana
Gnaphalium	*Gnaphalium lutcoalbum* (Linn)	Boro Kamra
Grangea	*Grangea maderespatana* (Linn)	Namuti
Helianthus	*Helianthus annus* (Linn)	Surjamukhi
Sonchus	*Sonchus wghtianus* (DC)	Bon Palong
Sphaeranthus	*Sphaeranthus indieus* (Linn)	Mundi Gach, Chhagol Nudi
Spilanthes	*Spilanthes acmella* (Murr.)	Bongenda
Tagetes	*Tagetes erecta* (Linn)	Genda Phul
Tridex	*Tridex procumbens* (Linn)	Trdhara
Xanthium	*Xanthium indicum* (Koenig)	Ghagra

B.3.1. Medicinally important plants of the Compositae family:

Most plants of the Compositae family are used as medicine in various diseases. Some plants of them are listed below[16]

Disease	Plants
Rheumatism	*Carthamus tinctorius* (Linn), *Grangea maderespatana* (Linn), *Tages erecta* (Linn)
Cold, Cough	*Spaeranthus indicus* (Linn), *Tagestes erecta* (Linn), *Helianthus, Eclipta alba* (Linn)
Bronchitis	*Tagestes erecta* (Linn), *Sonchus wightianus* (DC), *Helianthus annus* (Linn)
Diuretic	*Helianthus annus* (Linn), *Gnaphalium luteo-album*(Linn), *Xanthium indicum* (Koenig), *Blumaca lacera* (Linn), *Spilanthes calva* (DC)
Dysentry	*Spilanthes calva* (DC), *Elephentopus scraber* (Linn), *Tridax procumbens* (Linn), *Argentum conyzoides* (Linn)
Liver troubles	*Elephentopus scaber* (Linn), *Spilanthes calva* (DC), *Eclipta alba* (Linn)
Muscular pairs	*Grangea maderspatana* (Linn), *Tagets erecta* (Linn)
Diarrhoea	*Buumea lacera*(Linn), *Tridex procumbens* (Linn), *Ageratum conyzoides* (Linn)
Bleeding	*Tridex procumbens* (Linn), *Eclipta alba* (Linn)
Gastric disorders	*Sphaeranthus indicus* (Linn), *Eupatorium triplinerve* (Vahl.)
Asthma	*Sonchus wightianus* (DC), *Eupatorium odoratum* (Linn)
Scabies, Lithortriptic	*Spilanthes acmella* (Murr.)
Malarial fever	*Helianthus annus* (Linn), *Argentum conyzoides* (Linn)
Leucorrhoea	*Xanthium indicum* (Koenig)
Chest pain	*Sphaeranthus indicus* (Linn)
Hemorrhages	*Eupatorium triplinerve* (Vahl)
Paralysis	*Carthamus tinctorius* (Linn)
Filariases	*Elephentopus scaber* (Linn)
Piles	*Crangea maderspatana* (Linn), *Bhumea lacera* (Linn), *Elephentopus scaber* (Linn)
Dyspnea	*Argentum conyzoides* (Linn)
Amobie dysentery	*Elephentopus scaber* (Linn)
Jaundice	*Carthamus tinctures* (Linn)
Analgesic	*Elephentopus scaber* (Linn)
Ulcer	*Elephentopus scaber* (Linn)
Dysuria	*Elephentopus scaber* (Linn)
Sitosterols gllucoside	*Enhydra fluctuans* (Linn)

B.4.1. Economically [11, 12, 13] significant members of the Compositae family :

Common name	*Genus and Species*	Economic use
Lettuce	*Lactuca sativa* (Linn)	Food
Sunflower	*Helianthus annuus* (Linn)	Oil, Food, and Ornamental
Safflower	*Carthamus tinctorius* (Linn)	Oil, Food, and Ornamental
Endive	*Cichorium endivia* (Linn)	Food
Chicory	*Cichorium intybus* (Linn)	Food
Artichoke	*Cynara scolymus* (Linn)	Food
Cardoon Cynara	*Cardunculus* (Linn)	Food
Jerusalem Artichoke	*Helianthus tuberosus* (Linn)	Food
Noug	*Guizotia abysinnica* (Linn)	Oil and Food
Calendula	*Calendula officinalis* (Linn)	Oil, Ornamental, and Herb
Dimorphotheca Daisy	*Dimorphotheca pluvialis* (Linn)	Oil and Ornamental
Vernonia	*Vernonia* spp.	Oil
Crepis	*Crepis* spp.	Oil
Osteospermum	*Osteospermum* spp.	Oil and Ornamental
Stoke's Aster	*Stokesia laevis* (Linn)	Oil and Ornamental
Guayule	*Parthenium argentatum* (Linn)	Rubber
Pyrethrum Daisy	*Chrysanthemum cinerariifolium* (Linn)	Pesticide and Ornamental
Cone flowers	*Echinacea* spp.	Medicinal and Ornamental
Black-Eyed Susans	*Rudbeckia* spp.	Ornamental
Gerbera Daisies	*Gerbera* spp.	Ornamental
Marigolds	*Tagetes* spp.	Ornamental
Chrysanthemums	*Chrysanthemum* spp.	Ornamental
Cosmos	*Cosmos* spp.	Ornamental
Zinnias	*Zinnia* spp.	Ornamental
Lawn Daisy	*Bellis perennis* (Linn)	Ornamental and Herb
Tansy	*Tanacetum vulgare* (Linn)	Ornamental and Herb
Feverfew	*Tanacetum parthenium* (Linn)	Ornamental and Herb
Cotton Thistle	*Onopordum acanthium* (Linn)	Ornamental and Herb
Elecampane	*Inula helenium* (Linn)	Ornamental and Herb
Santolina	*Santolina chamaecyparissus* (Linn)	Ornamental and Herb
Curry Plant	*Helichrysum angustifolium* (Linn)	Ornamental and Herb
Sweet Joe Pye	*Eupatorium purpurea* (Linn)	Ornamental and Herb
Yarrow	*Achillea millefolium* (Linn)	Ornamental and Herb
Artemesias	*Artemesia* spp.	Ornamentals and Herbs
Tarragon	*Artemesia dracunculus* (Linn)	Herb
Costmary	*Chrysanthemum balsamita* (Linn)	Herb
Chamomile	*Anthemis nobilis* (Linn)	Herb
Coltsfoot	*Tussilago farfara* (Linn)	Herb
Dandelion	*Taraxacum officinale* (Linn)	Food and Weed
Ragweeds	*Ambrosia* spp.	Weeds
Hawkweeds	*Hieracium* spp.	Weeds
Thistles	*Cirsium* spp. and *Sonchus* spp.	Weeds
Groundsels	*Senecio* spp.	Weeds

C. The plant investigated

(*Enhydra fluctuans* Lour.)

C. The plant investigated (*Enhydra fluctuans* Lour.)

C.1.1. Classification of *Enhydra fluctuans* Lour:

Kingdom : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Order : Asterales
Family : Compositae
Genus : *Enhydra*
Species :*Enhydra fluctuans* Lour.

C.1.2. Description of the plant *Enhydra fluctuans* Lour[17]:

Common names : Helencha Shak, Hincha Shak, Harahach (Beng), water cress, marsh herb (Eng).

Habit and distribution : An edible semi aquatic herbaceous vegetable plant with serrate lanceolate leaves and small white flowers grows commonly in marshy and watery places all over the country. The leaves are slightly bitter. *Enhydra Fluctuans* Lour. are available throughout Bangladesh, India, Malaysia, Argentina, Vietnam, Assam and other warm countries.

Enhydra fluctuans var. *fluctuans* (Asteraceae) is a species common in South Asia.

Figure C.1: The plant *Enhydra Fluctuans* Lour.

C.1.3. Medicinal uses of *Enhydra fluctuans* Lour :

The leaves of this plant cure inflammation, leucoderma and good in small pox[18].

The leaves are also antibilous and used in nervous and have hypotensive activity[19]. The leaves of this floating herb were used in folk medicine in Malaysia and in India, for their laxative activities and for herpetic treatment[20]. The plant possesses nutritional value and its methanolic extract has been reported to have analgestic activity[21]. Chemical constituents like β-carotene [22,23], sesquiterpene lactones[24,25], terpenes[26] have been reported from this plant. Two new chlorinated melampolides[27] have been isolated and reported recently.Traditional medicines are still very commonly used in India for anthelmintic and antimicrobial purposes. In our way to investigate the local medicinal plants fortheir potential therapeutic uses, present study was aimed to investigate the anthelmintic and antimicrobial activity of petroleum ether, chloroform and ethanol extract of the aerial parts of *E. fluctuants* Lour.. More recently it has been shown to have folic acid activity[28].

C.1.4. Results of the previous phytochemical investigation on *Enhydra fluctuans* Lour.:

A novel bioactive isoflavone glycoside 4',5,6,7-tetrahydroxy-8-methoxyisoflavone-7-O-beta-D-galactopyranosyl-(1-->3)-O-beta-D-xylopyranosyl-(1-->4)-O-alpha-l-rhamnopyranoside from the methanolic extract of the leaves of *Enhydra fluctuans* Lour. Its structure was elucidated by various spectral analysis and chemical degradations[29].

The seasonal variation in the nutrient composition of *Enhydra fluctuans* Lour was studied in order to promote it`s consumption as green leafy vegetable. The plants had high crude protein content throughout all harvesting seasons.

Enhydra fluctuans Lour had a low ash content and was a good source of beta-carotene (3.7 to 4.2 mg /100 g on a fresh weight basis) [23].

The methanol extract of *Enhydra fluctuans* Lour., given orally at the dose of 250 and 500 mg /kg, was evaluated for its analgesic activity using the acetic acid induced writhing and the tail-flick methods. The extract showed promising activity in both tests[31].

The extract of the leaves of *Enhydra fluctuans* Lour. gave, in addition to three known sesquiterpene lactones, two new chlorine containing melampolides[27].

The investigation of the aerial parts of a variety of *Enhydra fluctuans* Lour afforded in addition to 4-hydroxyfarnesyl acetate and fluctuadin five new melampolides elucidated by high field ^{1}H-NMR spectroscopy[33].

Gibberellins A_9 and A_{13} were isolated from *Enhydra fluctuans* Lour[34].
and the structure of clerosterol was elucidated from *Enhydra fluctuans* Lour[26].
The structures of enhydrin and the co-occurring sesquiterpene lactones, viz. fluctuanin and fluctuadin have been isolated from *Enhydra fluctuans* Lour[25].

The petroleum ether extract of *Enhydra fluctuans* Lour. (Compositae) yielded three new diterpenoids, namely, 17-hydroxy-(−)-kaur-15-en-19-oic acid, 15α-isovalcroyloxy- and 15α-angeloyloxy-(−)-kaur-16-en-19-oic acid, in addition to the known (−)-kaur-16-en-19-oic acid, (−)-kauran-16α-ol, 16α-hydroxy-(−)-kauran-19-oic acid and 15α-hydroxy-(−)-kaur-16-en-19-oic acid. The steroidal components isolated are, stigmasterol, 4,22-stigmastadien-3-one, (24S)-24-ethyl-5,22,25-cholestatrien-3β-ol and the hitherto unreported (24S)-24-ethyl-5,22,25-cholestatrien-3β-yl stearate and (24S)-24-ethyl-4,22,25-cholestatrien-3-one. The other compounds isolated are β-amyrin, myricyl alcohol and myristic acid and three sesquiterpene lactones reported earlier[37].

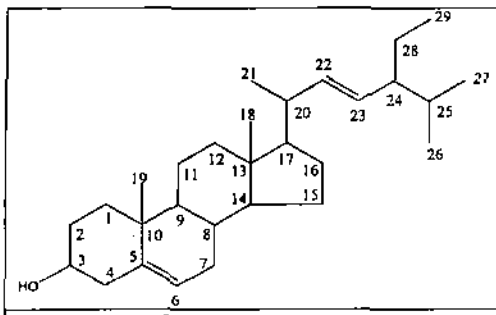
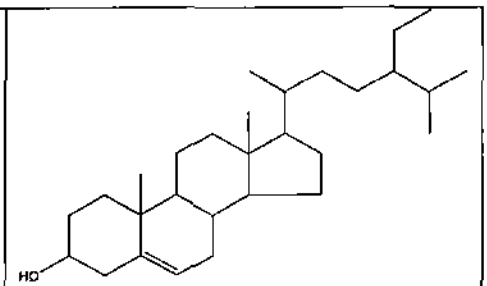
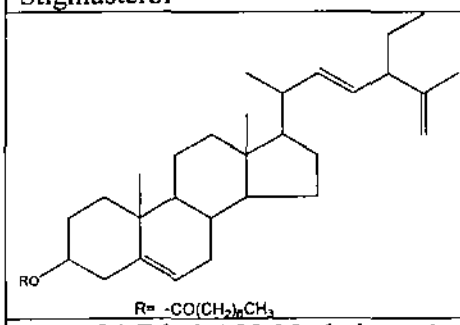
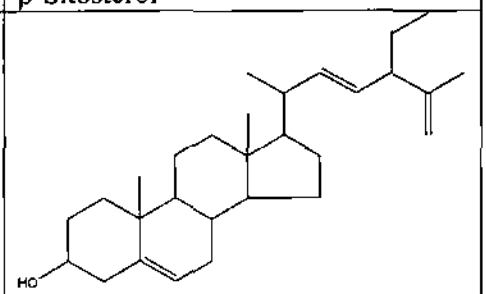
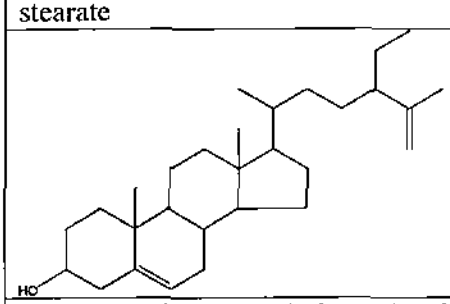
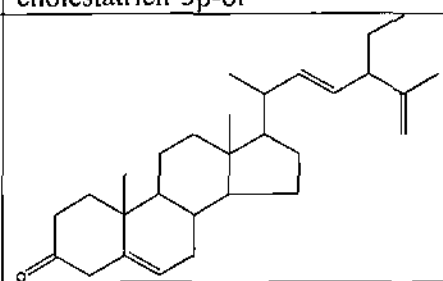
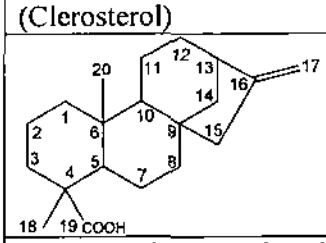
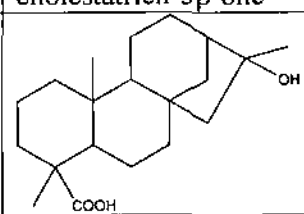
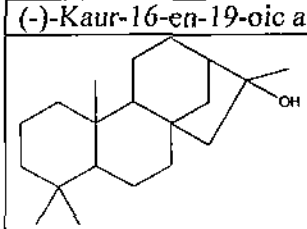
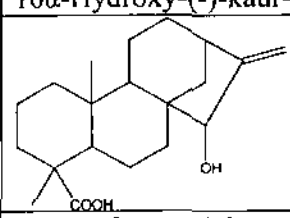
The chemical composition of *Enhydra fluctuans* Lour. essential oil from Vietnam was investigated by GC/retention indices and carbon-13 NMR spectroscopy. Myrcene, limonene, *trans*- and *cis*-dihydroperillaldehydes were the major components[20].

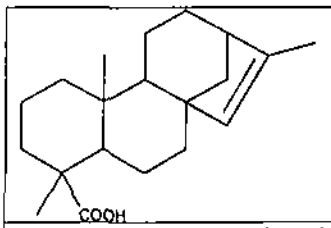
Diterpenoids from *Enhydra fluctuans* Lour was isolated [39].

The methanolic and aqueos extracts of *Enhydra fluctuans* Lour, was evaluated in experimental diarrhea, induced by castor oil in mice. Both methanolic and aqueous extracts, given orally at the dose, of 250 mg kg^{-1} body weight, showed significant anti-diarrhoeal activity[40].

From the petroleum ether extract of *Enhydra fluctuant* Lour, two new steroids have been isolated besides 4,22-stigmastadien-3-one. Spectral studies indicated them to be (24S)-24-ethyl -5,22,25-cholestatrien-3β-yl stearate and (24S)-24-ethyl-4,22,25-cholestatrien-3-one confirmed by their preparation from (24S)-24-ethyl-5,22,25-cholestatrien-3β-ol[41].

C.1.5. Structure of the compounds isolated from *Enhydra fluctuans* Lour:

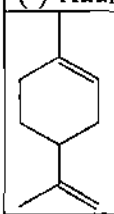
	
Stigmasterol	β-Sitosterol
	
(24S)-24-Ethyl-5,22,25-cholestatrien-3β-yl stearate	(24S)-24-Ethyl-5,22,25-cholestatrien-3β-ol
	
(24S)-24-Ethyl-5,25-cholestatrien-3β-ol (Clerosterol)	(24S)-24-Ethyl-5,22,25-cholestatrien-3β-one
	
(-)-Kaur-16-en-19-oic acid	16α-Hydroxy-(-)-kaur-19-oic acid
	
(-)-Kaur-16α-ol	15α-Hydroxy-(-)-kaur-19-oic acid



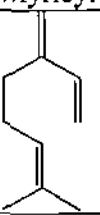
(-)-Kaur-15-en-19-oic acid

$CH_3(CH_2)_{12}CH_2OH$

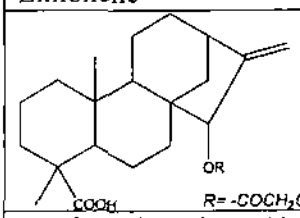
Myricyl alcohol



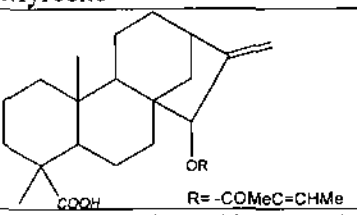
Limonene



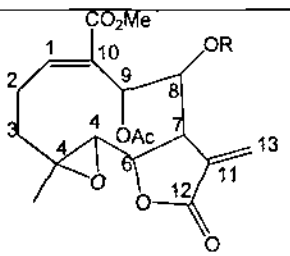
Myrcene

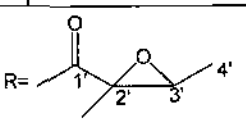


15α-isovaleroyloxy-(-)-kaur-19-oic acid

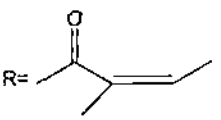


15α-angeloyloxy-(-)-kaur-19-oic acid

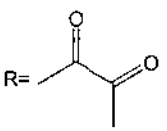




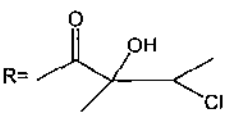
Enhydrin



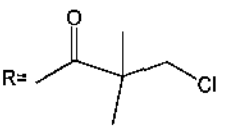
Fluctuanin



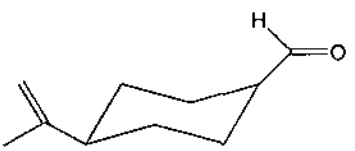
Fluctudin



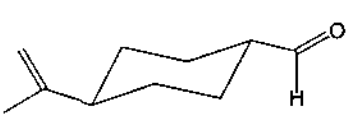
8-Deepoxangeloyl-8-[3-chloro-2-hydroxy-2-methylbutyroyl] enhydrin



8-Deepoxangeloyl-8-[2-hydroxy-3-chloro-isobutyroyl] enhydrin



***cis*-1,2-dihydroperillaldehyde**



***trans*-1,2-dihydroperillaldehyde**

D. The plant investigated

(*Spilanthes acmella* Murr.)

D. The plant investigated (*Spilanthes acmella* Murr.)

D.1. Information about the plant investigated :

D.1.1. Classification of *Spilanthes acmella* Murr.:

Kingdom : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Order : Asterales
Family : Compositae
Genus : *Spilanthes*
Species :*Spilanthes acmella*Murr.

D.1.2. Description of the plant *Spilanthes acmella* Murr.[42]

The *Spilanthes acmella* Murr. is a flowering herb and belongs to the plant family of Asteraceae. It is a small, erect plant that grows quickly and sends up gold and red flower inflorescences. It is very frost sensitive but perennial in the warmer climate. It has no particular odour, but when eaten it has an interesting flavour that slowly develops from pleasant and salty to a strong, tickling-burning pungency that leaves back a numb feeling in the mouth.

Its stem and branches are more or less hairy. Leaves are opposite, 2.5-5 by crenate-serrate or sometimes entire, glabrous or nearly so.

The yellow and brown cone shaped flower is actually a compact inflorescence composed of numerous tiny flowers. The inflorescence begins as a disk; the center

expands upward as the individual flowers develop. The yellow portion of the cone consists of those flowers which have opened and are shedding pollen.

Figure D.1: The plant *Spilanthes acmella* Murr.

D.1.3. Botanical[42,43] feature of *Spilanthes acmella* Murr. :

Botanical name : *Spilanthes Acmella* Murr.

Family name : Compositae

Common name : Toothache Plant, Spilanthes

Bengali name: Bongandha, Marhati-tiga

Part used : Leaves, flowers etc.

Habitat : Spread throughout Bangladesh, India, Ceylon and warm countries.

Product offered : Root

D.1.4. Medicinal uses of *Spilanthes acmella* Murr.[44] :

The most common and widespread use is to treat toothache and throat and gum infections. Worldwide the flower heads are used either fresh or dried and powdered, but the use of roots and leaves has been recommended as well. The plant is further recommended as a cure for dysentery and rheumatism, and to enhance the immune system. It increases the flow of saliva and is useful in fever especially during summers. It is used against blood parasites, especially against malaria, both prophylactic and curative.

The herb exhibits general immunomodulator properties when used internally, boosting production of leukocytes and antiviral interferon, as well as promoting phagocytosis. The leaves may also used to treat bacterial and fungal skin diseases. The internal use of this herb stimulates an increased rate of phagocytosis. It stimulates wound healing, protects the individual from colds and flu.

The leaves and flower heads contain analgesic, antifungal, anthelminthic, and antibacterial agents, but some of the compounds are destroyed by desiccation or freezing. As noted in the culinary uses section, *Spilanthes* is also a potent sialogogue.

The main active ingredient, spilanthol, has been reported poisonous to invertebrates (though harmless to warm-blooded creatures) and effective against blood parasites at even low concentration. The herb exhibits general immunomodulator properties when used internally, boosting production of leukocytes and antiviral interferon, as well as promoting phagocytosis.

The leaves may be used topically to treat bacterial and fungal skin diseases such as ringworm.

Therapeutic action: antifungal, antiviral, antiseptic. Enhance the immune system's resistance to infections. Stimulates wound healing.

Clinical indication: allergies, candidisis, herpes simplex, bacterial diseases, cold, ginvitis, influenza, infection, swollen glands, viral diseases.

D.1.5. Results of the previous phytochemical investigation on *Spilanthes acmella* Murr :

A literature survey of *Spilanthes acmella* Murr. revealed that some phytochemical works has been done on this plant. Reports of isolated compounds and other information available in the literature below:

More than 45 compounds have been identified including (E)-2-hexanol, 2-tridecanene germacrene, hexanol, β-caryophyllene and (Z)-3-hexanol[45].

A mixture[46] of C_{22}-C_{35} normal hydrocarbon has been isolated from the flower heads of *Spilanthes acmella* Murr. and identified by GC.

5-α-reductase inhibitors, antihistamins, *p*-menthane-3,8-diol and bioactive N-isobutylamides were isolated from *Spilanthes acmella* Murr[47].

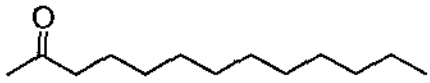
Spilanthol, undeca-2-E-7-Z-9-E-trienoic acid isobutylamides and undeca-2E-en-8-10-diyonoic acid isobutyl amide have been reported[48].

Characterization of the essential oil from flowers head of *Spilanthes acmella* Murr. has been reported[49].

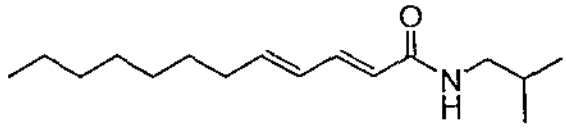
Phytochemical[50] studies *Spilanthes acmella* Murr. has been reported in the myericyl alcohol, palmitic acid, stearic acid, α-amyrin acetate, β-sitosterol, β-amyrin, α-amyrin, β-sitosterol D(+)glucoside and stigmasterol D(+)glucocide were isolated.

A report[51] on isolation α and β-aminoester and sitosterol glucoside from *Spilanthes acmella* Murr. with acitic, lauric, myristic and linoleic acid.

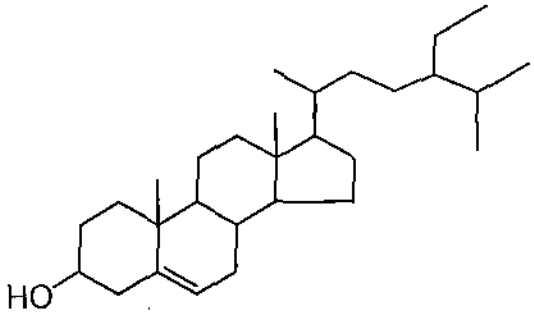
D.1.6. Structure of the compounds isolated from *Spilanthes acmella* Murr :



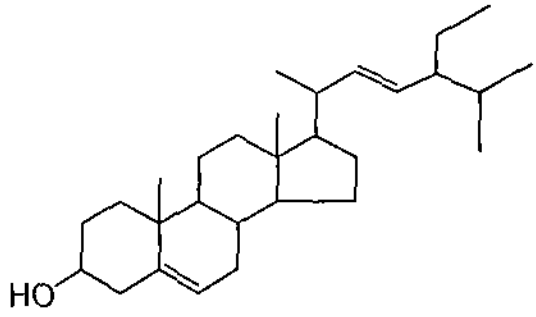
2-Tridecanone



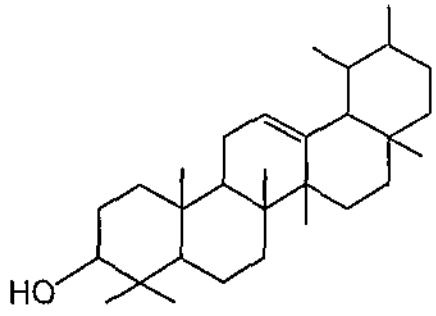
***iso*-Butylamide**



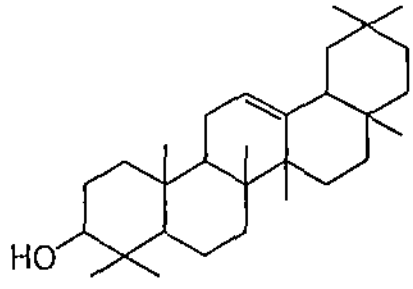
β-Sitosterol



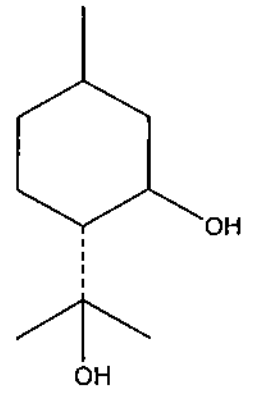
Stigmasterol



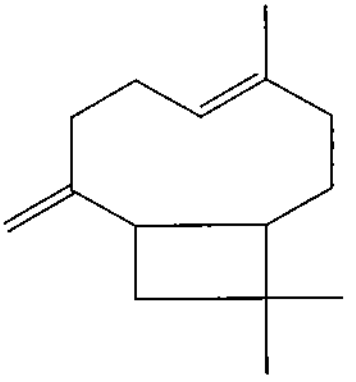
α-Amyrin



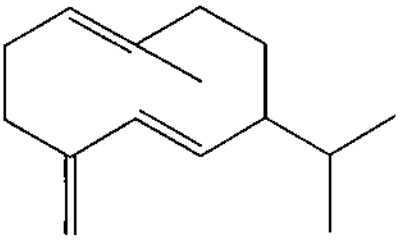
β-Amyrin



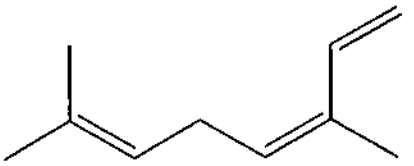
***p*-Menthane-3,8-diol**



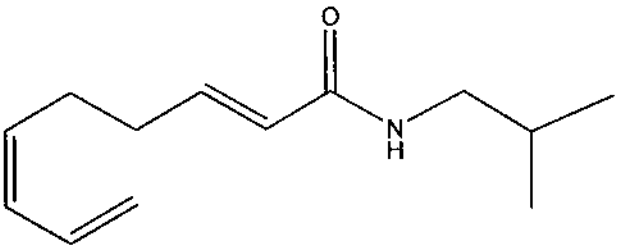
β-Caryophyllene



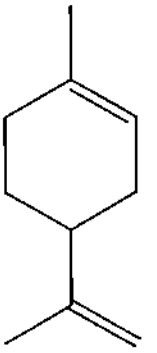
Germacrene



Ocimene



Spilanthol



Limonene

E. Methodology

E. Methodology

E.1. General methods:

E.1.1. Distillation of the solvents:

All solvents and analytical or laboratory grade reagents used during the investigation were procured E Merck (Germany) and BDH (England). All the solvents were distilled in a glass distillation set before use. Petroleum ether (40-60°C) was obtained by distilling petrol. Commercial grade hexane, dichloromethane, chloroform, ethyl acetate, acetone, methanol were used after distillation.

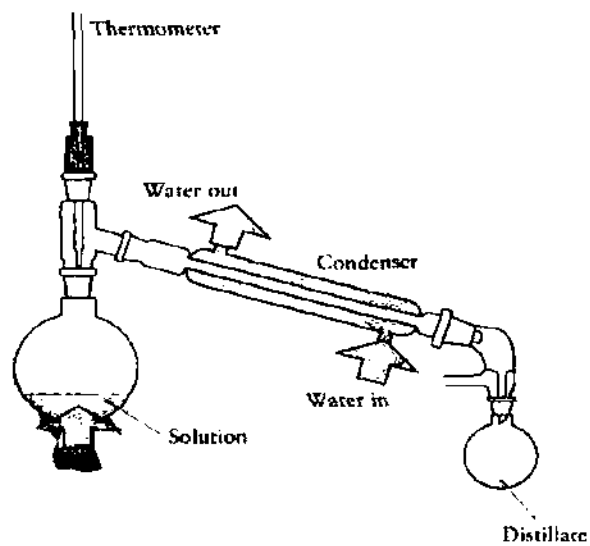


Figure E.1: Distillation of the solvents

E.1.2. Evaporation:

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

Figure E.2: Rotary vacuum evaporator

E.1.3. Preparation of extracts:

The plant was collected and washed with water to remove mud and dust particles. The plant was first dried in room temperature and then in the oven at 40°C. The dried plant was grinded to powder by a grinding machine. The powder was stored in air tight bottle for extraction.

E.1.4. Extraction procedure:

E.1.4.1. Initial extraction:

Extraction can be done in two ways such as

a) Cold extraction

b) Hot extraction

a) **Cold extraction** : In cold extraction the powdered plant materials is submerged in a suitable solvent or solvent systems in an air-tight flat bottomed container for several days, with occasional shaking and stirring. The major portion of the extractable compounds of the plant material will be dissolved in the solvent during this time and hence extracted as solution.

b) **Hot extraction**: In hot extraction the powdered plant material is successively extracted to exhaustion in a Soxhlet at elevated temperature with several solvents of increasing polarity.

The plant material extracted exhaustively in Soxhlet apparatus first with petroleum ether (boiling point, 60-80°C) and then with dichloromethane (CH_2Cl_2). All the extracts were filtered individually and then concentrated with a rotary evaporator (Buchii) at low temperature (40-50°C) under reduced pressure.

E.1.5. Chromatographic techniques:

Chromatography has been defined as primarily a separation process which is used for the separation of essentially molecular mixtures. It depends upon the redistribution of molecules of the mixture between two or more phases. It is the best and modern separation technique.

For separation of extracted compounds into individual pure ones, various types of chromatographic techniques were used, such as column chromatography, thin layer chromatography (TLC), preparative thin layer chromatography (PTLC) and vacuum liquid chromatography (VLC).

minutes[52, 53]. Table E.1.7 shows the amount of silica gel required for preparing plates of various thicknesses.

Table E.1.7. Amount of silica gel required for preparing TLC plates of various thicknesses:

Size (cm × cm)	Thickness (mm)	Amount of silica gel/plate (gm)
20 × 5	0.3 0.4 0.5	0.9 1.2 1.5

Cylindrical glass chamber (TLC tank) with airtight lid is used for the development of chromatoplates.

E.1.8. Preparation of TLC tank:

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

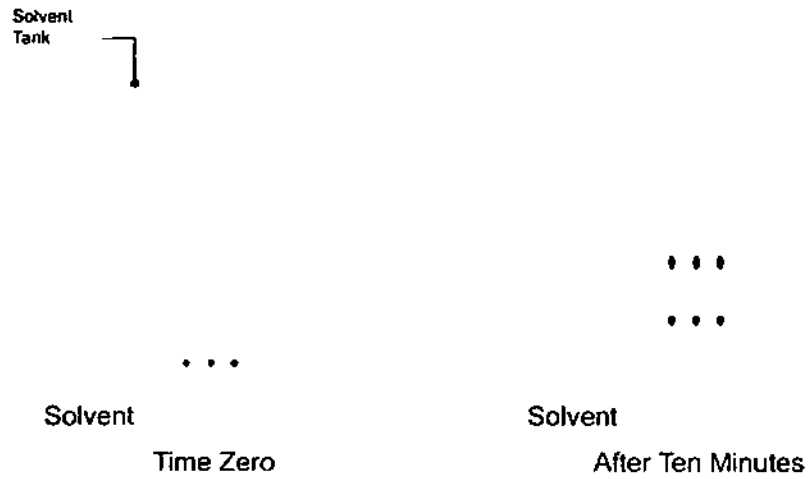


Figure E.4: TLC tank

E.1.9. Sample application (spotting the plates):

A small amount of dried extract is dissolved in a suitable solvent to get a solution (approximately 1%)[54,55]. The TLC plates were spotted with a small amount of the solution by using a narrow glass capillary. The capillary was washed with either acetone or ethanol before each sample was applied. The solution is applied on the activated silica plate with a capillary tube just 1 cm above the lower edge of the plate. The spot is dried with a hot air blower and a straight line is drawn 2 cm below the upper edge of the activated plate which marks the upper limit of the solvent flow.

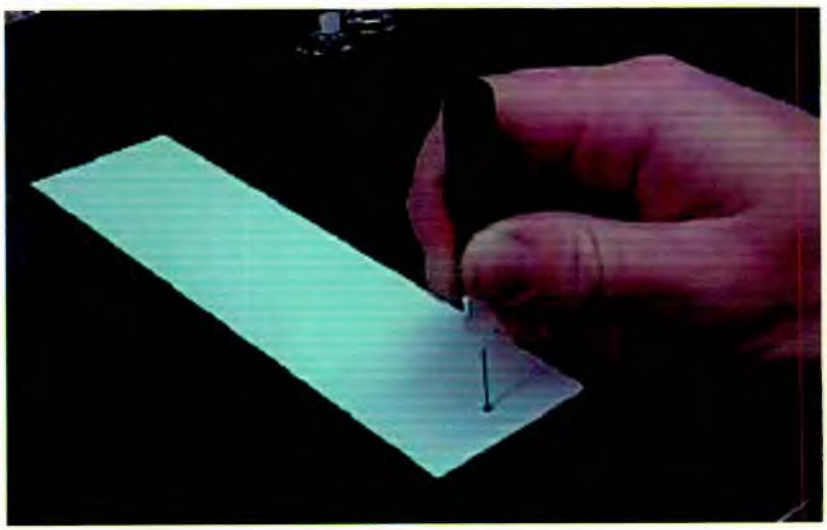


Figure E.5: Spotting TLC plate

E.1.10. Preparation of the reagents:

The properly developed plates are viewed under UV light of various wavelengths (254 nm and 366 nm) as well as treated with suitable reagents to detect the compounds.

E.1.10.1. Vanilin sulphuric acid reagent:

Sulphuric acid (400 ml) and absolute alcohol (100 ml) were mixed in a beaker (kept in an ice bath). Vanillin (0.25 g) was added to the mixture of alcohol and sulfuric acid, cooled and used for spraying the TLC plates.

Figure E.6: Vanillin H_2SO_4 sprayer

E.1.10.2. Dragendroff's reagent:

Solution A: Bismuth sub nitrate (0.6 g) was dissolved in water (2 ml)
Solution B: Potassium iodide (0.6 g) was dissolved in water (10 ml)
These two solutions were mixed and 22 ml of dilute HCl (7 ml of con. HCl + 15 ml water) was added to it .Then the mixture was diluted with water (400 ml).

E.1.11. Solvent systems:

The solvents used for TLC are given below:

(i) Hexane
(ii) Dichloromethane
(iii) Ethyl acetate
(iv) Methanol
(v) Hexane: Dichloromethane (in different ratio)
(vi) Hexane: Ethyl acetate (in different ratio)
(vii) Dichloromethane: Ethyl acetate (in different ratio)
(viii) Dichloromethane: Methanol (in different ratio)

E.1.12. Iodine chamber:

The developed chromatogram is placed in a closed chamber containing crystals of iodine and kept for few minutes. The compounds that appeared as brown spots are marked. Unsaturated compounds absorb iodine. Bound iodine is removed from the plate by air blowing.

E.1.13. Detection of spots :

For the location of the separated components, the plates were examined by the following methods:

1. Examination under UV lights in different wavelength, 254 and 350 nm.
2. The plates were exposed to iodine vapor for several minutes.
3. The plates were sprayed with vanillin-sulfuric acid regent (1.0%) followed by heating in an oven at 120°C for 15 minutes.

E.1.14. The R_f value:

Retardation factor (R_f) is the ratio of the distance travels by of the compound to the distance travels by the solvent front.

$$R_f = \frac{\text{distance travels by the compound}}{\text{distance travels by the solvent front}}$$

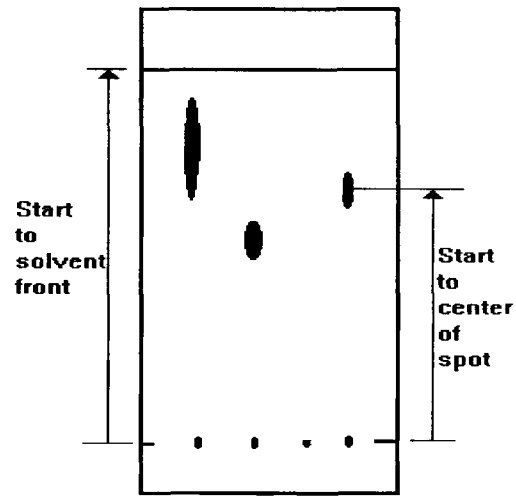


Figure E.7: R_f value

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

E.1.15. Vacuum liquid chromatography (VLC):

The concept of vacuum liquid chromatography is a relatively recent development in the field of chromatographic separation. It is a column

chromatography under reduced pressure and the column is packed with TLC grade silica gel. This technique was used for fractionating the crude extracts in order to obtain either pure compound or a mixture with a small number of compounds. A glass column of about 40 cm length having a system fitted with a water pump and collecting tube at the bottom was taken (Figure E.8). Silica gel (G-60PF_{254}) was used as adsorbent. Silica gel slurry in petroleum ether or any other suitable solvent was prepared. This slurry was packed into hard cake under an applied vacuum to give a column 3.15 cm in diameter and about 7.0 cm in height. The sample mixture or extract which to be separated, was dissolved in a minimum volume of suitable solvent. Then small amount of silica gel (around twice of the amount of sample) was mixed with sample solution. After through mixing, the solvent of the mixture of sample in silica gel was evaporated using rotary evaporator. To ensure complete removal of solvent, the sample-silica gel mixture was kept in vacuum desiccators. After drying, the mixture was converted into the powder by mortar. The powder was then poured on the top of the packed column to form a layer. Gradient elution with increasing polarity of eluting solvent was used until the more polar component was eluted. These elutes were collected manually in the fraction of different amounts and then the individual fractions were monitored by TLC.

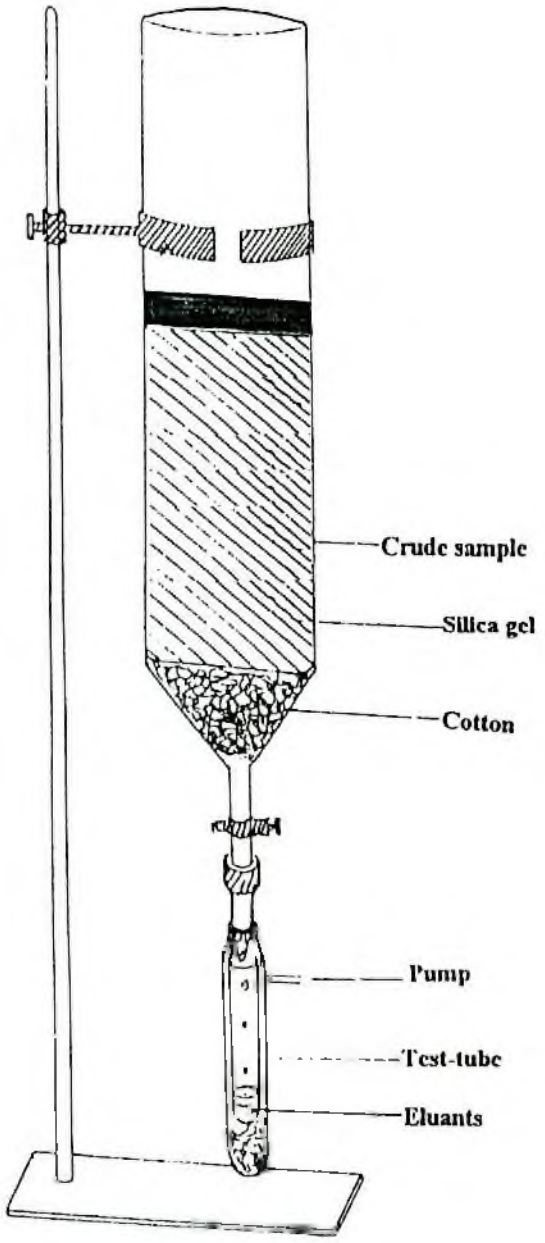


Figure E.8: Vacuum liquid chromatography (VLC) apparatus.

E.1.16. Crystallization:

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

E.1.17. Spectroscopic techniques:

E.1.17.1. Ultra-violet spectroscopy (UV):

Ultra-violet spectra were recorded using the Shimadzu UV–160A spectrophotometer .A small amount of the samples of different compounds were dissolved in chloroform or pyridine separately. The solution was taken in quartz cell (1 cm x 1 cm) to record the spectrum. The main bands (λ_{max}) were recorded as wavelength (nm).

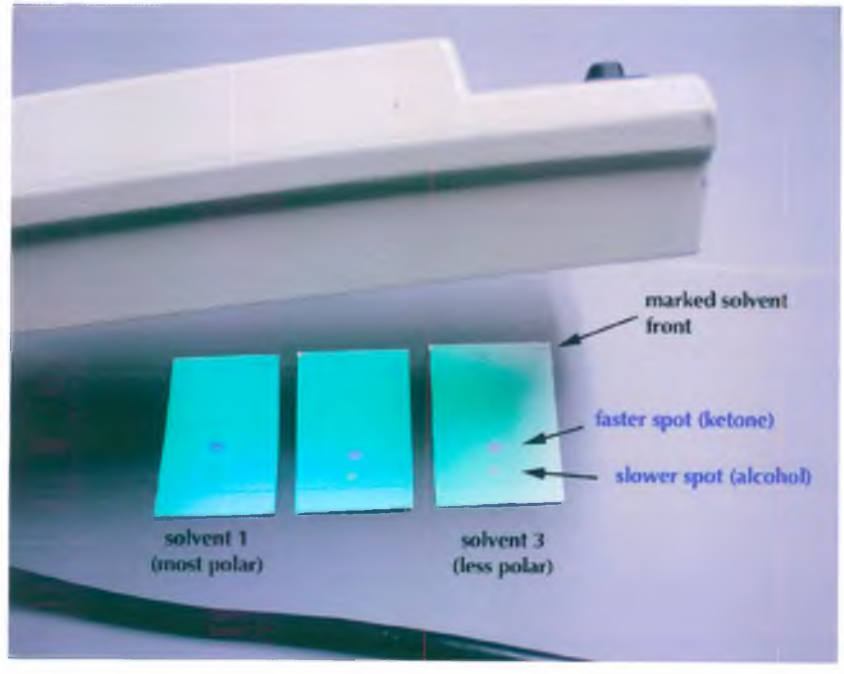


Figure E.9: Detection of the spots with UV light

E.1.17.2 Infra-red spectroscopy (IR):

A Shimadzu IR-470 spectrophotometer was used for recording infra-red spectrum. Major bands (ν_{max}) were recorded in wave number (cm^{-1}) as KBr pellets.

E.1.17.3. Nuclear magnetic resonance (NMR) spectroscopy:

^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra of pure sample were recorded by using an Ultra Shield Bruker DPX 400 spectrometer. The spectra were record using $CDCl_3$ with tetra methyl silane (TMS) as standard reference.

E.1.18. Melting point apparatus:

Melting point (m.p) was determined by using an electro thermal melting point apparatus (Mel-temp, OGAWA SEIKICO, and Japan).

E.1.19. Mass spectroscopy:

Mass spectrum of pure compound was recorded using GC-MS (Agilent Technologies 5975B EI/CI MSD, EU)

F. Experimental

F. Experimental

F.1. Investigation on *Enhydra fluctuans* Lour. and *Spilanthes acmella* Murr.

F.1.1. Collection of the plants:

The plants *Enhydra fluctuans* Lour. and *Spilanthes acmella* Murr. have been collected from the local market of Dhaka city and Curzon Hall area of Dhaka University respectively. These were identified from the Department of Botany, Dhaka University. The collected fresh plants were cleaned thoroughly. The plant materials (leaves and stems) were separated from its roots and dried under mild sunlight and then at 40°C in an oven. Afterwards the plants were powdered in a grinding machine (~200 meshes). These powders were used throughout the investigation.

F.1.2. Phytochemical screening of the plants:

Chemical tests were carried out on the aqueous extract and on the powdered specimens using standard procedures to identify the different constituents and the results are given in Table G.1.1 and Table H.1.1.

F.1.2.1. Qualitative determination[56]:

Test for tannins: The dried powder samples (~0.5 g) was boiled in water (20 ml) in a test tube and then filtered. A few drops of ferric chloride solution (0.1%w/v) were added and brownish green or a blue-black coloration was observed.

Test for phlobatannins: Deposition of a red precipitate (on boiling) extract of each plant sample was boiled with aqueous hydrochloric acid (1% w/v) indicated the presence of phlobatannins.

Test for saponin: The powdered sample (~ 2 g) was boiled in distilled water (20 ml) in a water bath and filtered. The filtrate (10 ml) was mixed with distilled water (5 ml) and shaken vigorously for a stable persistent froth. The frothing was mixed with olive oil (3 drops) and shaken vigorously, and then the formation of emulsion was observed.

Test for flavonoids: Three methods were used to determine the presence of flavonoids in the plant sample-

a) Dilute ammonia solution (5 ml) was added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H_2SO_4.

b) Few drops of aluminium ion (Al^{3+}) solution (1%) were added to a portion of each filtrate. A yellow coloration was observed indicating the presence of flavonoids.

c) A portion of the powdered plant sample in each case was heated with ethyl acetate (10 ml) over a steam bath for 3 min. The mixture was filtered and the filtrate (4 ml) was shaken with dilute ammonia solution (1 ml). A yellow colouration was observed indicating a positive test for flavonoids.

Test for steroids: Acetic anhydride (2 ml) was added to ethanolic extract (0.5 g) of each sample with H_2SO_4 acid (2 ml). The colour changed from violet to blue or green in some samples indicating the presence of steroids.

Test for terpenoids (Salkowski test): Each extract (5 ml) was mixed in chloroform (2 ml), and concentrated H_2SO_4 acid (3 ml) was carefully added to

form a layer. A reddish brown colouration at the interface was formed to show positive test for the presence of terpenoids.

Test for cardiac glycosides (Keller-Killani test): Each extract (5 ml) was treated with glacial acetic acid (2 ml) containing ferric chloride solution (1 drop). This was underlayed with concentrated H_2SO_4 acid (1 ml). A brown ring at the interface indicates a deoxysugar characteristic of cardinolides. A violet ring may appear below the brown ring, while in the acetic acid layer, greenish ring may from just gradually throughout thin layer.

F.1.3. Qantitative determination of the chemical constituent:

Alkaloid determination[57] *: Each sample (0.5 g) was taken into a conical flask* (100 ml) and acetic acid (20 ml, 10% in ethanol) was added. It was covered and allowed to stand for 12 hours. This was filtered and the extract was concentrated on water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to stand and the precipitate was collected and washed with dilute ammonium hydroxide solution and then filtered. The residue was the alkaloid, which was dried and weighed. The results are given in TableG.1.2. and Table H.1.2.

Flavonoid determination [58]**:** The plant sample (1 g) was extracted repeatedly with aqueous methanol (20 ml in 80%) (CH_3OH) at room temperature. The whole solution was filtered through Whitman filter paper No 42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness in a water bath and weighed to a constant weight. The results are given in Table G. 1.2. and Table H.1.2.

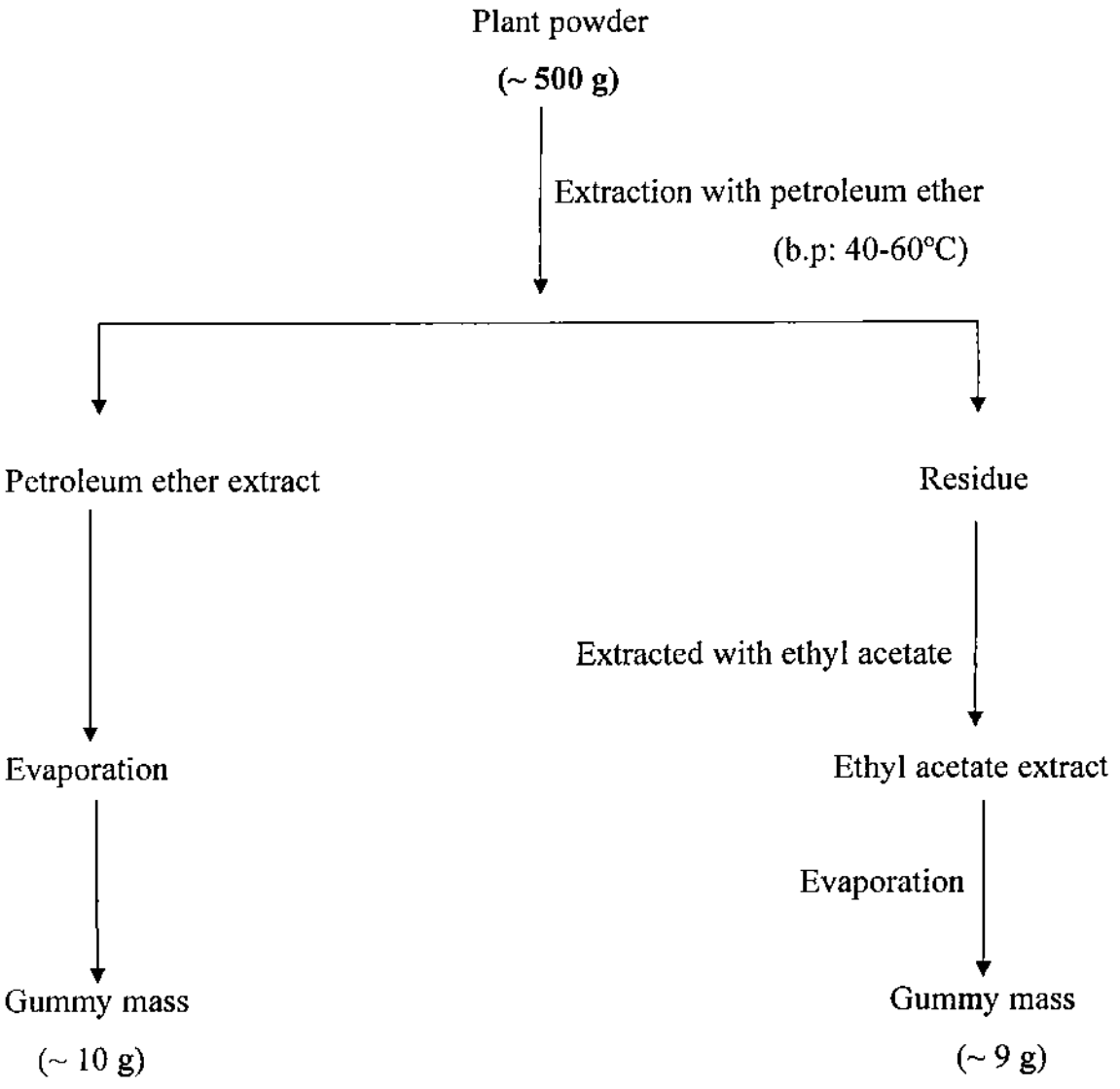
Saponin determination[59]**:** Each of the powder sample (5 g) was put into a conical flask and 20% aqueous ethanol (25 ml) was added. The sample was heated over a hot water bath for 4 hours with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with 20% aqueous ethanol (50 ml). The combined extracts were reduced to 10 ml over water bath at about 90°C. The concentrated extract (was transferred into a separatory funnel and diethyl ether (5 ml) was added and shaken vigorously. The aqueous layer was recovered while the other layer discarded. The purification process was repeated. *n*-butanol (15 ml) was added and *n*-butanol extract was washed twice with aqueous 5% NaCl (3 ml of) solution. The remaining solution was heated on a water bath. After evaporation the sample was dried in an oven to a constant weight; the saponin content calculated as percentage. The results are given in Table G. 1.2. and Table H.1.2.

F.2. Extraction, isolation and investigation of compounds from the plant *Enhydra fluctuans* Lour with different solvents:

F.2.1. Extraction:

The powder (~ 500 g) was extracted in a Soxhlet apparatus separately and successively with petroleum ether (b.p 40-60°C) and ethyl acetate. The extract was filtered and concentrated to a small volume separately using a 'Buchii' Rotary vacuum evaporator under reduced pressure. This was shown in Scheme F.2.2.

F.2.2. Extraction scheme of *Enhydra fluctuans* Lour.:



Scheme F.2.2: Solvent extraction scheme of *Enhydra fluctuans* Lour. plant

F.2.3. Investigation of petroleum ether (40-60°C) extract:

F.2.3.1 TLC (Thin Layer Chromatography) study:

TLC analysis of the petroleum–ether (b.p 40 - 60°C) extract showed several spots under UV–light, in iodine chamber and vanillin-sulphuric acid spray on TLC plate.

F.2.3.2. Fractionation by VLC (Vacuum liquid chromatography):

The petroleum ether extract was concentrated by rotary evaporator. The concentrated pet ether extract (~ 3.5 g) was mixed with TLC grade silica gel (60 GF_{254}).This sample was placed on the top of the bed of silica gel packed column(VLC). The column was then eluted with petroleum ether followed by mixtures of petroleum ether (PE) and dichloromethane (DCM), ethyl acetate (EA) and methanol (ME) of increasing polarity. Solvent system used as mobile phases in the analysis of petroleum ether extract was listed in Table F.2.1.

Table F.2.1: Number of fractions collected in test tubes from VLC of petroleum ether extract by using different solvent system:

Solvent system				Amount of Solvents (ml)	Number of test tubes
PE (%)	DCM (%)	EA (%)	ME (%)		
100	0	0	0	1600	1-84
98	2	0	0	500	85-104
96	4	0	0	300	105-119
94	6	0	0	400	120-132
92	8	0	0	300	133-143
90	10	0	0	400	144-158
88	12	0	0	300	159-171
86	14	0	0	350	172-181
84	16	0	0	200	182-190
81	19	0	0	300	191-203
78	22	0	0	250	204-212
75	25	0	0	300	213-226
70	30	0	0	300	227-237
65	35	0	0	400	238-254
60	40	0	0	300	255-168
55	45	0	0	300	169-279
50	50	0	0	250	280-288
40	60	0	0	250	289-296
30	70	0	0	200	297-306
20	80	0	0	200	307-318
10	90	0	0	200	319-326
0	100	0	0	200	327-334
0	95	5	0	100	335-338
0	90	10	0	150	339-345
0	85	15	0	100	346-349
0	80	20	0	100	350-352
0	75	25	0	100	353-356
0	70	30	0	100	357-360
0	65	35	0	100	361-364
0	60	40	0	100	365-368
0	50	50	0	100	369-372
0	40	60	0	100	373-376
0	30	70	0	100	377-381
0	20	80	0	100	382-386
0	10	90	0	100	387-391
0	0	100	0	100	392-396
0	0	90	10	100	397-400
0	0	80	20	100	401-404
0	0	70	30	100	405-408
0	0	60	40	100	409-412
0	0	50	50	100	413-416
0	0	40	60	100	417-420
0	0	0	100	100	421-423

F.2.3.3. Screening of the fractions:

Each of the fractions was monitored by TLC and the fractions of similar behaviors were combined together and marked as F_1, F_2, F_3, F_4, F_5, F_6, F_7, F_8, F_9, F_{10}, F_{11}, F_{12}, F_{13}, F_{14} and F_{15} and given in Table F.2.2.

Table F.2.2 : Screening of the fractions by the similar TLC pattern:

Test Tube Numbers	Fractions	TLC Pattern
1-14	F_1	One spot with tailing.
15-33	F_2	One spot with no tailing.
34-50	F_3	Two spots with tailing.
51-78	F_4	Mixture.
79-104	F_5	Three spots.
105-130	F_6	Two spots with tailing.
131-150	F_7	One spot with no tailing.
151-170	F_8	Two spots.
171-210	F_9	One spot with tailing.
211-278	F_{10}	One spot with no tailing.
279-316	F_{11}	Mixture
317-334	F_{12}	Mixture
335-365	F_{13}	Mixture
366-396	F_{14}	Mixture
397-423	F_{15}	Mixture

F.2.3.4. Analysis of the fractions:

TLC analysis of the fractions showed that F_2 and F_7 were single compounds but other fractions (F_3, F_4, F_5, F_6 and F_1) were mixture. So F_2 and F_7 were analyzed.

a) Analysis of fraction F_2:

Fraction F_2 (30 ml) was concentrated to a small volume under reduced pressure and was left undisturbed at room temperature. After several days, an white crystalline substance was obtained. The crystals were washed

with hexane very well. After washing, the crystals were dissolved in chloroform and the solution was kept in a vial for recrystallization at room temperature and nice crystals were obtained. Its TLC study showed a single spot in different solvent systems. It was designated as **EF_3**.

b) Analysis of fraction F_7 :

Fraction F_7 (70 ml) was concentrated in a rotary evaporator and was left undisturbed at room temperature for several days. A white crystalline powder was obtained. The crystals were separated from mother liquor and purified by washing with different solvents. The powder was washed with hexane very well. After washing, it was dissolved in chloroform and the solution was kept in a vial for recrystallization at room temperature and nice crystals were obtained. Its TLC study also showed a single spot in different solvent systems. It was designated as **EF_4**.

F.2.3.5. Properties of the isolated compounds:

F.2.3.5.1.The compound EF_3 :

F.2.3.5.2. Properties of EF_3 :

Physical state: white crystalline.
Solubility: Soluble in chloroform and hexane.
R_f value: 0.25 (over silica gel, GF_{254}, petroleum ether : dichloromethane = 3:2 as the mobile phase).
Melting point: 170-172°C
Amount: 10 mg.

F.2.3.5.3. Spectral characteristics:

F.2.3.5.3. 1. IR spectrum (in KBr pellet):

IR spectrum of compound EF_3 (Figure F.1) had important frequencies of absorption (ν_{max}) at 3450, 3050, 2900, 1680, 1650, 1460, 1350, 1210, 870 and 800 cm^{-1} .

F.2.3.5.3.2. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$):

^{1}H-NMR spectrum of compound EF_3 (Figure F.2, F.3 and F.4) had important chemical shift (δ_H value) at 0.94 (s), 1.23 (s), 2.62 (m), 4.72 (s), 4.78 (s) and 1.39-2.13 (m,d,t) ppm.

F.2.3.5.3.3. ^{13}C-NMR spectrum (100 MHz, $CDCl_3$) :

^{13}C-NMR spectrum of compound EF_3 (Figure F.5, F.6 and F.7) had important chemical shift (δ_C value) at 185.0, 155.7, 103.0, 57.0, 55.1, 48.9, 44.2, 43.8, 43.7, 41.2, 40.7, 39.9, 37.7, 33.1, 28.9, 21.8, 19.0, 18.4 and 15.5 ppm.

F.2.3.5.3. 4. EIMS (70 eV) m/z:

Mass spectrum of compound EF_3 (Figure F.8) showed important ion peaks at (m/z) (relative intensity %) at 302 (73), 287 (66), 259 (79), 257 (33), 243 (68), 241 (71), 213 (50) and 187 (29), 131 (87), 105 (78), 91 (100) and 79 (65).

F.2.3.5.4. The compound EF_4 :

F.2.3.5.5. Properties of EF_4:

Physical state: White needle shape crystal.

Solubility: Readily soluble in chloroform.

R_f value: 0.70 (over silica gel, GF_{254}, pet-ether-dichloromethane = 3:2 as the mobile phase).

Melting point: 146-148°C

Amount: 7 mg.

F.2.3.5.6. Spectral characteristics:

F.2.3.5.6.1. IR spectrum (KBr pellet):

IR spectrum of compound EF_4 *(Figure F.9) had important frequencies of absorption* (ν_{max}) at 3450, 3050, 2920, 2850, 1650, 1460, 1370, 1050, 960, 880 and 800 cm^{-1}.

F.2.3.5.6.2. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$):

^{1}H-NMR spectrum of compound EF_4 (Figure F.10, F.11 and F.12) had important chemical shift (δ_H value) at 0.68 (s), 0.82 (m), 1.00 (3), 1.01 (d), 1.66 (br. s), 2.41 (br. q), 3.52 (m), 5.17 (m), 5.24 (m), 5.34 (d) and 1.07-2.29 (m,d,t) ppm.

F.2.3.5.6.3. ^{13}C-NMR spectrum (100 MHz, $CDCl_3$) :

^{13}C-NMR spectra of compound EF_4 (Figure F.13, F.14 and F.15) had important chemical shift (δ_C value) at 12.0, 19.0, 19.4, 20.2, 20.8, 21.0, 24.3, 28.7, 31.6, 31.8, 31.9, 36.5, 37.2, 39.7, 40.1, 42.3, 50.1, 55.9, 56.8, 71.8, 121.7 130.0, 137.2 and 140.3 ppm.

F.3. Investigation of ethyl acetate extract:

F.3.1. TLC (Thin layer chromatography) study:

TLC analysis of the ethyl acetate extract showed several spots under UV–light, in iodine chamber and vanillin-sulphuric acid spray on TLC plate.

F.3.2. Fractionation by VLC (Vacuum liquid chromatography):

The ethyl acetate extract was concentrated by rotary evaporator. The concentrated ethyl acetate extract (~ 9 g) was mixed with TLC grade silica gel (60 GF_{254}).This sample was placed on the top of the bed of silica gel packed column(VLC). The column was then eluted with petroleum ether followed by mixtures of petroleum

ether (PE) and dichloromethane (DCM), ethyl acetate (EA) and methanol (ME) with increasing polarity. Solvent system used as mobile phases in the analysis of ethyl acetate extract was listed in Table F.3.1.

Table F.3.1: Number of fractions collected in test tubes from VLC of ethyl acetate extract by using different solvent system:

Solvent system				Total amounts of solvents (ml)	Numbers of test Tubes
PE (%)	DCM (%)	EA (%)	ME (%)		
100	0	0	0	900	1-46
99	1	0	0	100	47-50
98	2	0	0	100	51-55
97	3	0	0	300	56-70
96	4	0	0	100	71-74
95	5	0	0	100	75-90
94	6	0	0	100	91-94
93	7	0	0	200	95-104
92	8	0	0	100	105-113
91	9	0	0	150	114-117
90	10	0	0	200	118-123
88	12	0	0	100	124-132
86	14	0	0	100	133-137
84	16	0	0	100	138-142
82	18	0	0	100	143-147
80	20	0	0	200	148-157
78	22	0	0	100	158-162
75	25	0	0	100	163-166
70	30	0	0	200	167-177
65	35	0	0	100	178-182
60	40	0	0	100	183-185
55	45	0	0	200	186-189
50	50	0	0	100	190-195
45	55	0	0	100	196-200
40	60	0	0	100	201-206
30	70	0	0	100	207-210
20	80	0	0	100	211-215
10	90	0	0	100	216-220
0	100	0	0	200	221-229
0	98	2	0	100	230-234
0	96	4	0	100	235-240
0	94	6	0	100	241-244
0	92	8	0	100	245-248
0	90	10	0	100	249-252
0	88	12	0	100	253-257
0	85	15	0	100	285-261

Solvent system				Total amounts of solvents (ml)	Numbers of test Tubes
PE (%)	DCM (%)	EA (%)	ME (%)		
0	82	18	0	100	262-266
0	80	20	0	100	267-272
0	75	25	0	100	273-277
0	70	30	0	100	278-282
0	68	32	0	100	283-286
0	65	35	0	100	287-291
0	60	40	0	100	292-295
0	55	45	0	100	296-300
0	50	50	0	100	301-305
0	45	55	0	100	306-309
0	40	60	0	100	310-314
0	30	70	0	100	315-318
0	25	75	0	100	319-323
0	20	80	0	100	324-328
0	10	90	0	100	329-333
0	0	100	0	100	334-338
0	0	98	2	100	339-343
0	0	95	5	100	344-348
0	0	92	8	100	349-354
0	0	90	10	100	355-359
0	0	85	15	100	360-364
0	0	80	20	100	365-369
0	0	75	25	100	370-374
0	0	70	30	100	375-380
0	0	70	35	100	381-385
0	0	60	40	100	386-390
0	0	30	70	100	391-395
0	0	20	80	100	396-400

F.3.3. Screening of the fractions:

Each of the fractions was monitored by TLC and the fractions of similar behaviors were combined together and marked as F_1, F_2, F_3, F_4, F_5, F_6, F_7, F_8, F_9, F_{10}, F_{11} and it is given in Table F.3.2.

Table F.3.2: Screening of the fractions by the similar TLC pattern:

Test Tube Numbers	Fractions	TLC Pattern
1-16	F_1	One spot with tailing.
17-24	F_2	One spot with no tailing.
25-50	F_3	Two spots with tailing.
51-91	F_4	Mixture.
92-137	F_5	One spot with no tailing.
138-180	F_6	One spot with tailing.
181-215	F_7	Two spots with tailing.
216-270	F_8	Two spots.
271-310	F_9	Three spots
311-378	F_{10}	Mixture
379-400	F_{11}	Mixture

F.3.4. Analysis of the fractions:

TLC analysis of the fractions showed that F_2 and F_5 were single compounds but F_1, F_3, F_4, F_6, F_7, F_8, F_9, F_{10}, F_{11} and F_{12} were found as a mixture of compounds. So F_2 and F_5 were analyzed.

a) Analysis of fraction F_2:

Fraction F_2 (40 ml) was concentrated to a small volume under reduced pressure and was left undisturbed at room temperature. After several days a white crystalline substance was obtained. The crystals were washed with hexane very well. After washing, the crystals were dissolved in chloroform and the solution was kept in a vial for recrystallization at room temperature when nice crystals were obtained. Its TLC study showed a single spot in different solvent systems. It was designated as **EFA**.

F.3.5. Properties of the isolated compounds:

F.3.5.1 The compound EFA:

F.3.5.2. Properties of EFA :

Physical state: white crystalline.

Solubility: Soluble in chloroform and hexane.

R_f value: 0.25 (over silica gel, GF_{254}, pet-ether-dichloromethane = 3:2 as the mobile phase).

Melting point: 142-146°C

Amount: 10 mg.

F.3.5.3. Spectral characteristics:

F.3.5.3.1. IR spectrum (KBr pellet):

IR spectrum of compound EFA (Figure F.16) had important frequencies of absorption (ν_{max}) at 3450, 3050, 1720, 1640, 1450, 1360, 1240, 1030, 970, 890, 880 and 800 cm^{-1}.

F.3.5.3.2. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$):

^{1}H-NMR spectrum of compound EFA (Figure F.17, F.18 and F.19) had important chemical shift (δ_H value) at 0.69 (s) 0.83 (t), 0.99 (d), 1.00 (s), 1.6 (m), 2.4 (m) 3.47 (m), 4.58 (m), 4.68 (m), 5.17 (m), 5.22 (m), 5.36 (br.d) and 1.1-2.4 (m,d,t) ppm.

F.3.5.3.3. ^{13}C-NMR spectrum (400 MHz, $CDCl_3$) :

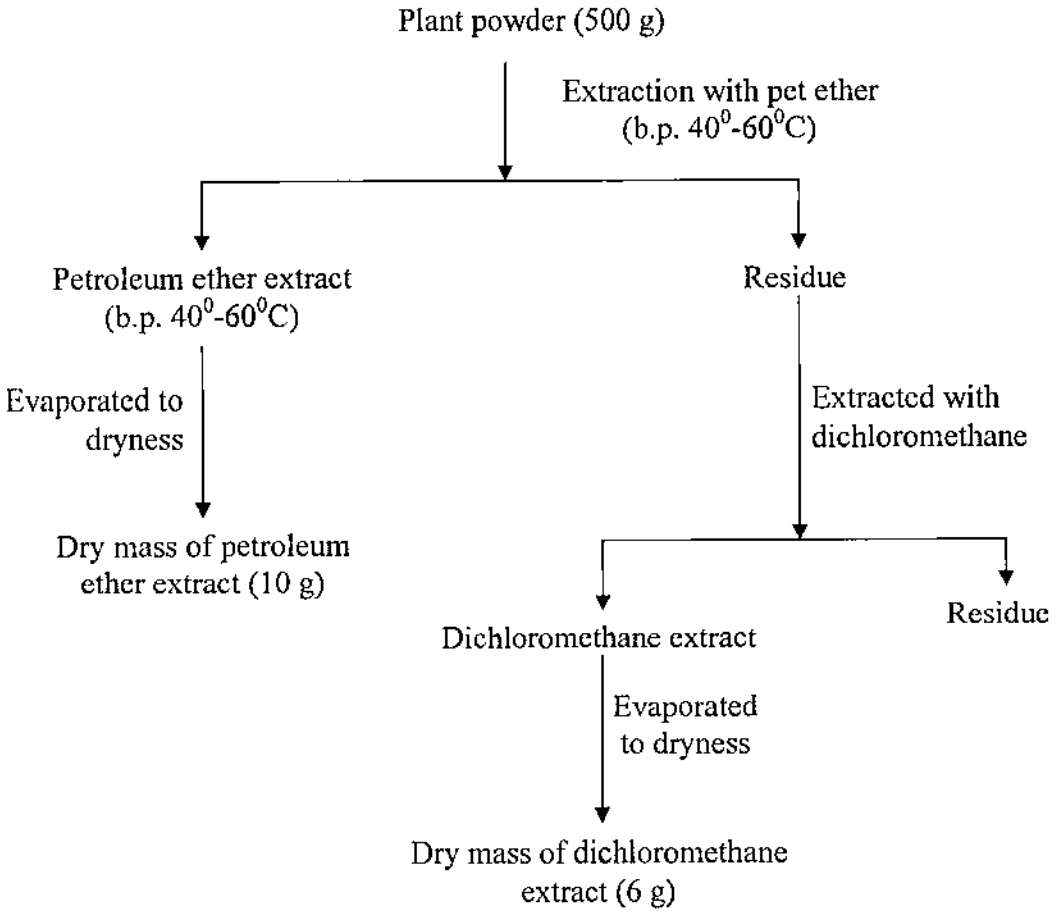
^{13}C-NMR spectrum of compound EFA (Figure F.20, F.21, F.22, F.23 and F.24) had important chemical shift (δ_C value) at 12.1, 19.3, 20.2,21.0, 21.4, 24.4, 25.7, 27.9, 28.6, 29.2, 31.8, 31.9, 36.6, 37.0, 38.1, 40.1, 42.2 , 50.1, 52.0, 55.9, 56.8, 74.0, 109.5, 122.6, 130.0, 137.1, 139.6, and 148.6 ppm.

F.4. Extraction, isolation and investigation of compounds from the plant *Spilanthes acmella* Murr. with different solvents :

F.4.1 Extraction:

The leaves' powder (500 gm) of *Spilanthes acmella* Murr. (Section F.1.1) was extracted in a Soxhlet apparatus separately and successively with petroleum ether (b.p 60-80°C) and dichloromethane (CH_2Cl_2). The two extracts were filtered individually and concentrated to a small volume using a conventional distillation set and then a rotary evaporator (Buchii) under reduced pressure. This was shown in Scheme F.4.2.

F.4.2 Extraction of scheme of *Spilanthes acmella* Murr:



Scheme F.4.2: Extraction of *Spilanthes acmella* Murr

F.4.3. Isolation and characterization of compounds from petroleum ether extract of leaves powder of *Spilanthes acmella* Murr. :

F.4.3.1. Thin layer chromatography of the petroleum ether extract:

A TLC study (Petroleum ether: EtOAc = 9:1) of petroleum ether extract showed the presence of three spots under day-light. Upon exposure to iodine vapour it

gave two deep brown colored spots that were visualized under daylight. Again, TLC study showed the presence of six spots by spraying with vanillin-sulfuric acid followed by heating for 10 minutes. Of these six spots two were violet, three were pink and one was green. The presence of pink colour was thought to be an indication of the presence of either steroid or fatty acid material or both. However, its Salkowski and Libermann-Burchard reaction gave positive result confirming the presence of steroids.

F.4.3.2. Fractionation of the extract by vacuum liquid chromatography (VLC):

The pet-ether extract was concentrated by rotary evaporator. The concentrated pet-ether extract (10 g) was mixed with column grade silica gel. This sample was placed on the top of the bed of column (VLC) packed with column grade silica gel.

The column was then eluted with 100% petroleum ether (b.p. 40-60°C), petroleum ether followed by mixtures of petroleum ether (PE) and dichloromethane (DCM), and methanol (ME) with increasing polarity.

The fractions were collected in an amount of about 20 ml in a series of test tubes. Solvent systems used as mobile phases in the analysis of petroleum ether extract were listed in Table F.4.1.

Table F.4.1: Number of fractions collected in test-tubes from VLC of petroleum ether extract by using different solvent systems:

Solvent systems			Amount of solvent (ml)	Number of test tubes
PE (%)	DCM (%)	ME (%)		
100	0	0	800	1-33
98	2	0	200	34-42
99	1	0	200	43-50
96	4	0	200	51-58
93	7	0	200	59-68
90	10	0	100	69-80
85	15	0	100	81-84
80	20	0	100	85-89
75	25	0	100	90-93
70	30	0	100	94-98
60	40	0	100	99-103
50	50	0	100	104-107
40	60	0	100	108-111
30	70	0	100	112-115
20	80	0	100	116-124
10	90	0	100	125-128
0	100	0	100	129-136
0	98	2	100	137-140
0	95	5	100	141-143
0	92	8	100	144-147
0	90	10	100	148-150
0	50	50	100	151-155
0	0	100	100	156-160

F.4.3.3. Screening of the fractions:

Each of the fractions was monitored by TLC (over silica gel PF_{254}). Fractions of the similar TLC behavior were combined together and were designated as F_1, F_2, F_3, F_4, F_5, F_6 , F_7 , F_8 , F_9 and F_{10} .

Table F.4.2. TLC behaviors of fractions are listed in table:

Test Tube Numbers	Fractions	TLC Pattern
1-19	F_1	No spots
20-29	F_2	Two spots with tailing
30-45	F_3	Three spots with tailing
46-65	F_4	Tailing
66-79	F_5	Three spots.
80-110	F_6	Mixture
111-119	F_7	Three spots.
120-135	F_8	Three spots with tailing
136-144	F_9	Single spots
145-166	F_{10}	Mixture

F.4.3.4. Analysis of the fractions by TLC:

TLC analysis of the fractions was further carried out when fraction F_9 was found to contain a single compound. The rest of the fractions were found to contain mixture of compounds. So attempt was taken to characterize the fraction F_9.

F.4.3.5. Analysis of the fraction F_9 :

Fraction F_9 was left undisturbed at room temperature for several days. White needle shaped crystals were obtained. The crystals were separated from mother liquor and washed with different solvents. Its TLC study showed a single spot. It was designated as **SA-1**.

F.4.3.6. Properties of the compound isolated from petroleum ether extract:

F.4.3.6.1. The compound SA-1:

F.4.3.6.2. Properties of SA-1 :

Physical state	: White crystal
Solubility	: Soluble in dichloromethane and chloroform
R_f value	: 0.5 (solvent system, Petroleum ether: EtOAc = 9:1)
Melting point	: 148-150°C
Amount	: 4 mg

F.4.3.6.3. Spectroscopic characteristics:

F.4.3.6.3.1. IR Spectrum:

IR Spectrum (Figure F.25) ν_{max} cm^{-1} (in KBr pellet):

The compound SA-1 had important frequencies of absorption at 3450, 2920, 2850, 1655, 1455, 1365 and 1040 cm^{-1}.

F.4.3.6.3.2. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) (Figure F.26, F.27 and F.28):

Important chemical shift value (δ) of SA-1 were found at 5.35(d), 5.15(dd), 5.03(dd), 3.51(m), and 1.00(s), 91(d), 0.85(d), 0.84(d), 0.80(t) and 0.69(s) ppm.

F.4.3.6.3.3. ^{13}C-NMR spectrum (400 MHz, $CDCl_3$) (Figure F.29):

Important chemical shift value (δ) of SA-1 were found at 141.0, 138.0, 128.5, 121.7, 71.8, 56.9, 56.1, 42.4, 37.3, 31.9, 31.7, 36.2, 29.7, 21.3, 21.1, 19.4 and 19.0 ppm.

F.5. Isolation and characterization of compounds from dichloromethane extract of leaves' powder of *Spilanthes acmella* Murr. :

The dichloromethane crude extract was subjected to TLC screening to find out the type of compounds present in the extract. The whole portion of the dichloromethane extract (5.0 gm) was subjected to column chromatography for rapid fractionation. The column fractions obtained from column were monitored by TLC.

F.5.1. Column chromatography (CC) of dichloromethane crude extract:

The column was packed with fine silica gel (Kieselgel 60, mesh 70-230) was used as the packing material. Slurry of silica gel in *n*-hexane was added into a glass column having the length and diameter of 62 cm and 4 cm respectively. When the desired height of the adsorbent bed was obtained, the column was washed with few hundred milliliter of *n*-hexane to facilitate proper packing. The sample was prepared by adsorbing 3.5 gm of methanol soluble extract onto silica gel (Kieselgel 60, mesh 70-230), allowed to dry and subsequently applied on top of the adsorbent layer. The column was then eluted with *n*-hexane, followed by mixtures of *n*-hexane (HE) and dichloromethane (DCM) with increasing polarity, then by DCM and finally with DCM and methanol (ME) mixtures with increasing polarity. Solvent systems used as mobile phases in the analysis of *n*-hexane soluble extract were listed in Table F.5.1. A total of 20 fractions were collected.

Table F.5.1: Different solvent systems used for column chromatography (CC) analysis of dichloromethane crude extract:

Solvent systems			Volume collected (ml)	Fraction No.
HE (%)	DCM (%)	ME (%)		
100	0	0	300	1
75	25	0	200	2
70	30	0	200	3
65	35	0	200	4
60	40	0	200	5
55	45	0	200	6
50	50	0	200	7
45	55	0	200	8
40	60	0	200	9
35	65	0	200	10
30	70	0	200	11
25	75	0	200	12
20	80	0	200	13
10	90	0	200	14
0	100	0	200	15
0	98	2	200	16
0	95	5	100	17
0	90	10	100	18
0	50	50	100	19
0	0	100	100	20

F.5.2. Analysis of column fractions by TLC:

All the column fractions were screened by TLC under UV light and a number of similar fractions were mixed together and identified them by new codes which are given in Table F.5.2.

Table F.5.2. List of new fraction codes:

Column fraction	New code
1 – 4	C-1
5 – 6	C-2
7 – 9	C-3
10 – 11	C-4
12 – 13	C-5
14 – 19	C-6
19 – 20	C-7

F.5.3. Attempt of purification and characterization of the fractions:

All the fractions obtained by column chromatography (CC) were monitored by TLC under UV light and by spraying with vanillin-sulfuric acid reagent followed by heating at 110°C. On the basis of the TLC behavior, fractions C-1, C-4, C-5 and C-6 were selected for further investigations.

F.5.4. Analysis of fraction C-1:

Fraction C-1 left undisturbed at the room temperature for several days. A white needle shaped crystal (40 mg) was obtained. The crystals were separated from the mother liquor and washed with different solvents. Its TLC study (solvent system, PE : EtOAc= 80:20) showed three spots of which two were found to be UV active and other was UV inactive. The UV inactive spot was identified by using vanillin-

sulfuric acid spray reagent. The latter compound was separated by preparative thin layer chromatography (PTLC).

F.5.5. Preparative thin layer chromatography:

The crystals (~40 mg) were dissolved in chloroform to make a solution. This solution was used to separate the component by TLC using the solvent system pet-ether and ethyl acetate (80:20). After drying, the plate was covered with a paper except an edge and the uncovered part was sprayed with vanillin-sulfuric acid reagent and heated by hot air blower. When reddish pink color was observed on the edge which became violet colored by heating for long time. Then the plate was marked accordingly.

F.5.6. Separation and elution of the identified band :

The band was separated with the help of a clean spatula and was taken in a previously cleaned beaker. Then ethyl acetate was added to the beaker and shaken for several minutes.

An elution tube was cleaned and dried well. It was then plugged with cotton and fixed with a stand. The content of the beaker was then introduced into the elution tube. The filtrate was collected in a beaker. Then another 30 ml of ethyl acetate was passed through the elution tube and the filtrate was collected in the beaker. After removal of the solvent, white crystal was obtained which was designated as **SA-2**.

F.5.7. Column chromatography (CC) of combined column fractions (C-4, C-5, C-6) :

The column was packed with fine silica gel (Kieselgel 60, mesh 70-230) as the packing material. Slurry of silica gel in n-hexane was added into a glass column having the length and diameter 62 cm and 4 cm respectively. When the desired height of the adsorbent bed was obtained, the column was washed with a few hundred milliliter of n-hexane to facilitate proper packing.

The sample was prepared by adsorbing 0.6 gm of combined column fraction into silica gel (Kieselgel 60, mesh 70-230), allowed to dry and subsequently applied on top of the adsorbent layer.

The column was then eluted with n-hexane, followed by mixtures of *n*-hexane (HE) and ethyl acetate (EA) with increasing polarity, then by ethyl acetate and finally with ethyl acetate and methanol (ME) mixtures of increasing polarity. Solvent systems used as mobile phases in the analysis of column fractions 4, 5 and 6 were listed in Table F.5.3. A total of 20 fractions were collected.

Table F.5.3. Different solvent systems used for column fractions of dichloromethane extract:

Solvent systems			Volume collected (ml)	Fraction No.
HE (%)	EA (%)	ME (%)		
100	0	0	200	1
90	10	0	50	2
80	20	0	50	3
70	30	0	50	4
60	40	0	50	5
55	45	0	50	6
50	50	0	50	7
45	55	0	50	8
40	60	0	50	9
35	65	0	50	10
30	70	0	50	11
25	75	0	50	12
20	80	0	50	13
15	85	0	50	14
10	90	0	50	15
5	95	0	50	16
0	100	0	50	17
0	95	5	50	18
0	50	50	50	19
0	0	100	50	20

F.5.8. Analysis of column fractions by TLC :

All the column fractions were monitored by TLC under UV light and the similar fractions were mixed together and marked them by new codes which are given in Table F.5.4.

Table F.5.4 List of new fraction codes:

Column fraction	New code
1 – 5	F-1
6 – 9	F-2
10 – 15	F-3
16 – 20	F-4

F.5.9. Isolation and purification of compounds from selected fractions:

TLC analysis of the fractions showed that F-3 was single compound but F-1, F-2 and F-4 were found as a mixture of compounds. So, F-3 was analyzed.

F.5.9.1. Isolation and purification of compound F-3:

F-3 was found to yield colored crystals. The crystals were washed with petroleum ether carefully .Finally these crystals were dissolved in chloroform and transferred to a vial for recrystallization and was designated as **SA-3** (3 mg).

F.5.9.2. Properties of the compound isolated from dichloromethane extract :

The compound SA-2:

F.5.9.2.1. Properties of SA-2:

Similar to SA-1 (Section: F.4.3.6.2)

F.5.9.2.2. Spectroscopic characteristics:

F.5.9.2.2.1. IR Spectrum:

Similar to SA-1 (Section: F.4.3.6.3.1)

F.5.9.2.2.2. ^{1}H-NMR Spectrum (400 MHz, $CDCl_3$):

Similar to SA-1 (Section: F.4.3.6.3.2)

F.5.9.2.3. The compound SA-3 :

F.5.9.2.4. Properties of SA-3:

Physical state	: White crystalline powder
Solubility	: Soluble in dichloromethane and chloroform
R_f value	: *0.45 (solvent system, toluene: EtOAc = 95:5)*
Amount	: 3 mg

F.5.9.2.5. Spectroscopic characteristics:

F.5.9.2.5.1. IR Spectrum ν_{max} cm^{-1} (in KBr pellet) (Figure F.30):

The compound SA-3 had important frequencies of absorption at 3450, 2920, 2850, 1655, 1455, 1365, 1040 cm^{-1}.

F.5.9.2.5. 2. ^{1}H-NMR Spectrum (400 MHz, $CDCl_3$) (Figure F.31 and F.32):

The compound has important ^{1}H-NMR chemical shift value (δ) of SA-3 were observed at 5.71(s), 1.16 (s), 0.69(s), 0.90(d) and 0.84(d) ppm.

F.5.9.2.6.Chemical test for SA-3:

Chemical test for >C=O group was carried out and it was found to be positive.

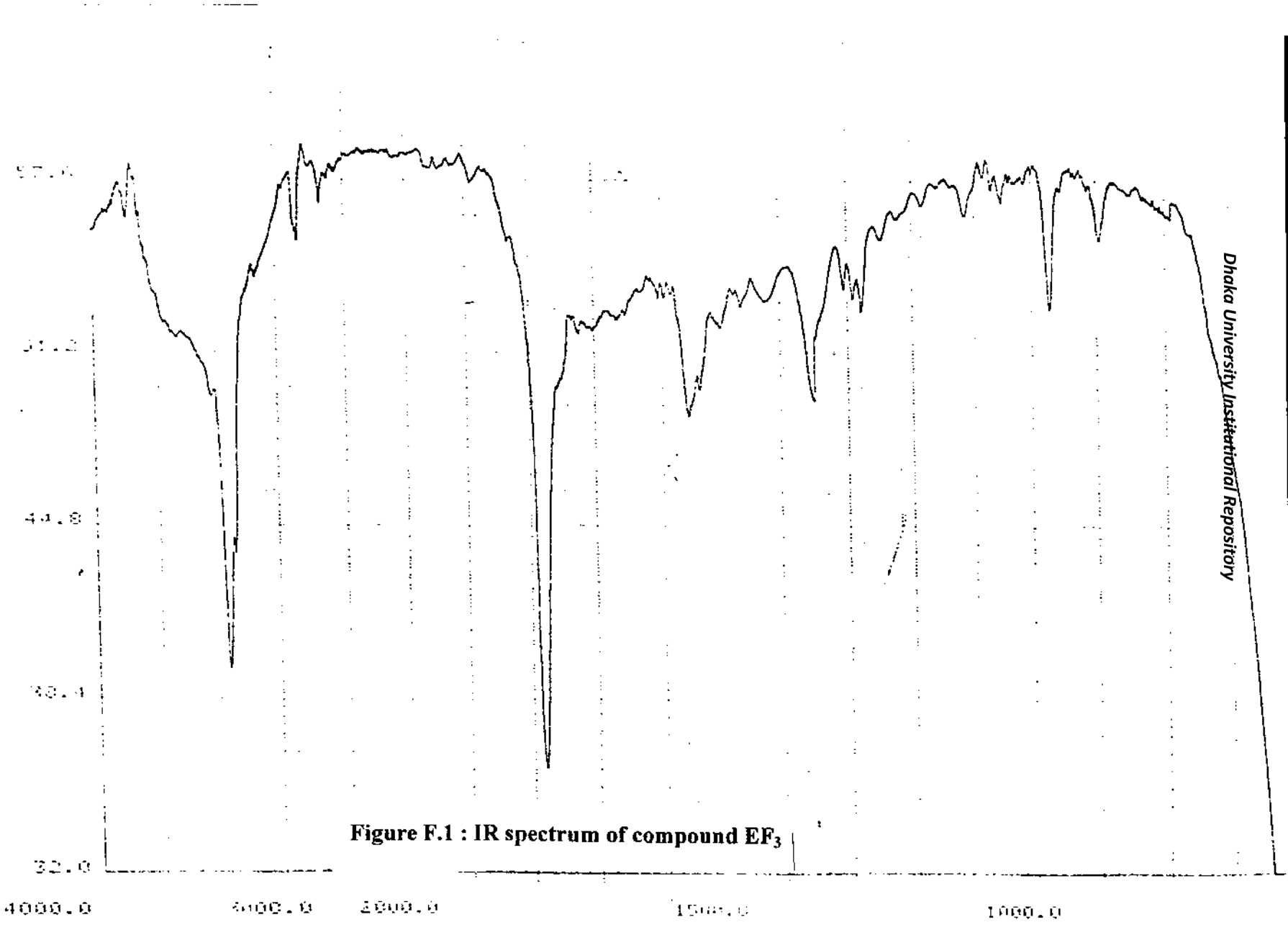


Figure F.1 : IR spectrum of compound EF_3

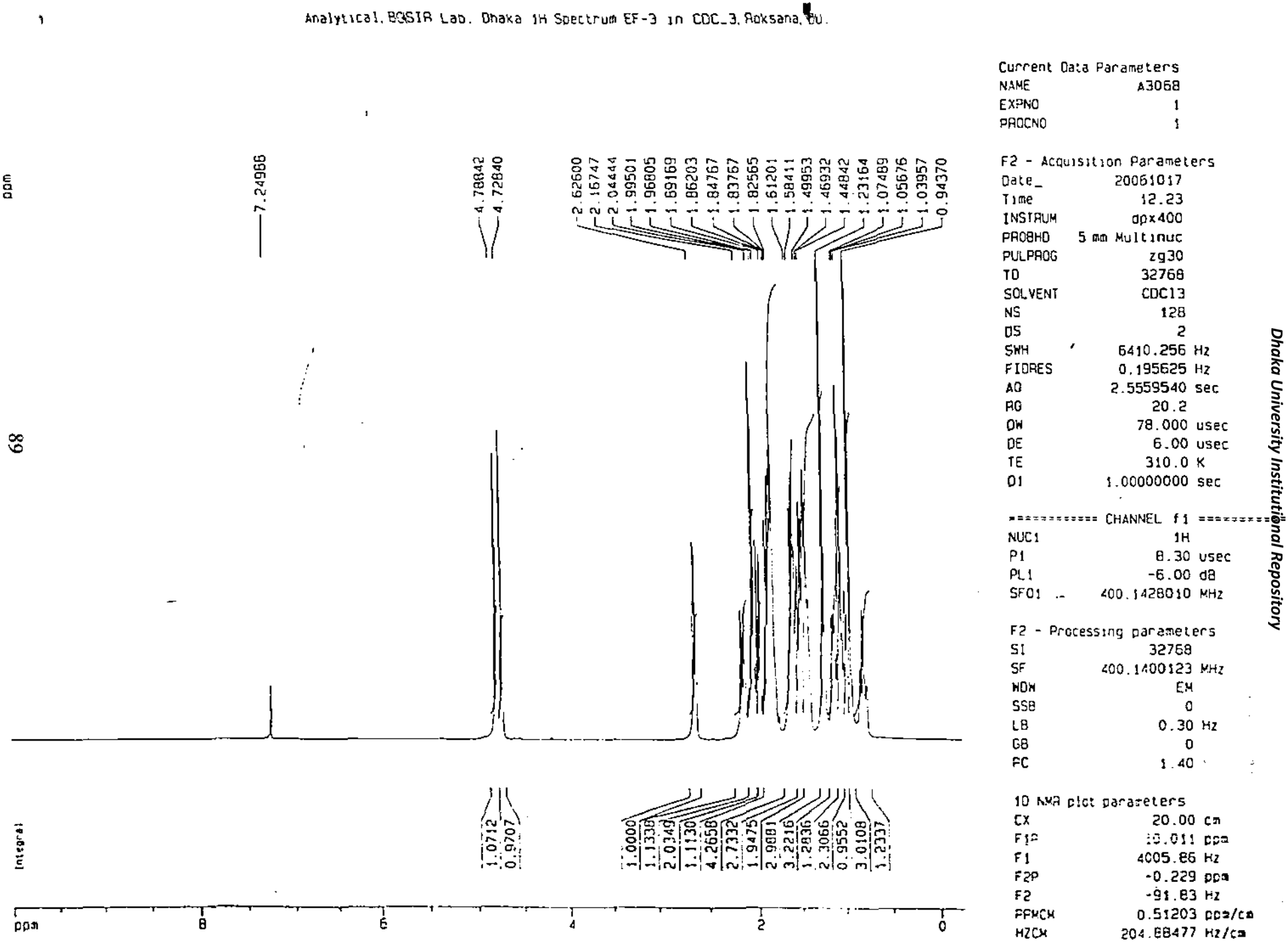


Figure F.2 : ^{1}H-NMR spectrum of compound EF_3

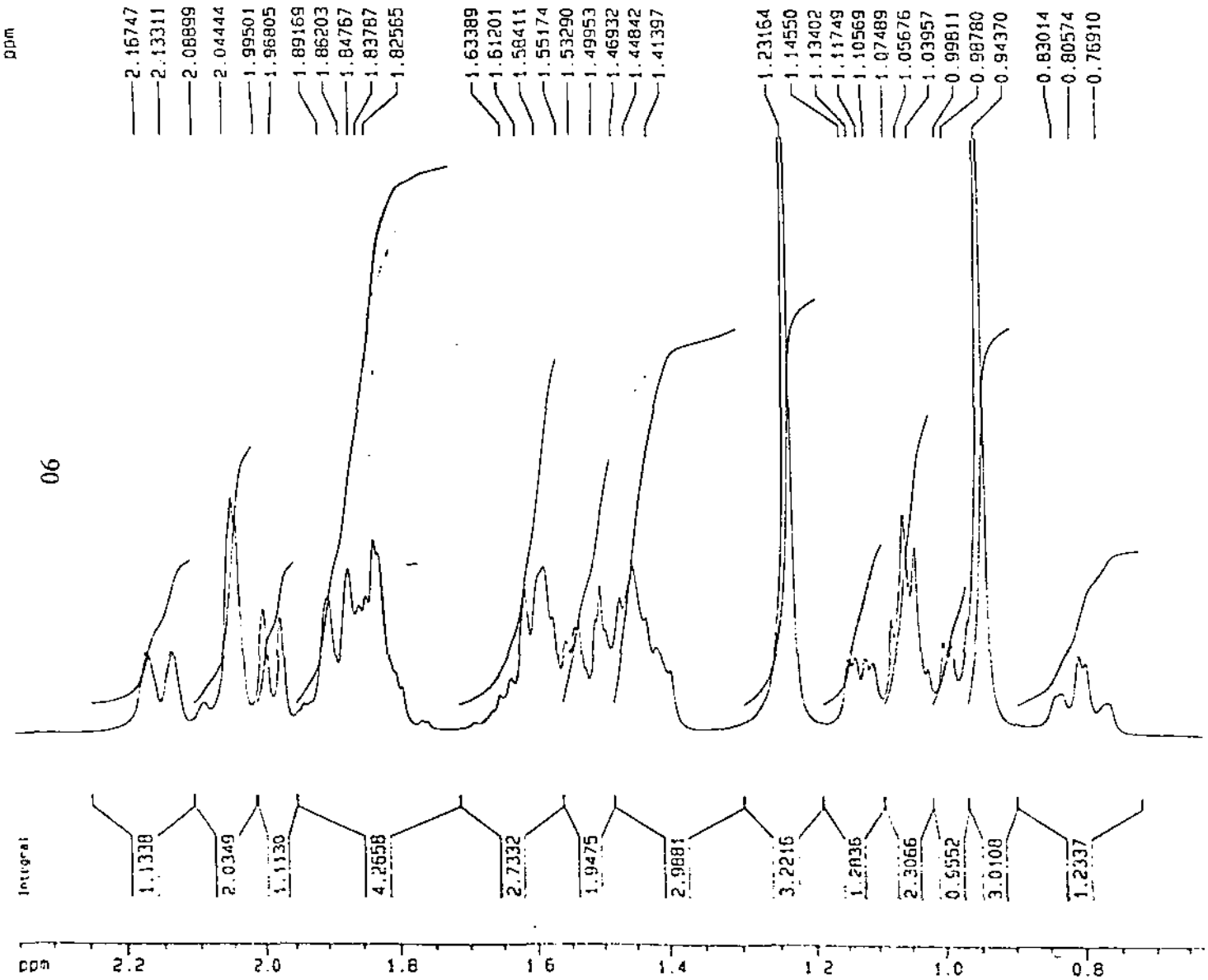


Figure F.3 : Expanded ^{1}H-NMR spectrum of compound EF_3

Current Data Parameters

NAME	A3068
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20061017
Time	12.23
INSTRUM	dpx400
PROBHD	5 mm Multinuc
PULPROG	zg30
TD	32768
SOLVENT	CDCl3
NS	128
DS	2
SWH	6410.256 Hz
FIDRES	0.195625 Hz
AQ	2.5559540 sec
RG	20.2
DW	78.000 usec
DE	6.00 usec
TE	310.0 K
D1	1.00000000 sec

========== CHANNEL f1 ==========

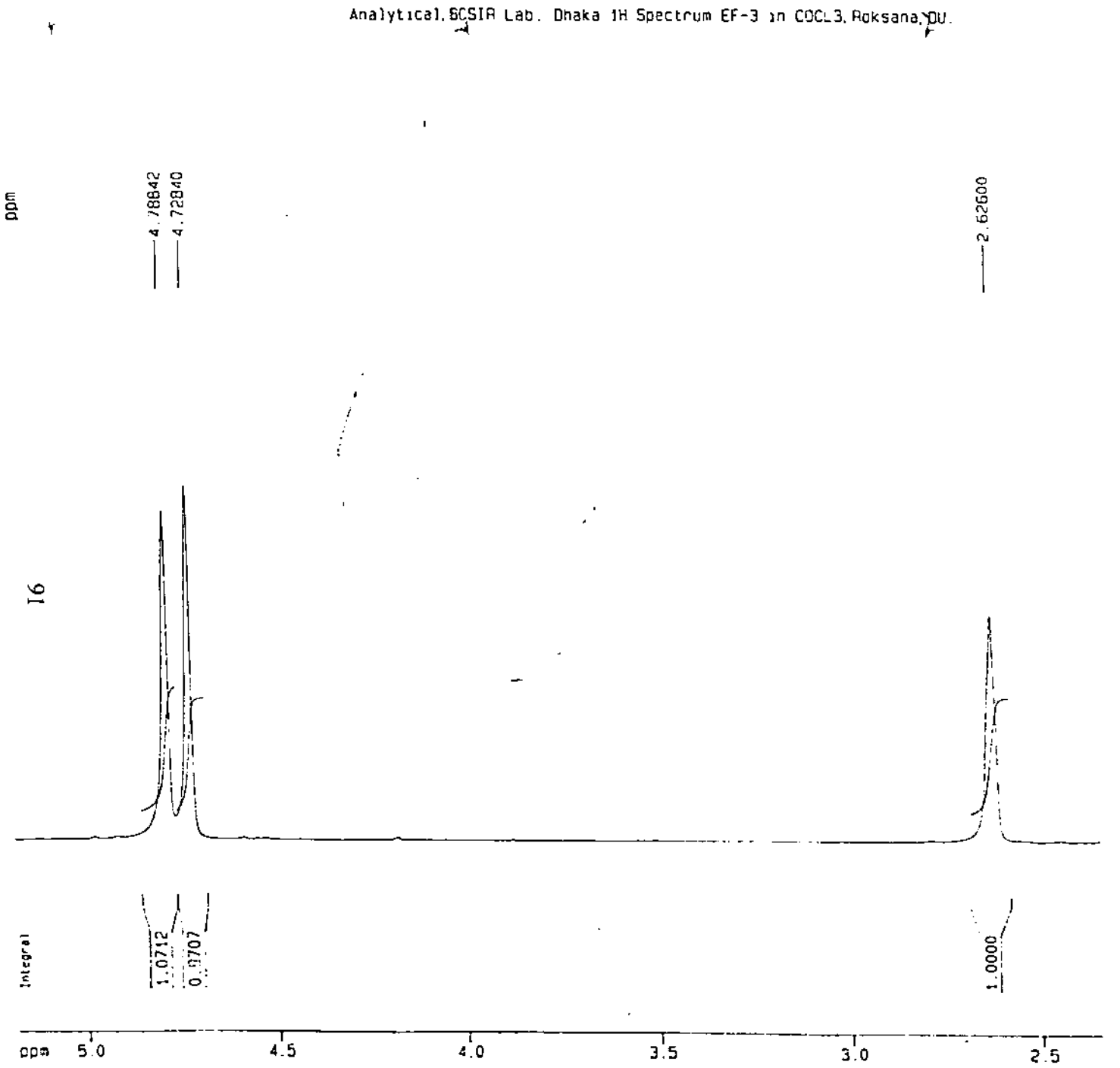
NUC1	1H
P1	8.30 usec
PL1	-6.00 dB
SFO1	400.1428010 MHz

F2 - Processing parameters

SI	32768
SF	400.1400123 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	2.353 ppm
F1	941.45 Hz
F2P	0.625 ppm
F2	250.13 Hz
PPMCM	0.08638 ppm/cm
HZCM	34.56560 Hz/cm



Current Data Parameters

NAME	A3068
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20061017
Time	12.23
INSTRUM	dpx400
PROBHD	5 mm Multinuc
PULPROG	zg30
TD	32768
SOLVENT	CDCl3
NS	128
DS	2
SWH	6410.256 Hz
FIDRES	0.195625 Hz
AQ	2.5559540 sec
RG	20.2
DW	78.000 usec
DE	6.00 usec
TE	310.0 K
D1	1.00000000 sec

========== CHANNEL f1 ==========

NUC1	1H
P1	8.30 usec
PL1	-6.00 dB
SFO1	400.1428010 MHz

F2 - Processing parameters

SI	32768
SF	400.1400123 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	5.182 ppm
F1	2073.72 Hz
F2P	2.352 ppm
F2	941.24 Hz
PPMCM	0.14151 ppm/cm
HZCM	56.62429 Hz/cm

Figure F.4 : Expanded ^{1}H-NMR spectrum of compound EF_3

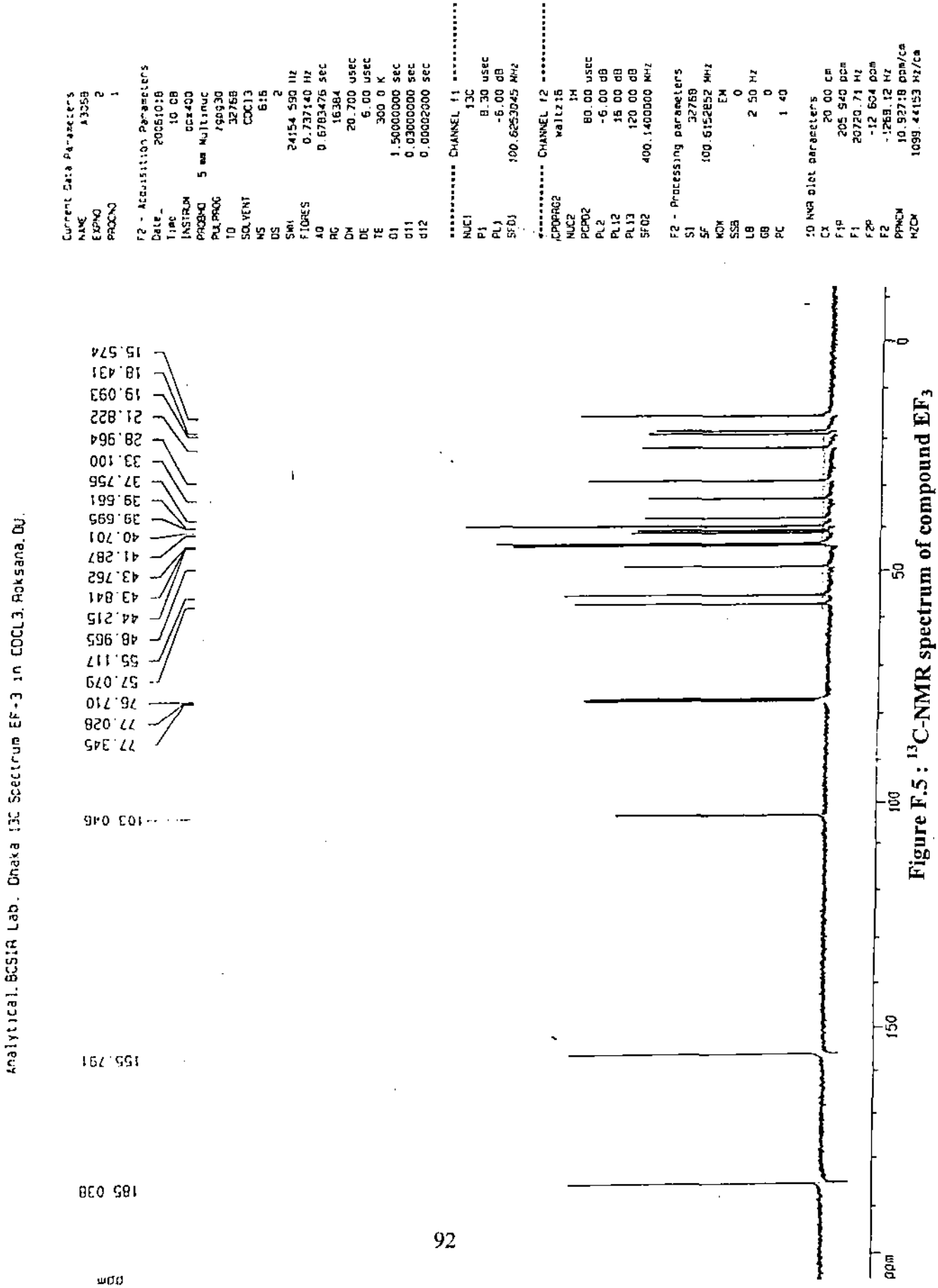


Figure F.5 : ^{13}C-NMR spectrum of compound EF_3

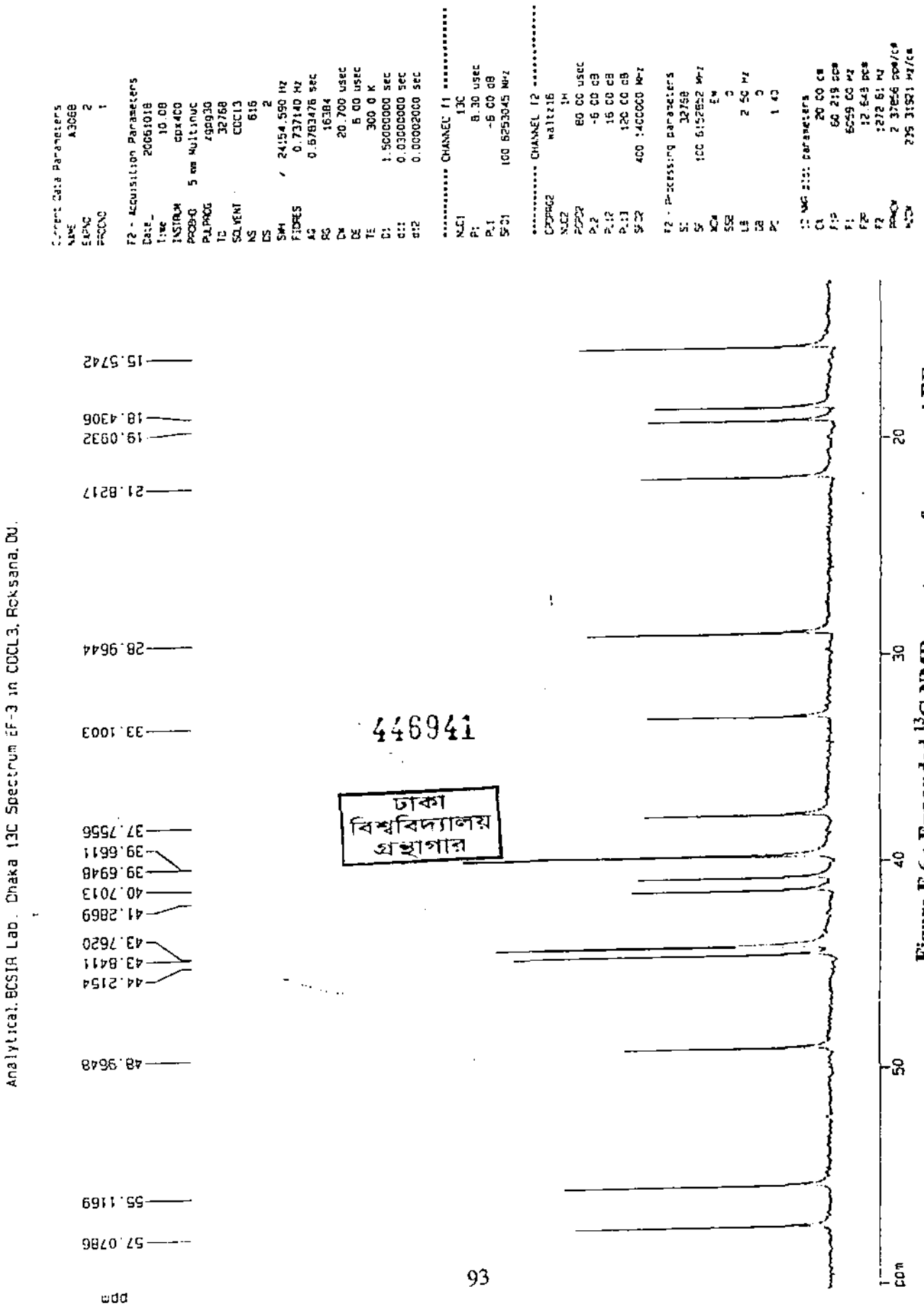


Figure F.6 : Expanded ^{13}C-NMR spectrum of compound EF_3

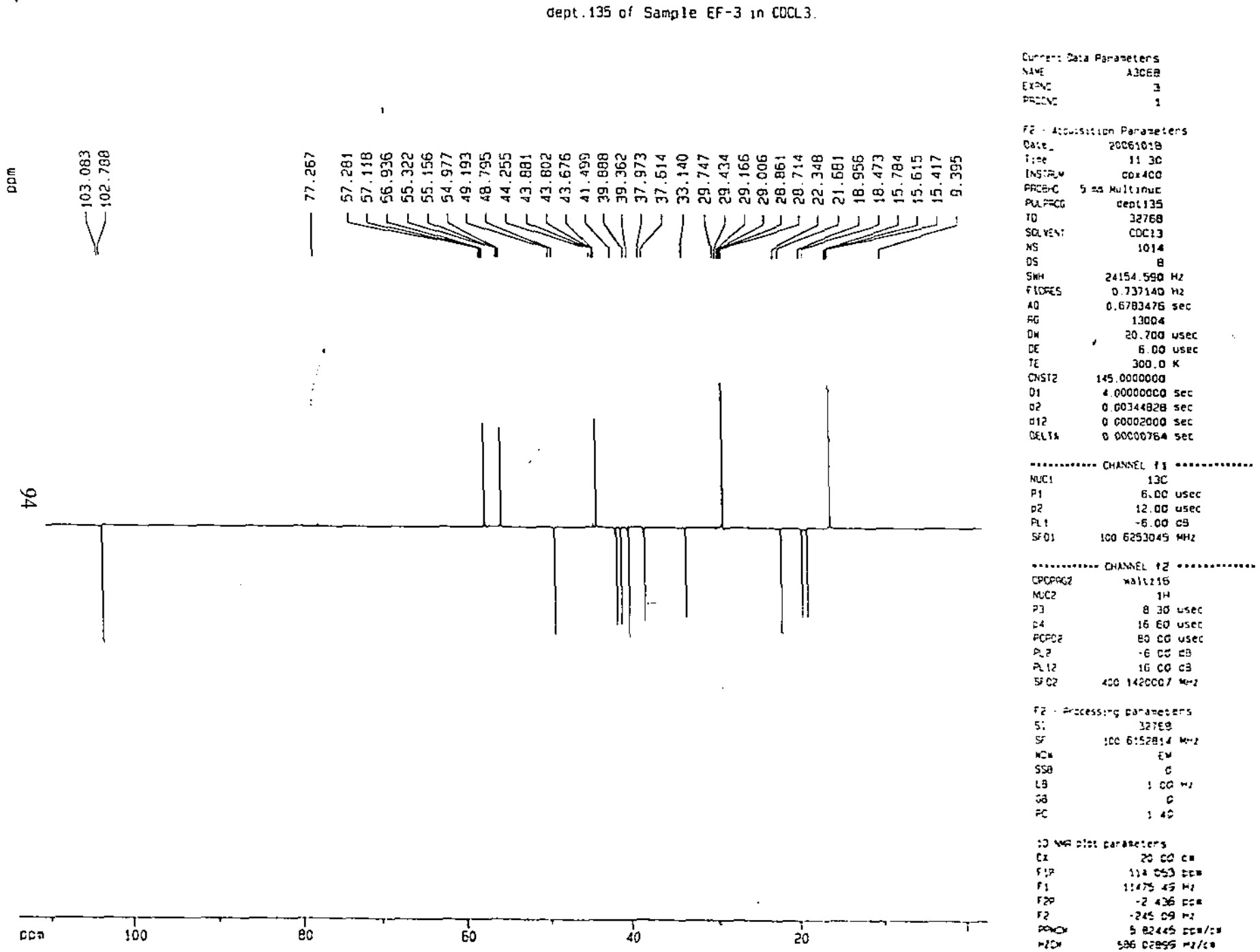


Figure F.7 : DEPT 135 ^{13}C-NMR spectrum of compound EF$_3$

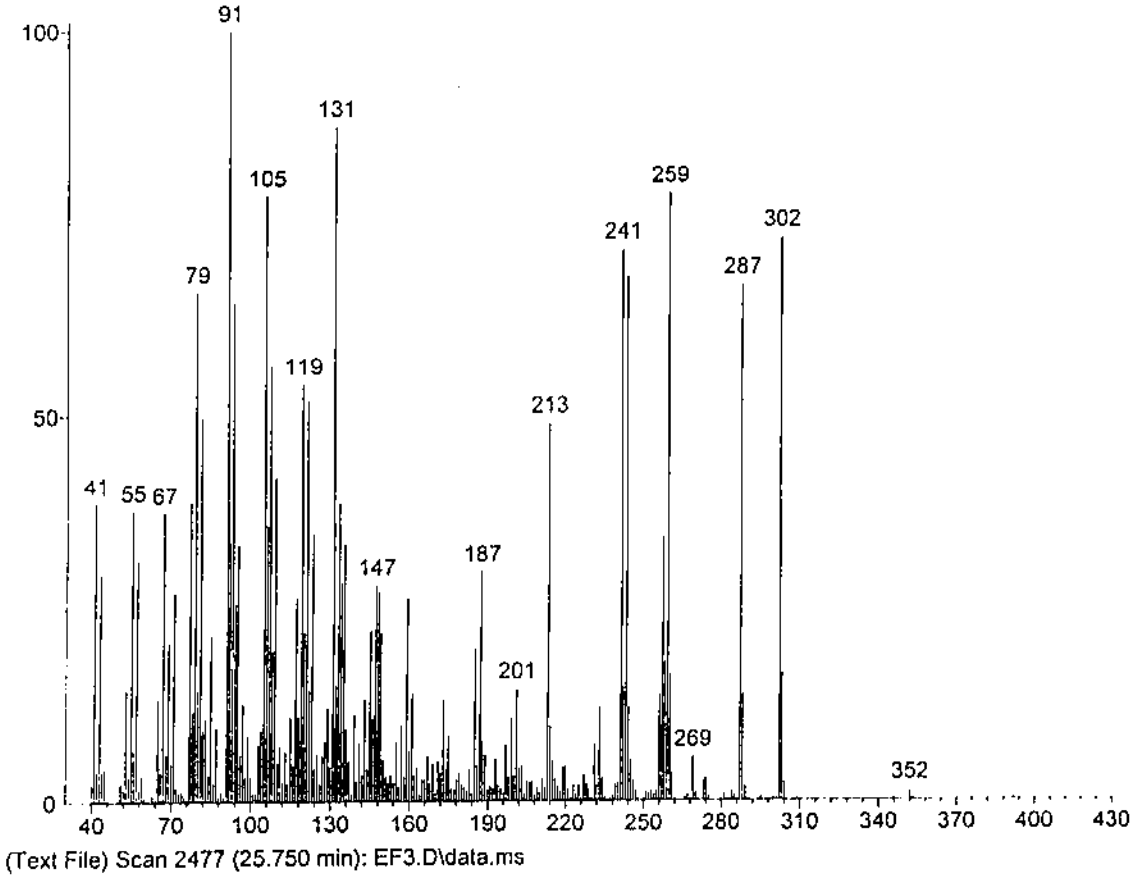


Figure F.8 : Mass spectrum of compound EF_3

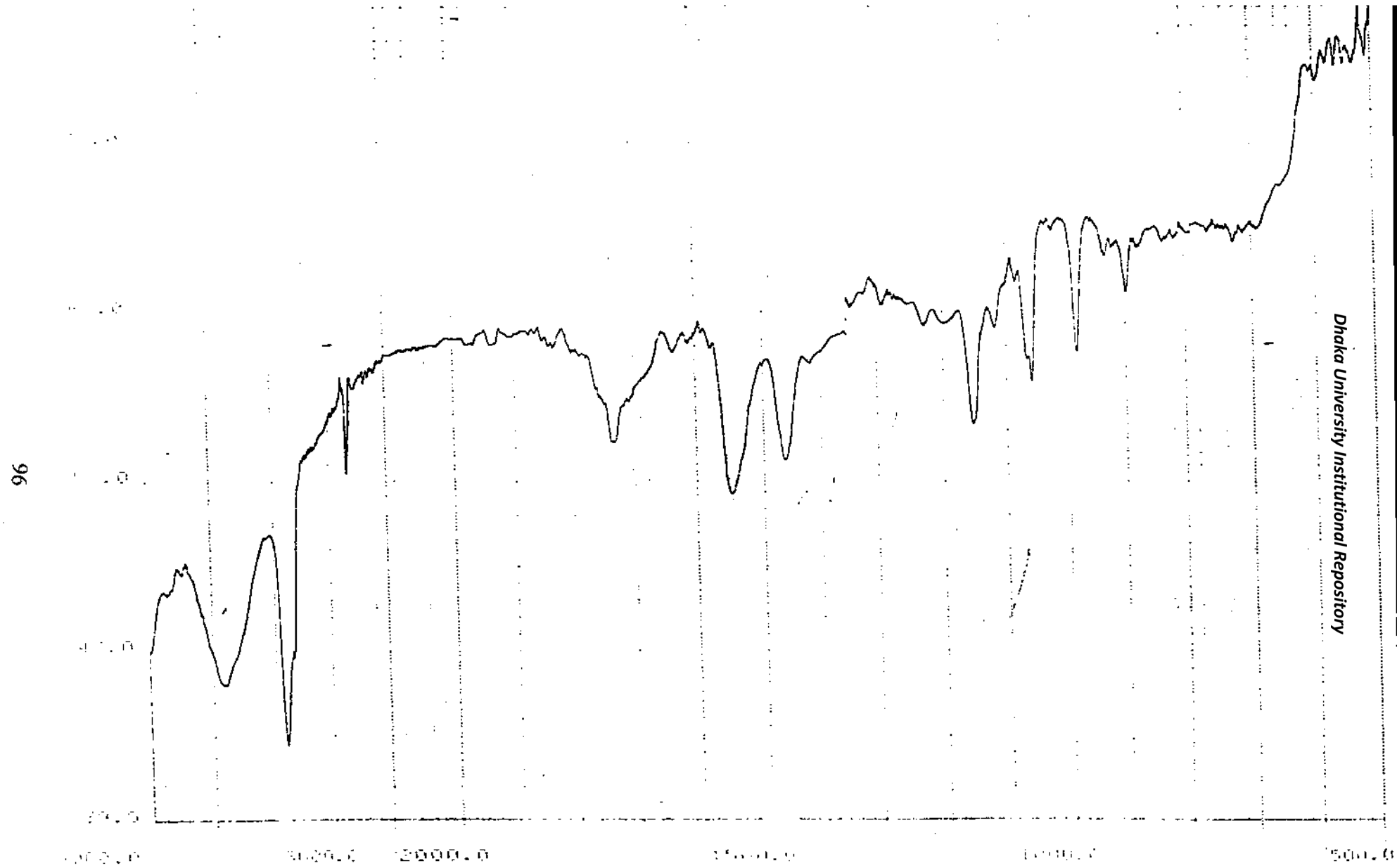


Figure F.9 : IR spectrum of compound EF_4

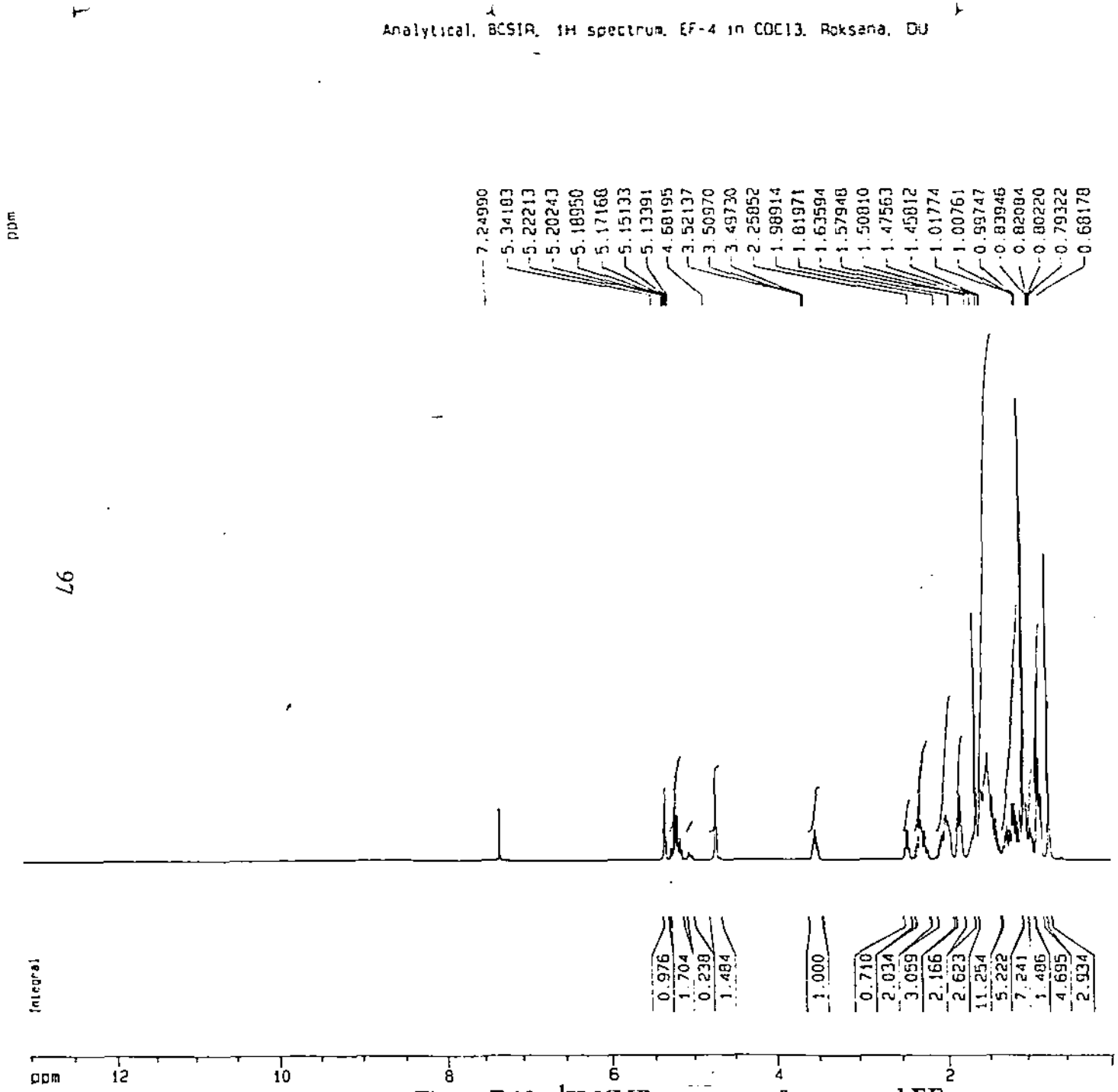


Figure F.10 : ^{1}H-NMR spectrum of compound EF_4

Current Data Parameters

NAME	A3000
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20060921
Time	11.17
INSTRUM	dpx400
PROBHD	5 mm Multinuc
PULPROG	zg30
TD	32768
SOLVENT	CDCl3
NS	93
DS	2
SWH	6410.256 Hz
FIDRES	0.195625 Hz
AQ	2.5559540 sec
RG	228.1
DW	78.000 usec
DE	6.00 usec
TE	310.0 K
D1	1.00000000 sec

========== CHANNEL f1 ==========

NUC1	1H
P1	8.30 usec
PL1	-6.00 dB
SFO1	400.1428010 MHz

F2 - Processing parameters

SI	32768
SF	400.1400125 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	13.019 ppm
F1	5209.28 Hz
F2P	-0.027 ppm
F2	-10.89 Hz
PPMCM	0.65229 ppm/cm
HZCM	261.00833 Hz/cm

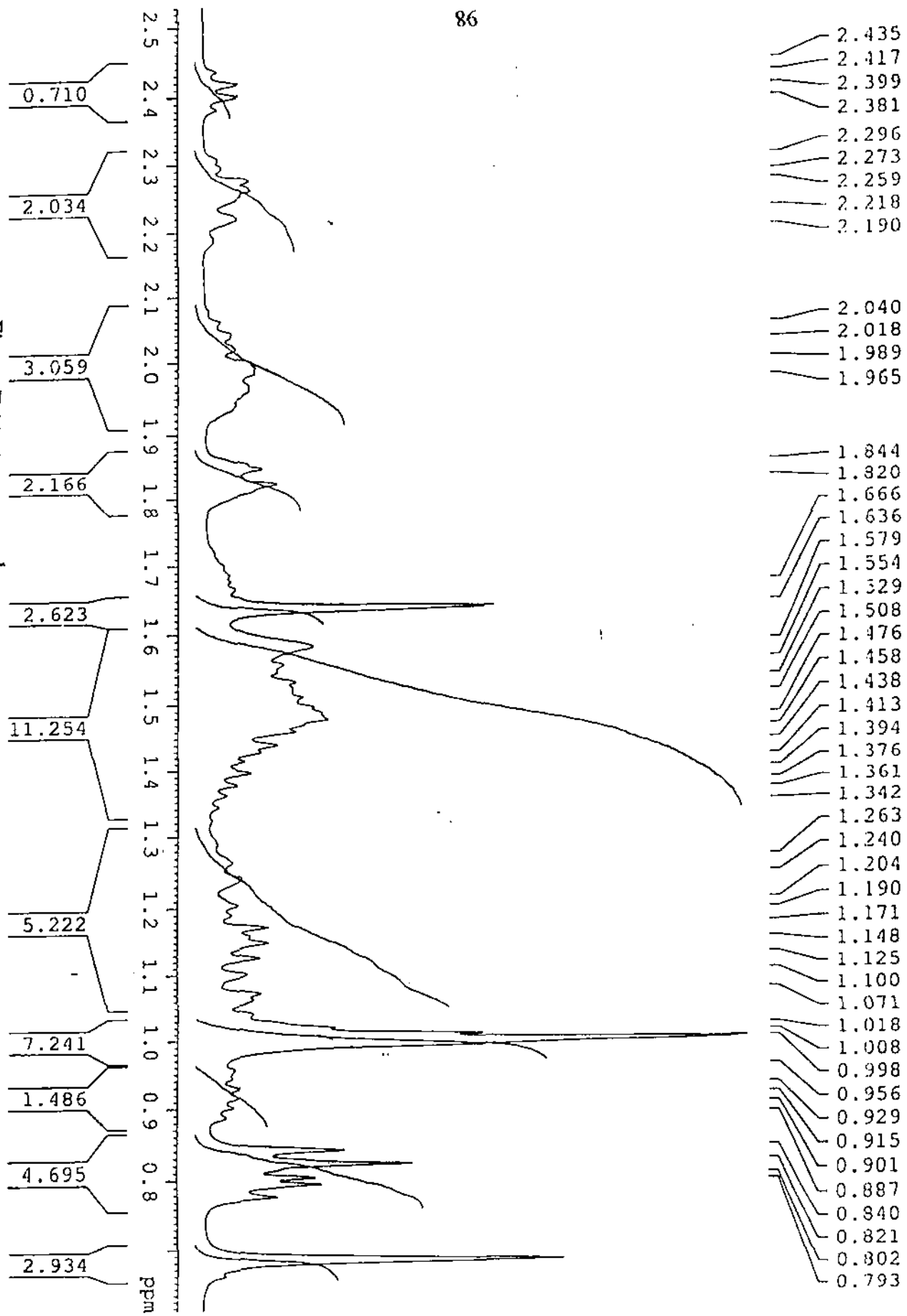


Figure F.11 : Expanded ^{1}H-NMR spectrum of compound EF$_4$

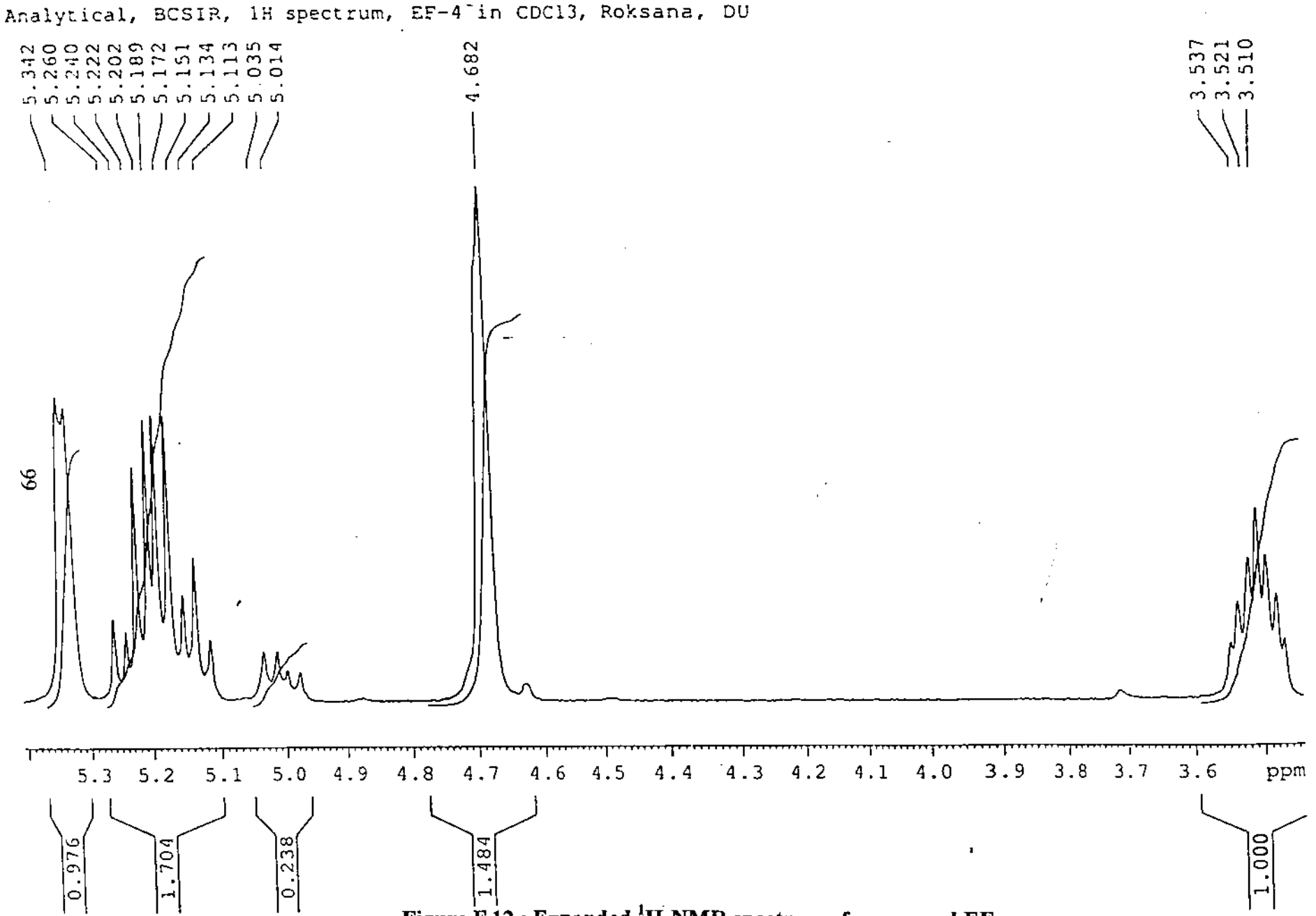


Figure F.12 : Expanded ^{1}H-NMR spectrum of compound EF_4

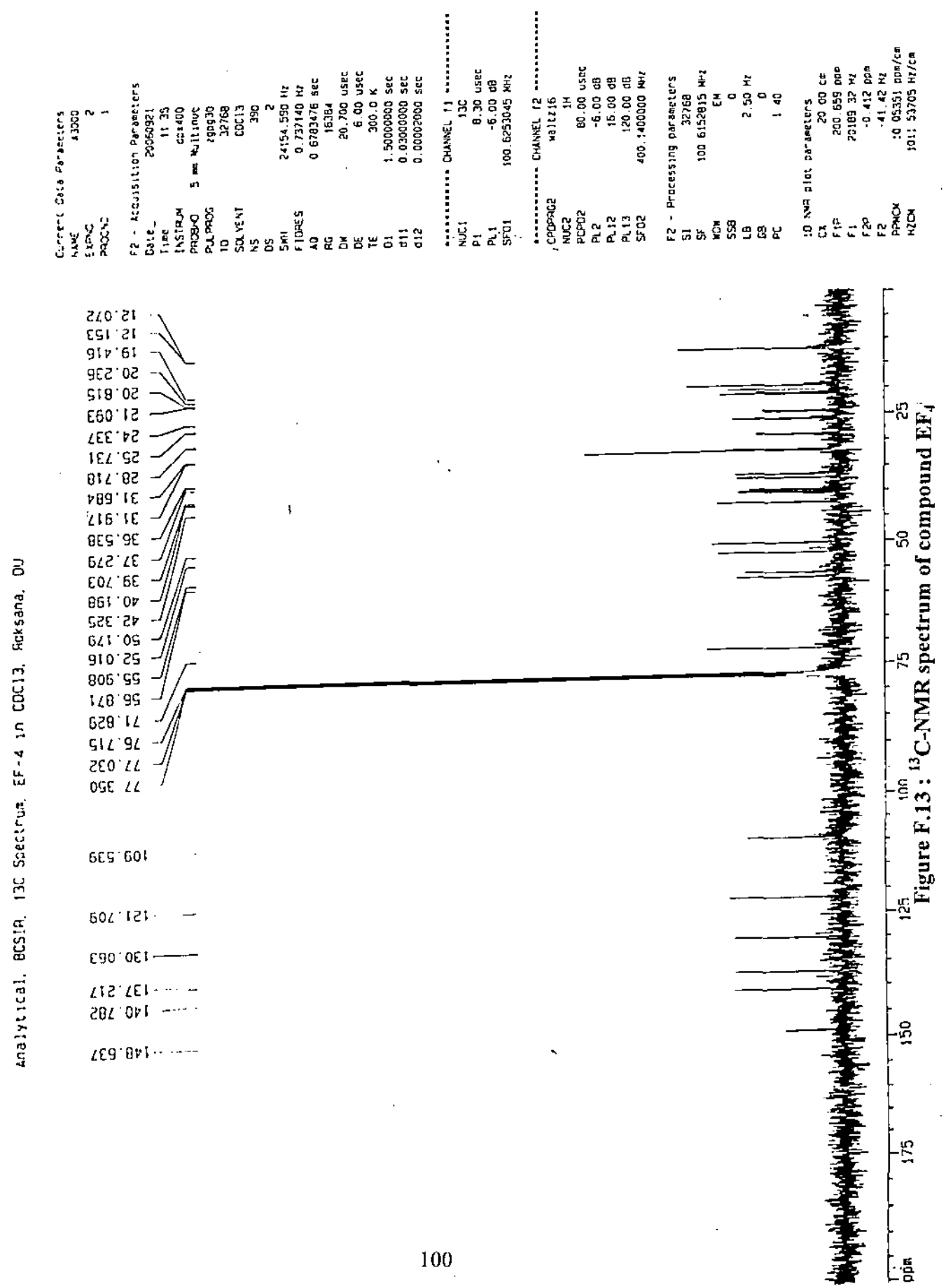


Figure F.13 : ^{13}C-NMR spectrum of compound EF$_4$

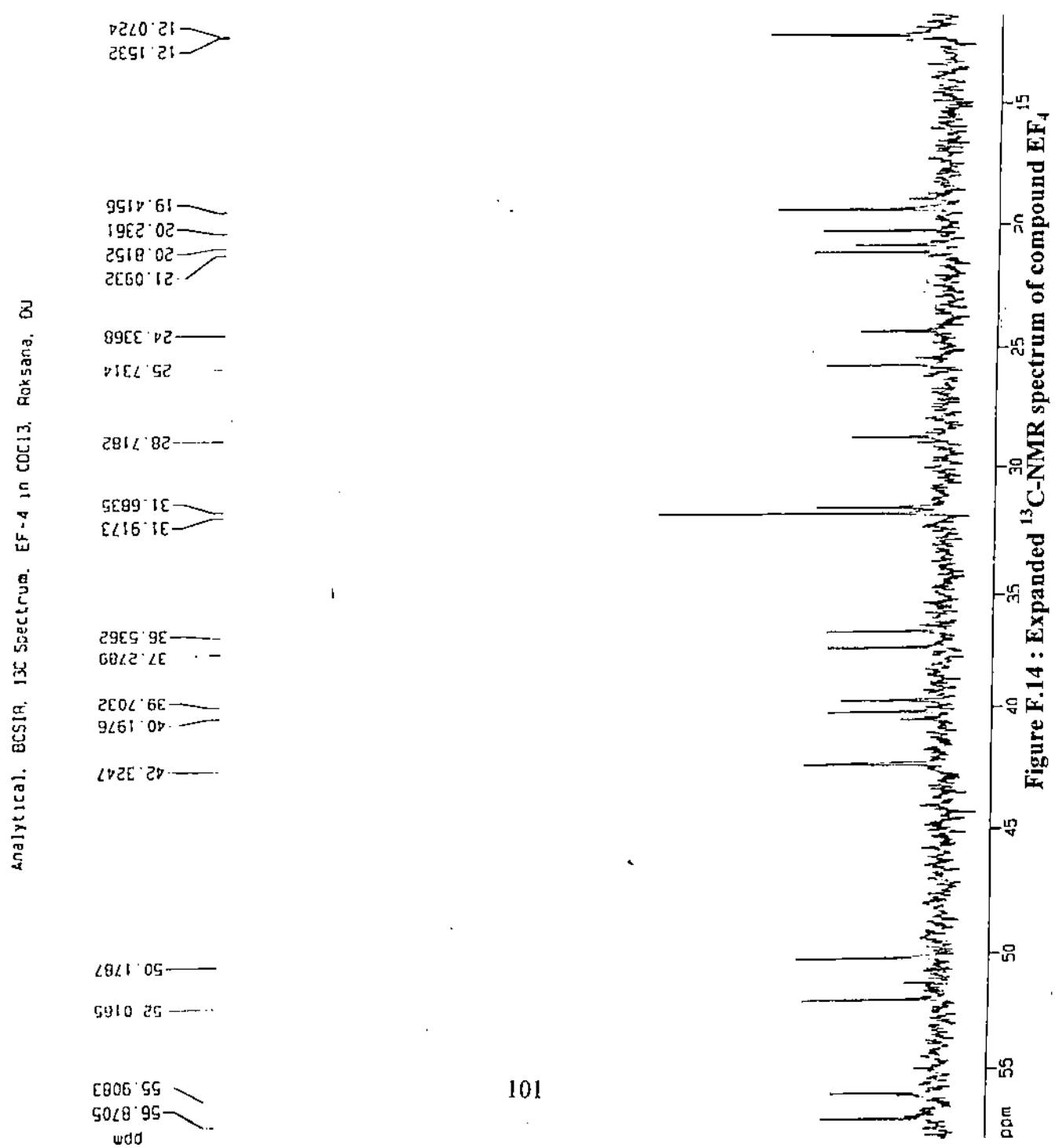


Figure F.14 : Expanded ^{13}C-NMR spectrum of compound EF_4

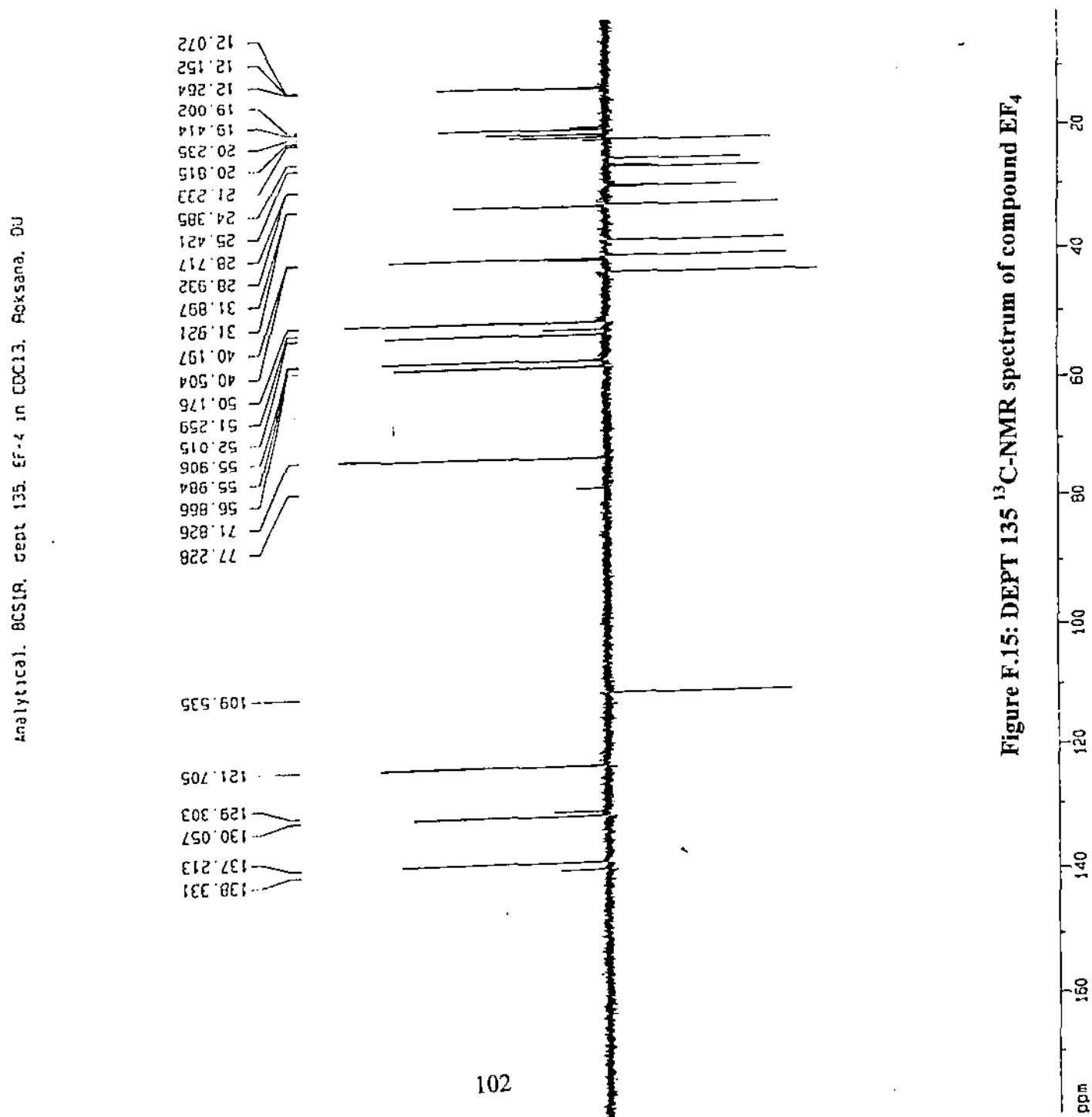


Figure F.15: DEPT 135 ^{13}C-NMR spectrum of compound EF$_4$

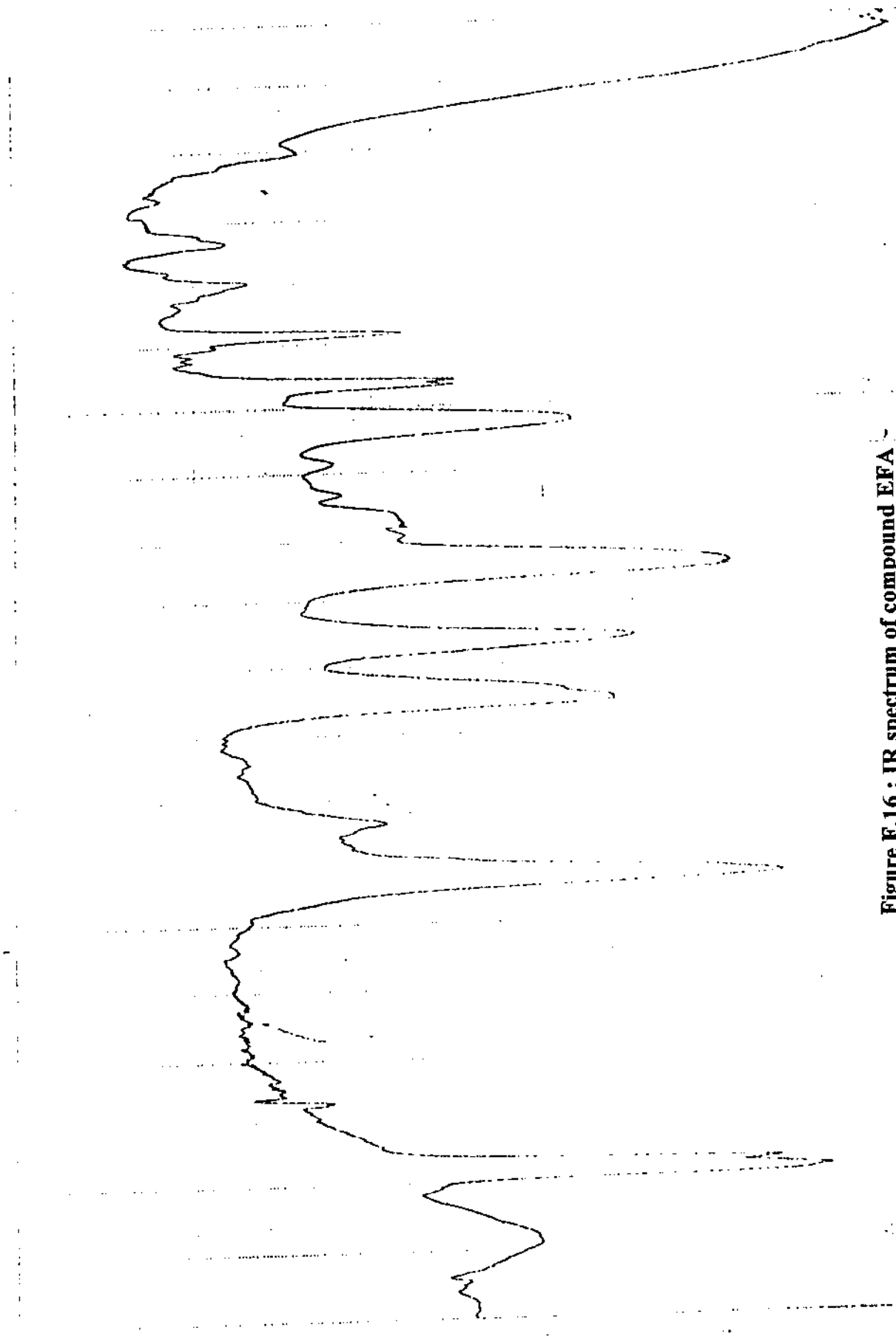


Figure F.16 : IR spectrum of compound EFA

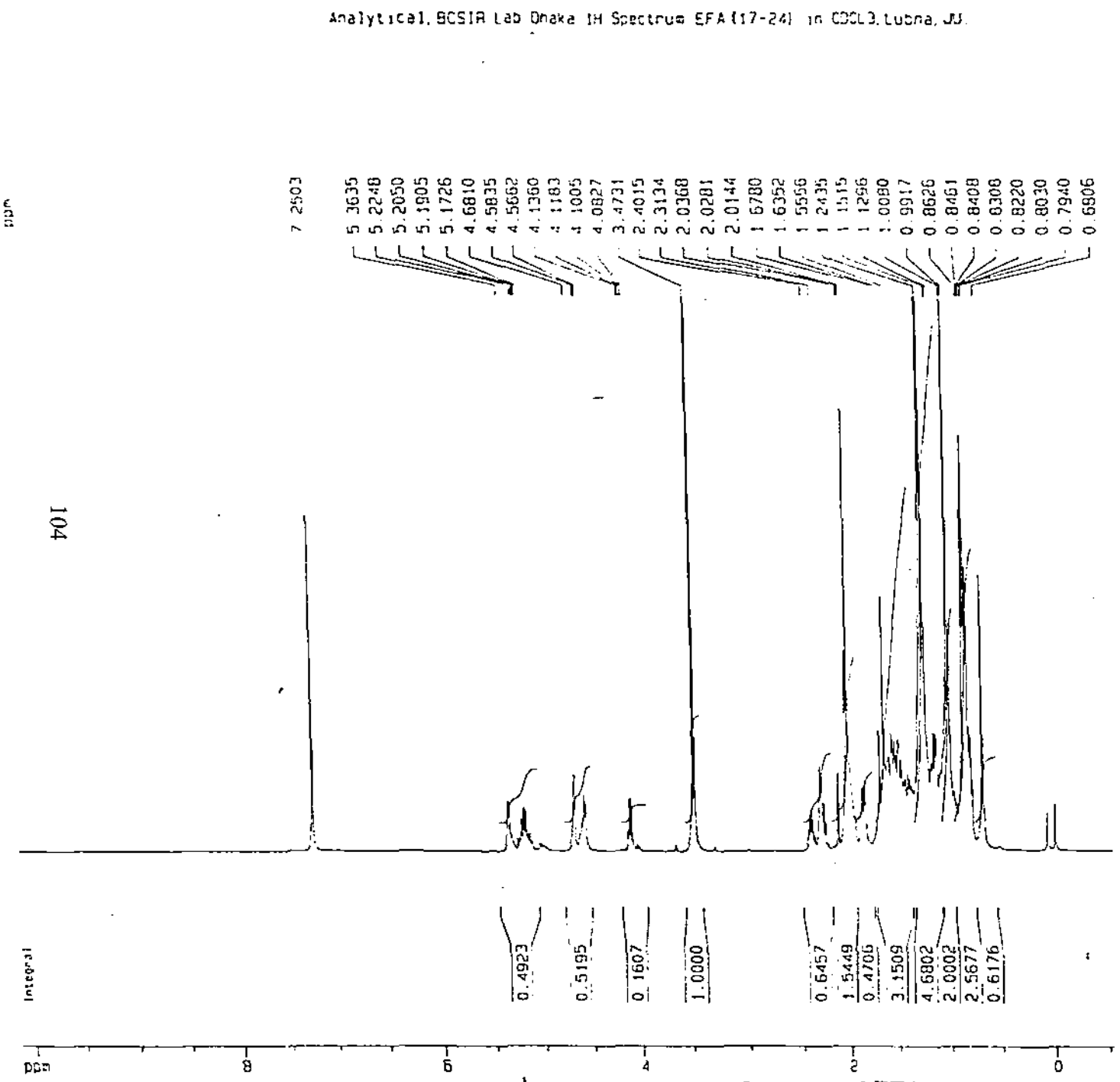


Figure F.17 : ^{1}H-NMR spectrum of compound EFA

Current Data Parameters

NAME	A3912
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20071128
Time	12.31
INSTRUM	dpx400
PROBHD	5 mm Multinuc
PULPROG	zg30
TD	32768
SOLVENT	CDCl3
NS	99
DS	2
SWH	6410.258 Hz
FIDRES	0.195625 Hz
AQ	2.5559540 sec
RG	90.5
DW	78.000 usec
DE	6.00 usec
TE	310.0 K
D1	1.00000000 sec

========== CHANNEL f1 ==========

NUC1	1H
P1	8.30 usec
PL1	-6.00 dB
SFO1	400.1428010 MHz

F2 - Processing parameters

SI	32768
SF	400.1400126 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	10.162 ppm
F1	4066.33 Hz
F2P	-0.535 ppm
F2	-213.94 Hz
PPMCM	0.53485 ppm/cm
HZCM	214.01329 Hz/cm

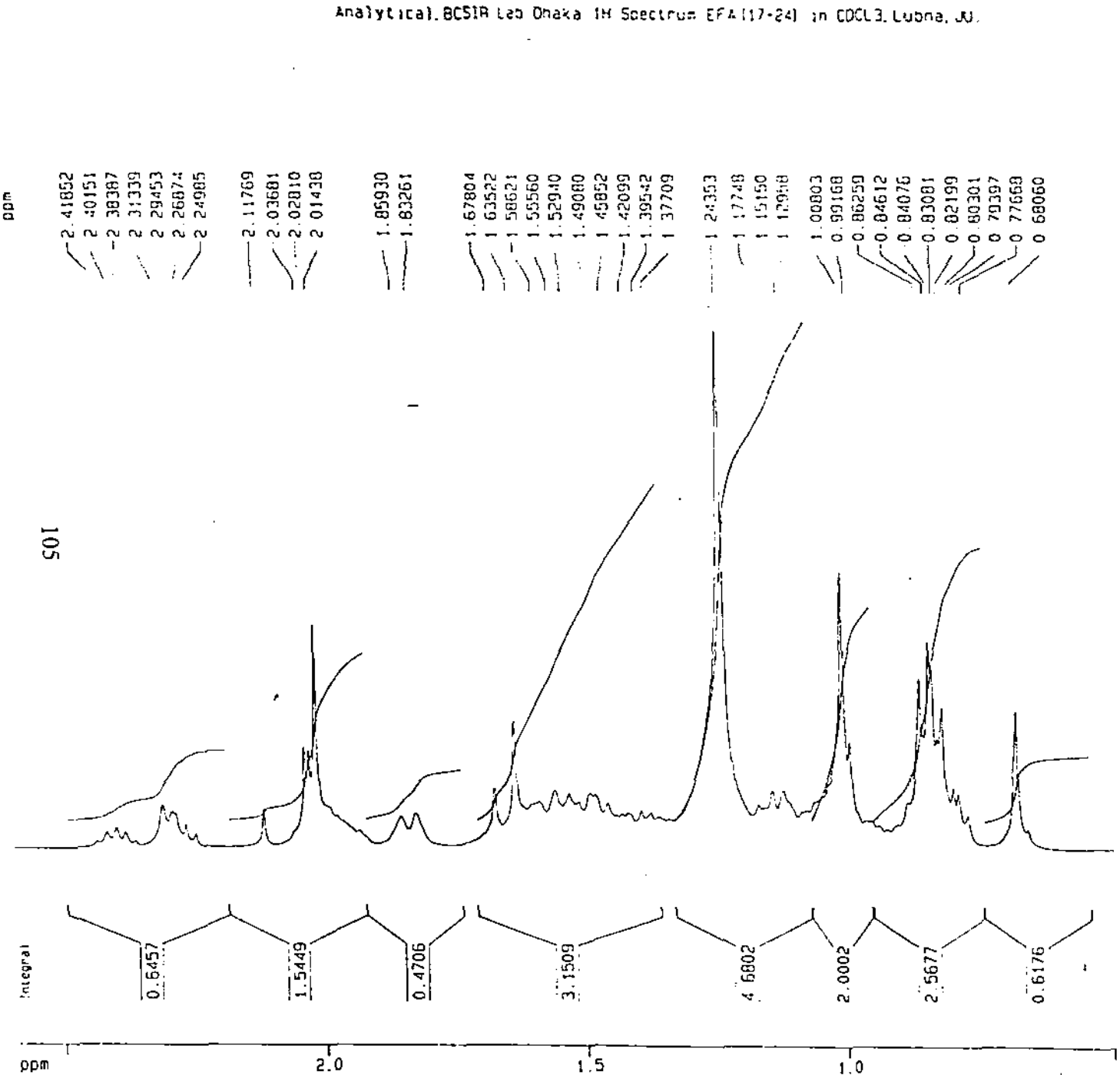


Figure F.18 : Expanded ^{1}H-NMR spectrum of compound EFA

Current Data Parameters

NAME	A3912
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20071128
Time	12 31
INSTRUM	dpx400
PROBHD	5 mm Multinuc
PULPROG	zg30
TD	32768
SOLVENT	CDCl3
NS	99
DS	2
SWH	6410.256 Hz
FIDRES	0.195625 Hz
AQ	2.5559540 sec
RG	90.5
DW	78.000 usec
DE	6.00 usec
TE	310.0 K
D1	1.00000000 sec

========== CHANNEL f1 ==========

NUC1	1H
P1	8.30 usec
PL1	-6.00 dB
SFO1	400.1428010 MHz

F2 - Processing parameters

SI	32768
SF	400.1400126 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	2.588 ppm
F1	1035.38 Hz
F2P	0.498 ppm
F2	199.15 Hz
PPMCM	0.10449 ppm/cm
HZCM	41.81163 Hz/cm

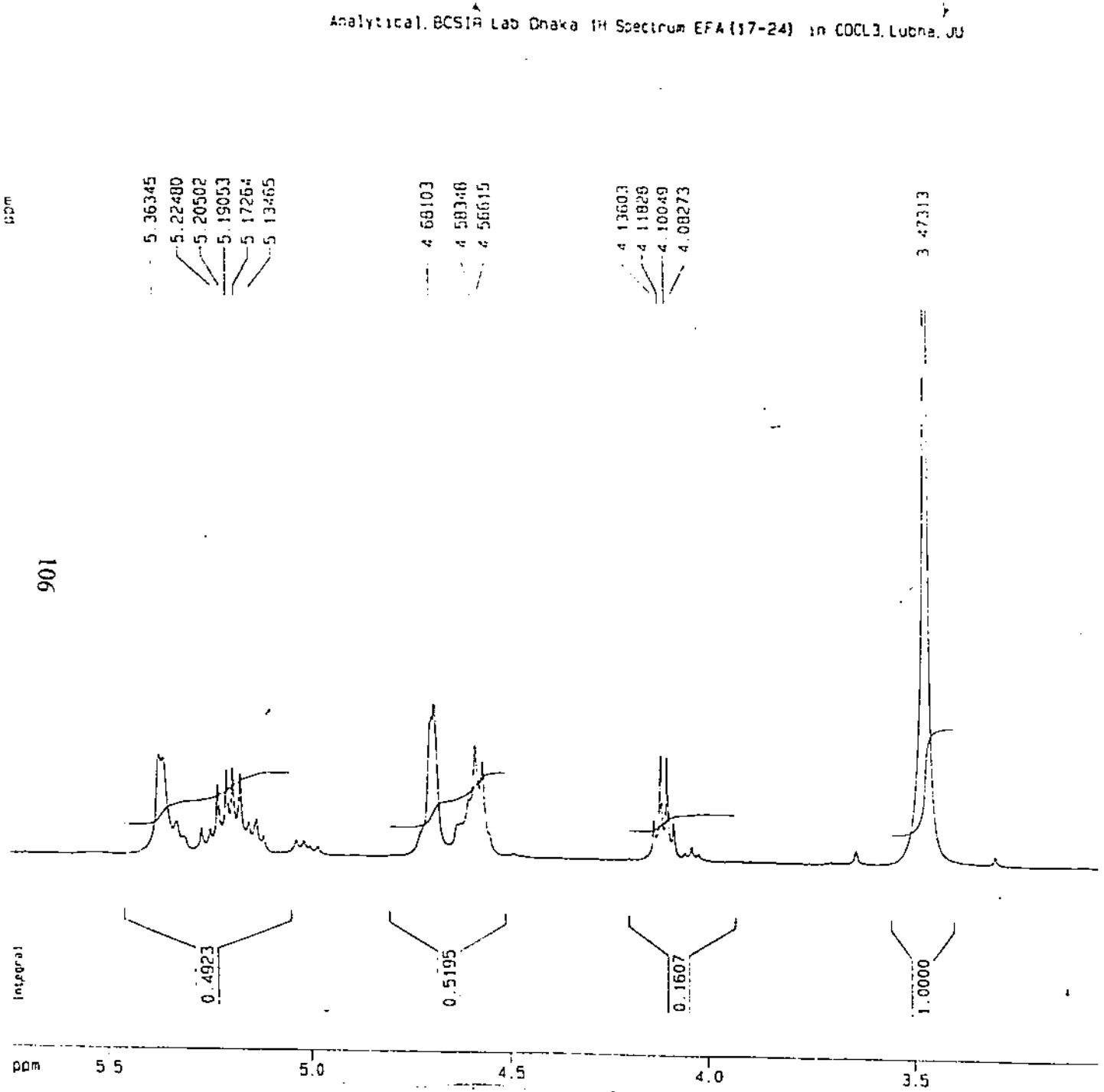


Figure F.19 : Expanded ^{1}H-NMR spectrum of compound EFA

```
Current Data Parameters
NAME                  A3912
EXPNO                    1
PROCNO                   1

F2 - Acquisition Parameters
Date_              20071128
Time                 12 31
INSTRUM              dpx400
PROBHD     5 mm Multinuc
PULPROG              zg30
TD                  32768
SOLVENT              CDCl3
NS                      99
DS                       2
SWH              6410.256 Hz
FIDRES           0.195625 Hz
AQ               2.5559540 sec
RG                    90.5
DW                78.000 usec
DE                 6.00 usec
TE                310.0 K
D1             1.00000000 sec

========== CHANNEL f1 ==========
NUC1                  1H
P1                 8.30 usec
PL1               -8.00 dB
SFO1          400.1428010 MHz

F2 - Processing parameters
SI                  32768
SF            400.1400126 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.40

1D NMR plot parameters
CX                 20.00 cm
F1P                5.744 ppm
F1                2298.26 Hz
F2P                3.052 ppm
F2                1221.42 Hz
PPMCM           0.13456 ppm/cm
HZCM            53.84195 Hz/cm
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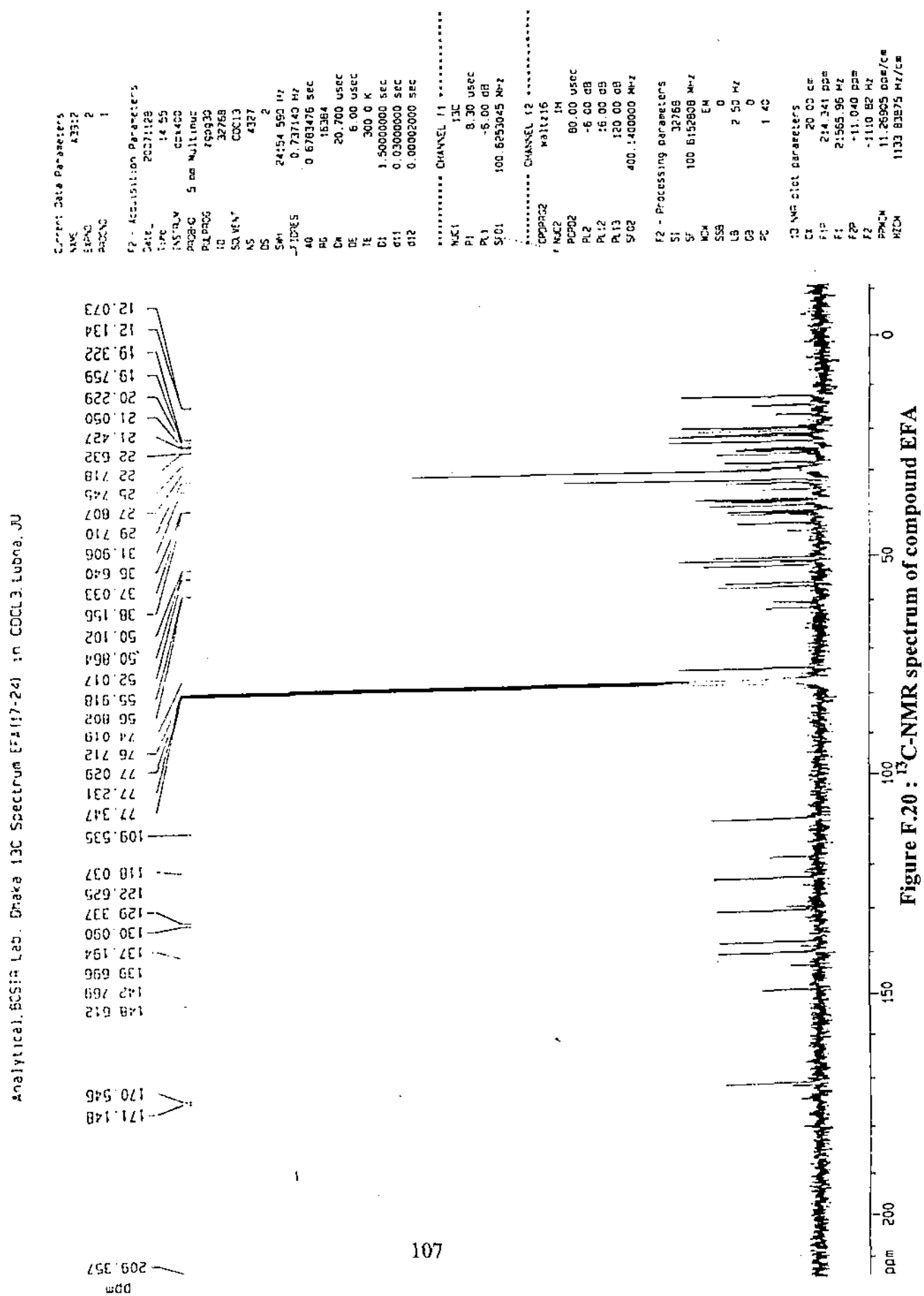

Figure F.20 : ^{13}C-NMR spectrum of compound EFA

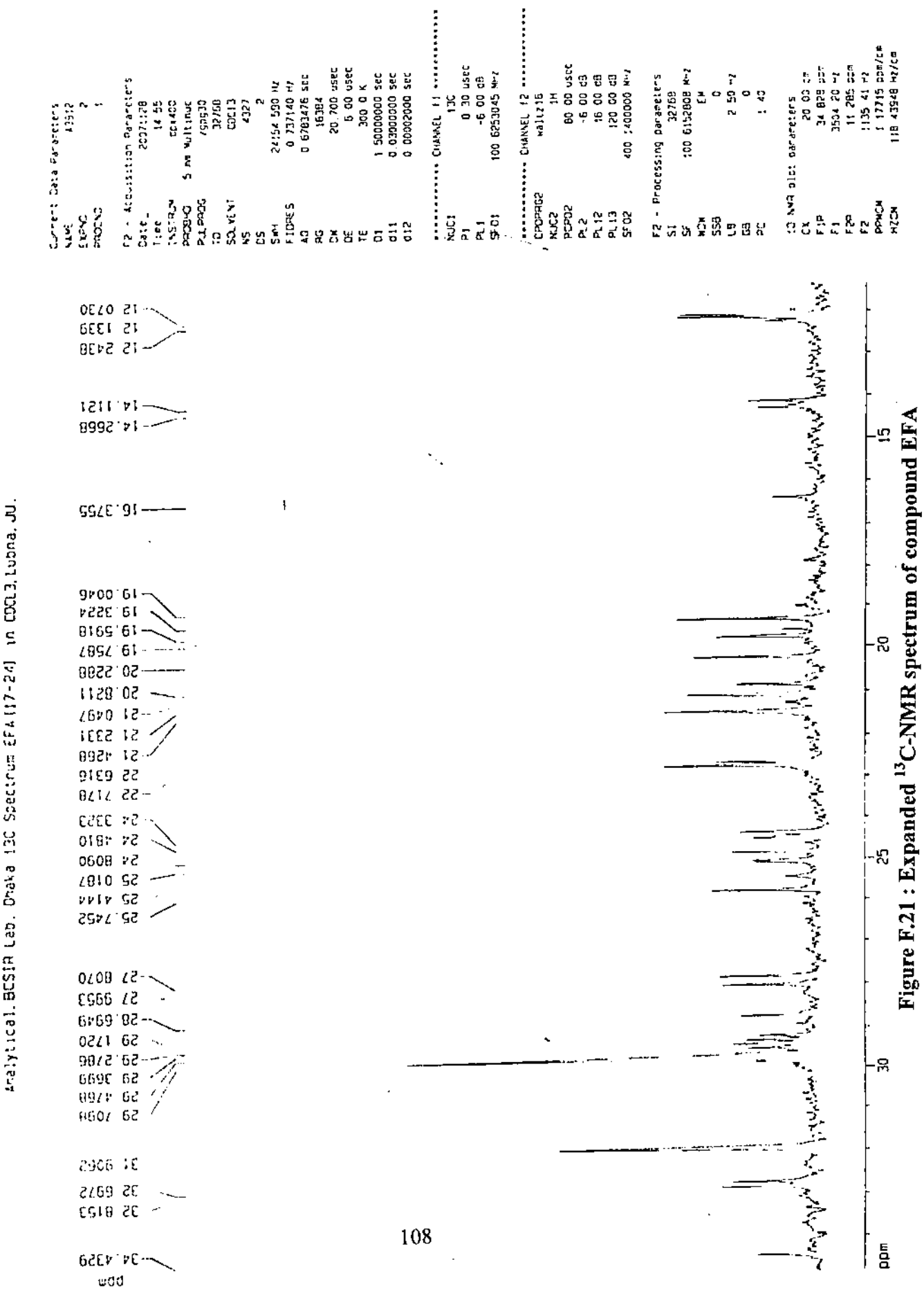

Figure F.21 : Expanded ^{13}C-NMR spectrum of compound EFA

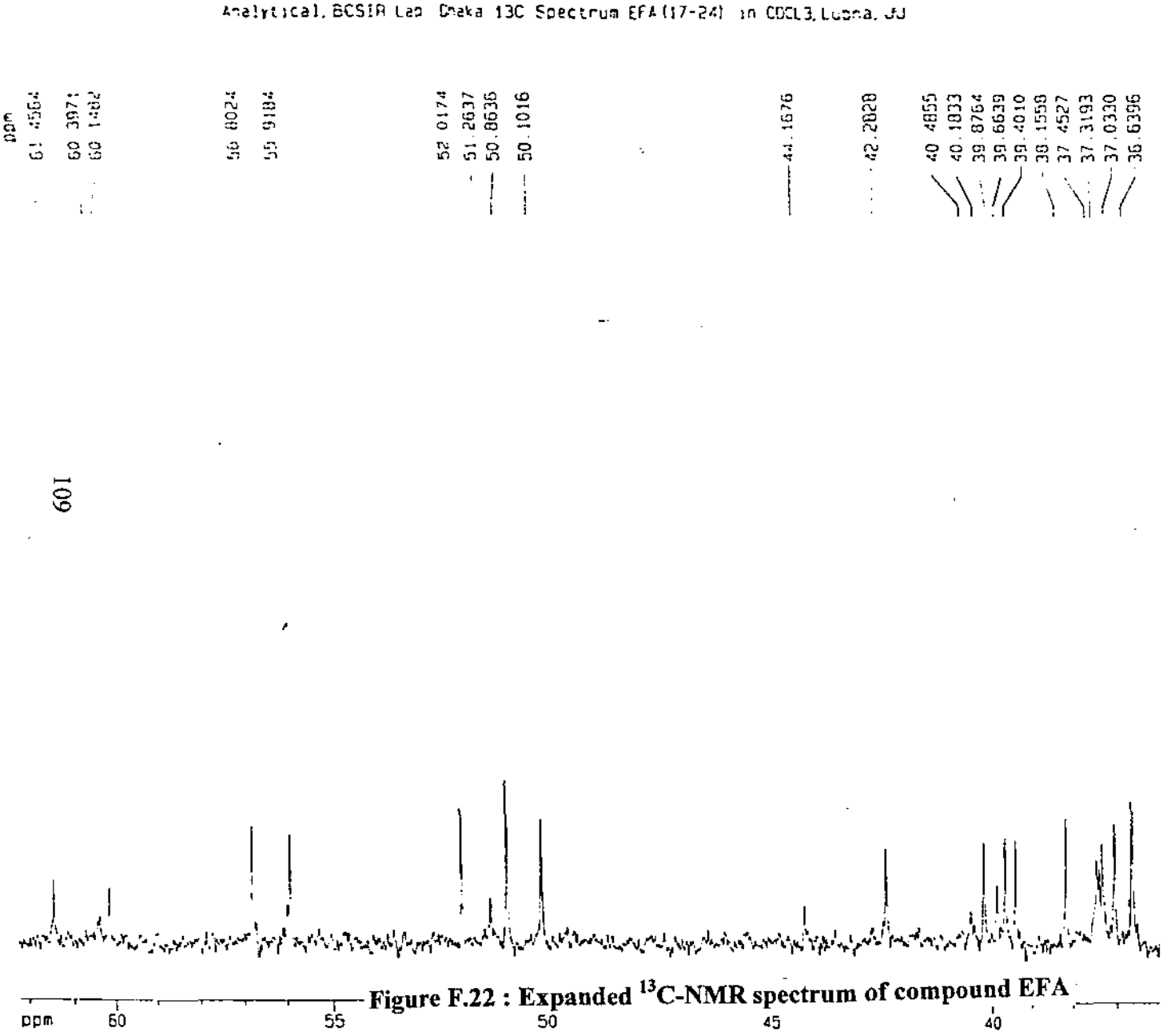

Figure F.22 : Expanded ^{13}C-NMR spectrum of compound EFA

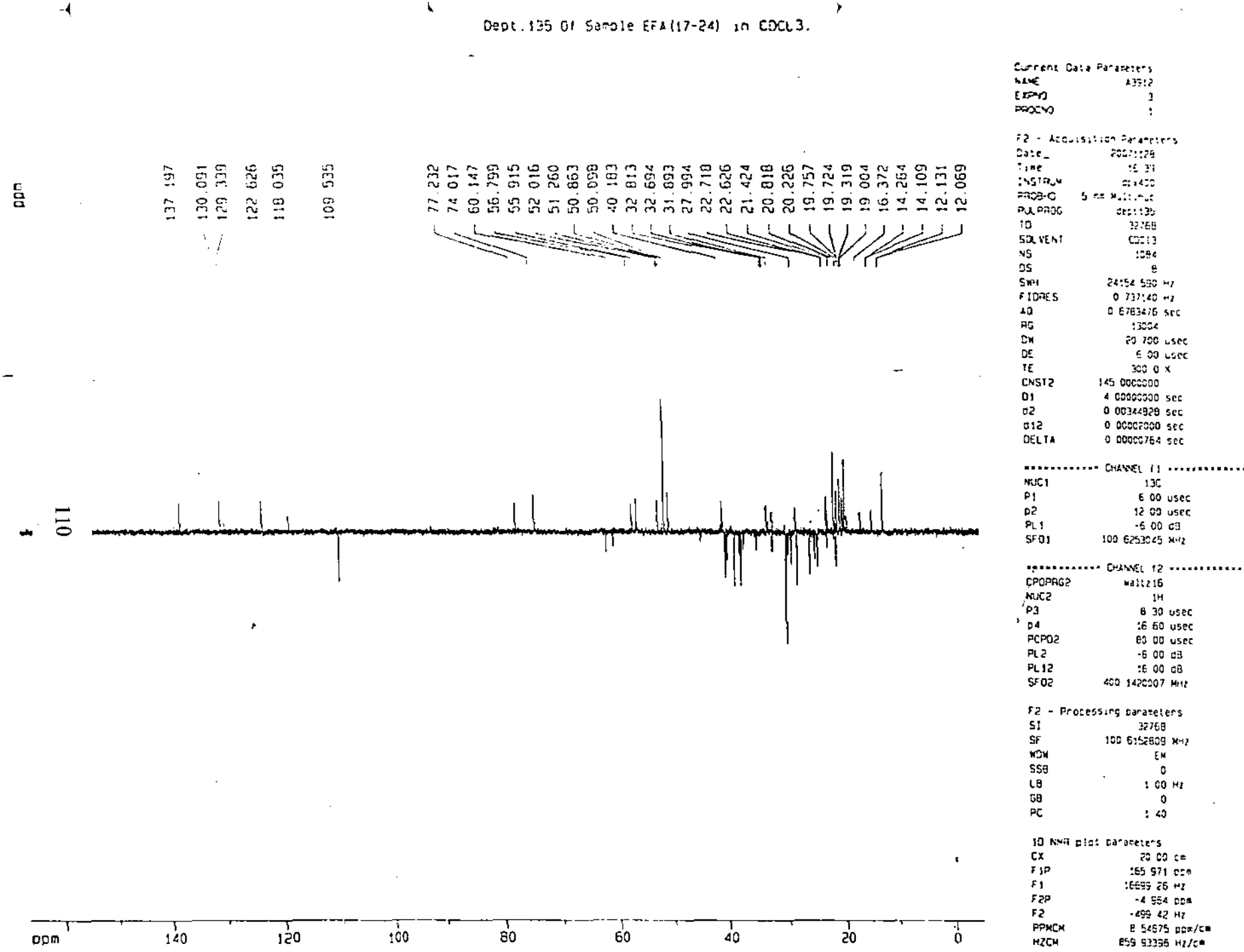

Figure F.23 : DEPT 135 ^{13}C-NMR spectrum of compound EFA

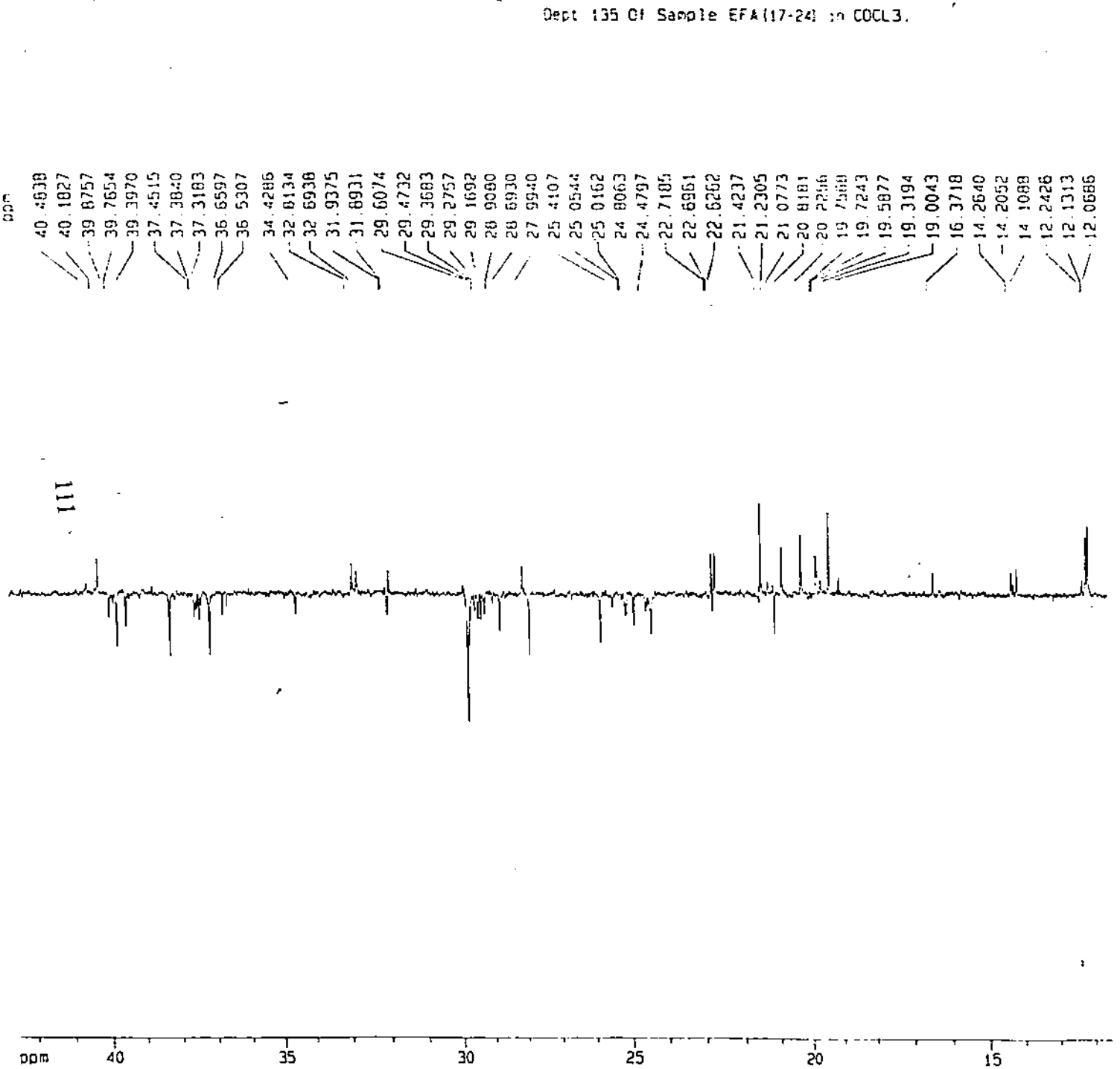

Figure F.24 : DEPT 135 ^{13}C-NMR spectrum of compound EFA

Current Data Parameters
NAME A3512
EXPNO 3
PROCNO 1

F2 - Acquisition Parameters
Date_ 20071128
Time 16.39
INSTRUM dpx400
PROBHD 5 mm Multinuc
PULPROG dept135
TD 32768
SOLVENT CDCl3
NS 1084
DS 8
SWH 24154.590 Hz
FIDRES 0.737140 Hz
AQ 0.6783476 sec
RG 13004
DW 20.700 usec
DE 6.00 usec
TE 307.0 K
CNST2 145.0000000
D1 4.00000000 sec
d2 0.00344828 sec
d12 0.00002000 sec
DELTA 0.00000764 sec

========== CHANNEL f1 ==========
NUC1 13C
P1 6.00 usec
p2 12.00 usec
PL1 -6.00 dB
SF01 100.6253045 MHz

========== CHANNEL f2 ==========
CPDPRG2 waltz16
NUC2 1H
P3 8.30 usec
p4 16.60 usec
PCPD2 80.00 usec
PL2 -6.00 dB
PL12 16.00 dB
SF02 400.1420007 MHz

F2 - Processing parameters
SI 32768
SF 100.6152809 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

1D NMR plot parameters
CX 20.00 cm
F1P 42.577 ppm
F1 4283.95 Hz
F2P 11.496 ppm
F2 1156.64 Hz
PPMCM 1.55409 ppm/cm
HZCM 156.36526 Hz/cm

Figure F.25 : IR spectrum of compound SA-1

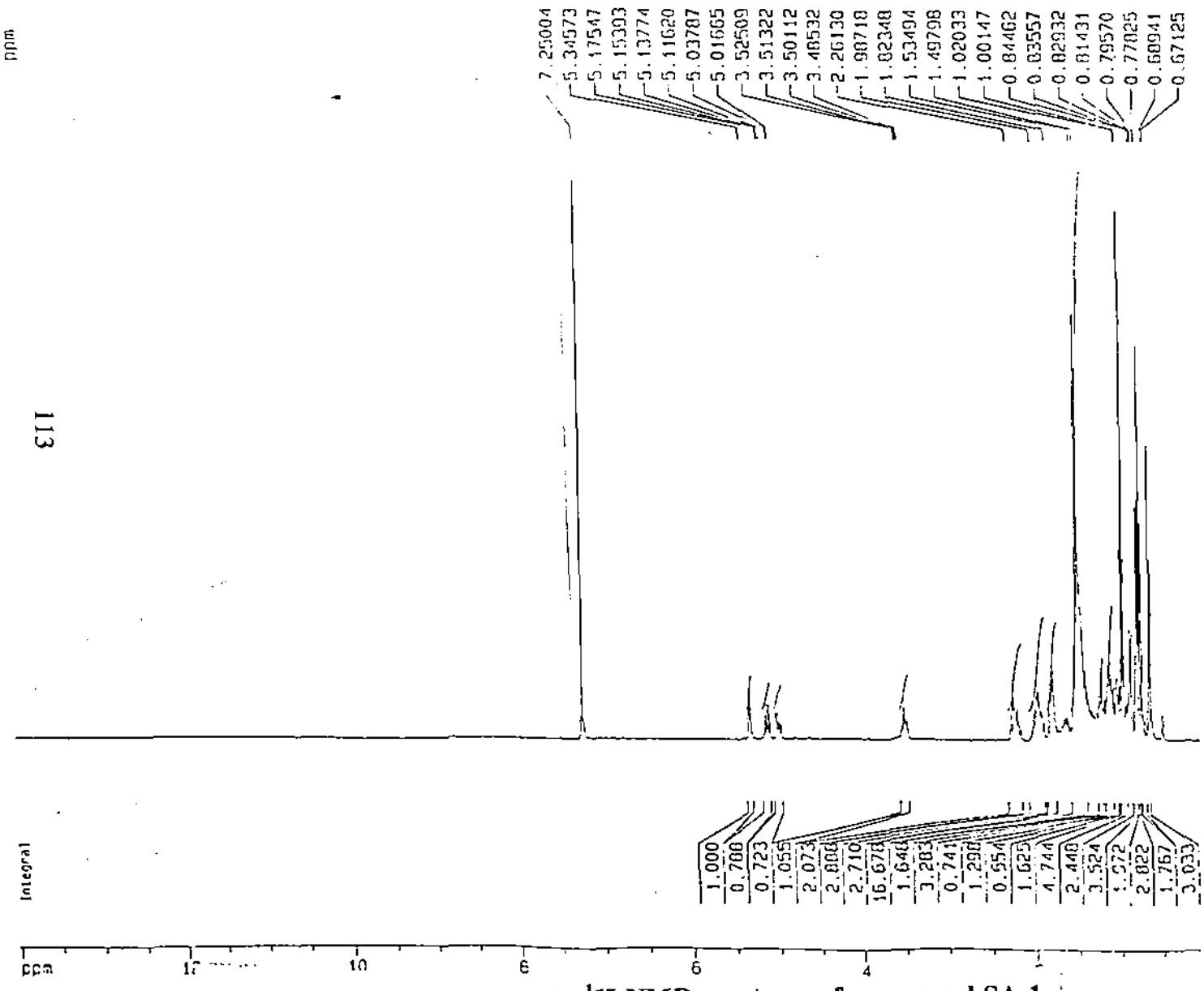

Figure F.26 : ^{1}H-NMR spectrum of compound SA-1

```
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EXPNO                     1
PROCNO                    1

F2 - Acquisition Parameters
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Time                  20.03
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PULPROG                zg30
TD                    32768
SOLVENT               CDCl3
NS                      164
DS                        2
SWH               6410.256 Hz
FIDRES            0.195625 Hz
AQ                2.5559540 sec
RG                      512
DW                 78.000 usec
DE                  6.00 usec
TE                  310.0 K
D1                1.00000000 sec

============ CHANNEL f1 ============
NUC1                    1H
P1                   8.30 usec
PL1                  -6.00 dB
SFO1            400.1428010 MHz

F2 - Processing parameters
SI                    32768
SF              400.1400126 MHz
WDW                      EM
SSB                       0
LB                   0.30 Hz
GB                        0
PC                    1.40

1D NMR plot parameters
CX                   20.00 cm
F1P                 14.032 ppm
F1                5614.60 Hz
F2P                 0.074 ppm
F2                  29.49 Hz
PPMCM             0.69792 ppm/cm
HZCM            279.26339 Hz/cm
```

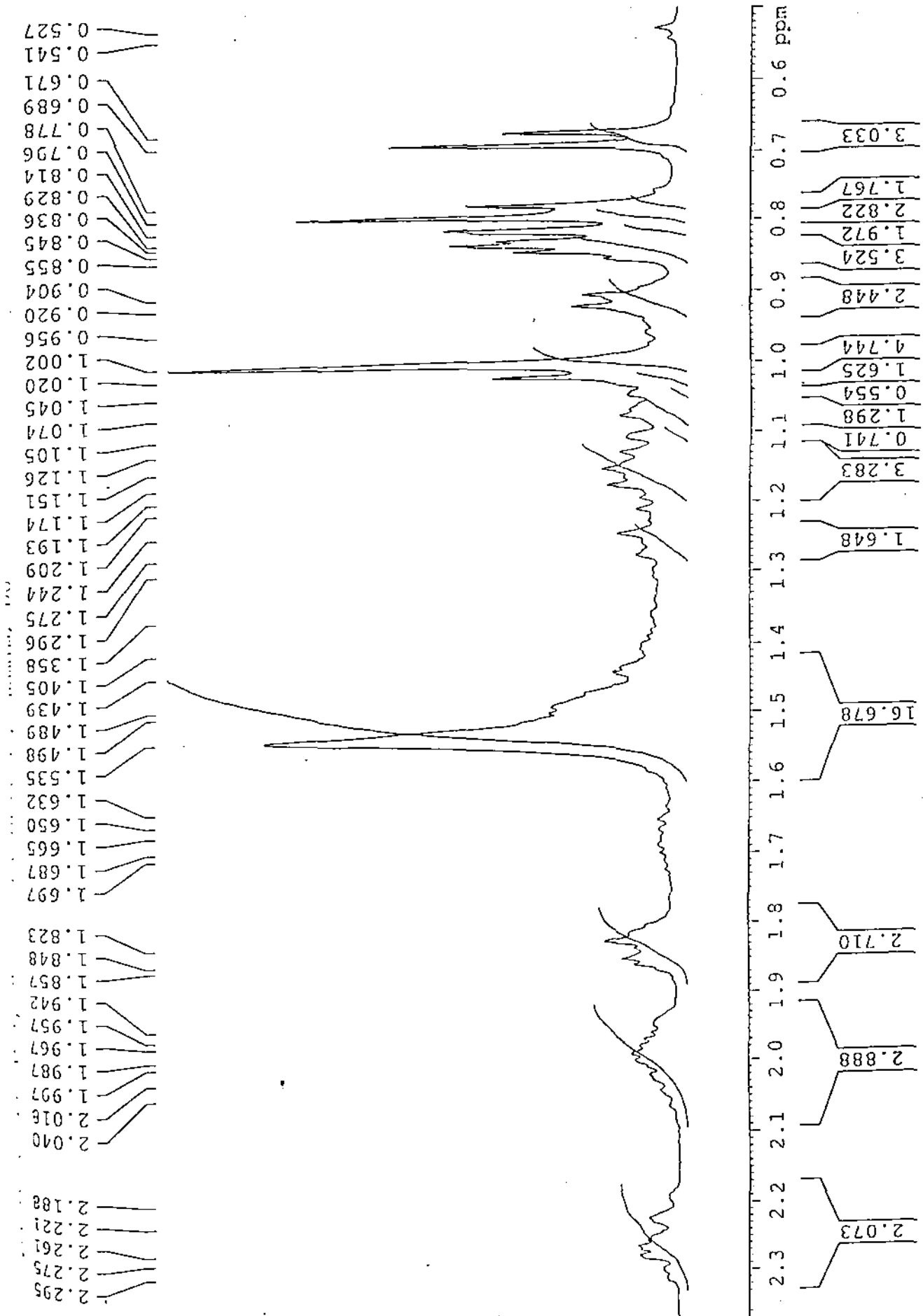


Figure F.27 : Expanded ^{1}H-NMR spectrum of compound SA-1

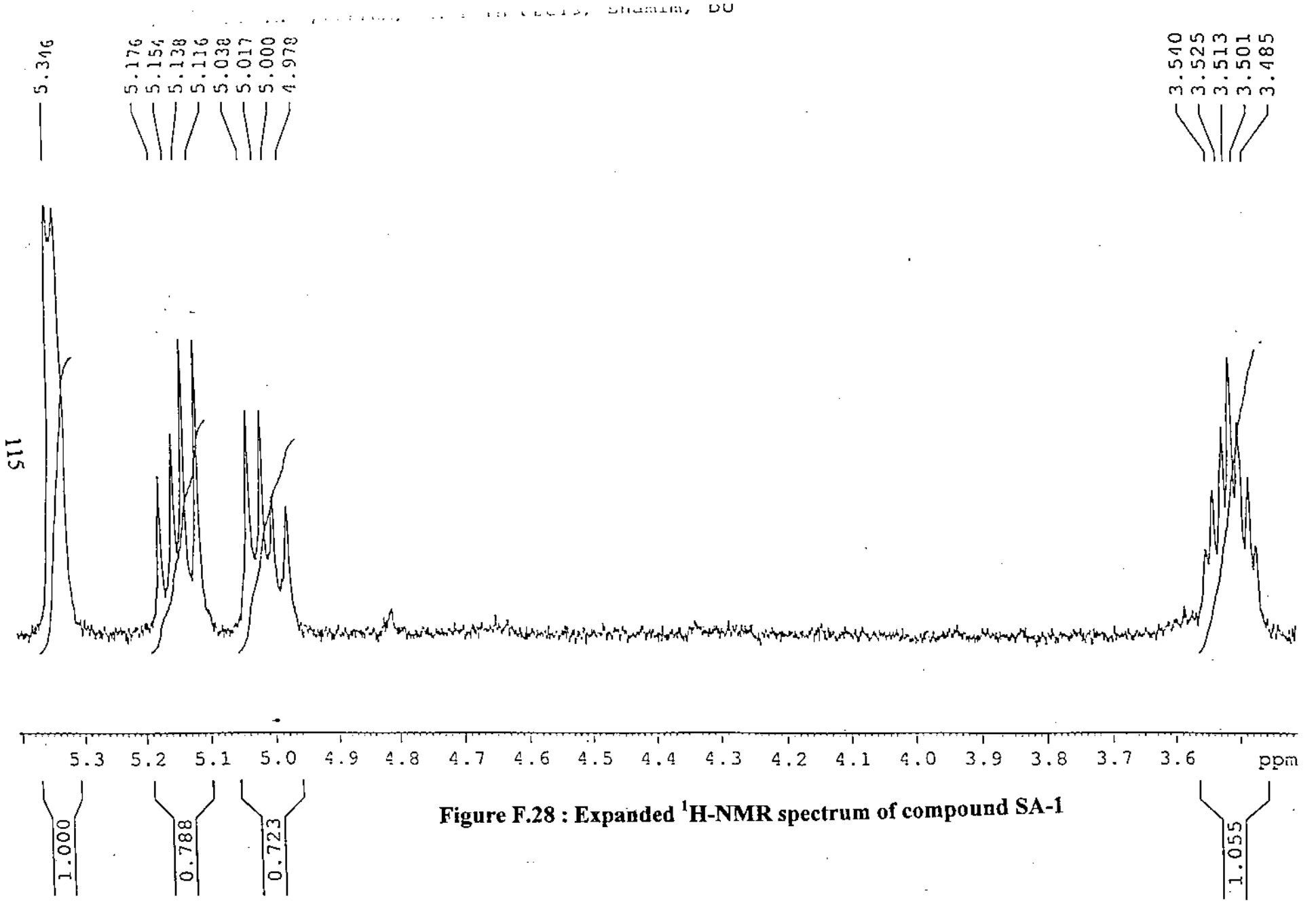


Figure F.28 : Expanded ^{1}H-NMR spectrum of compound SA-1

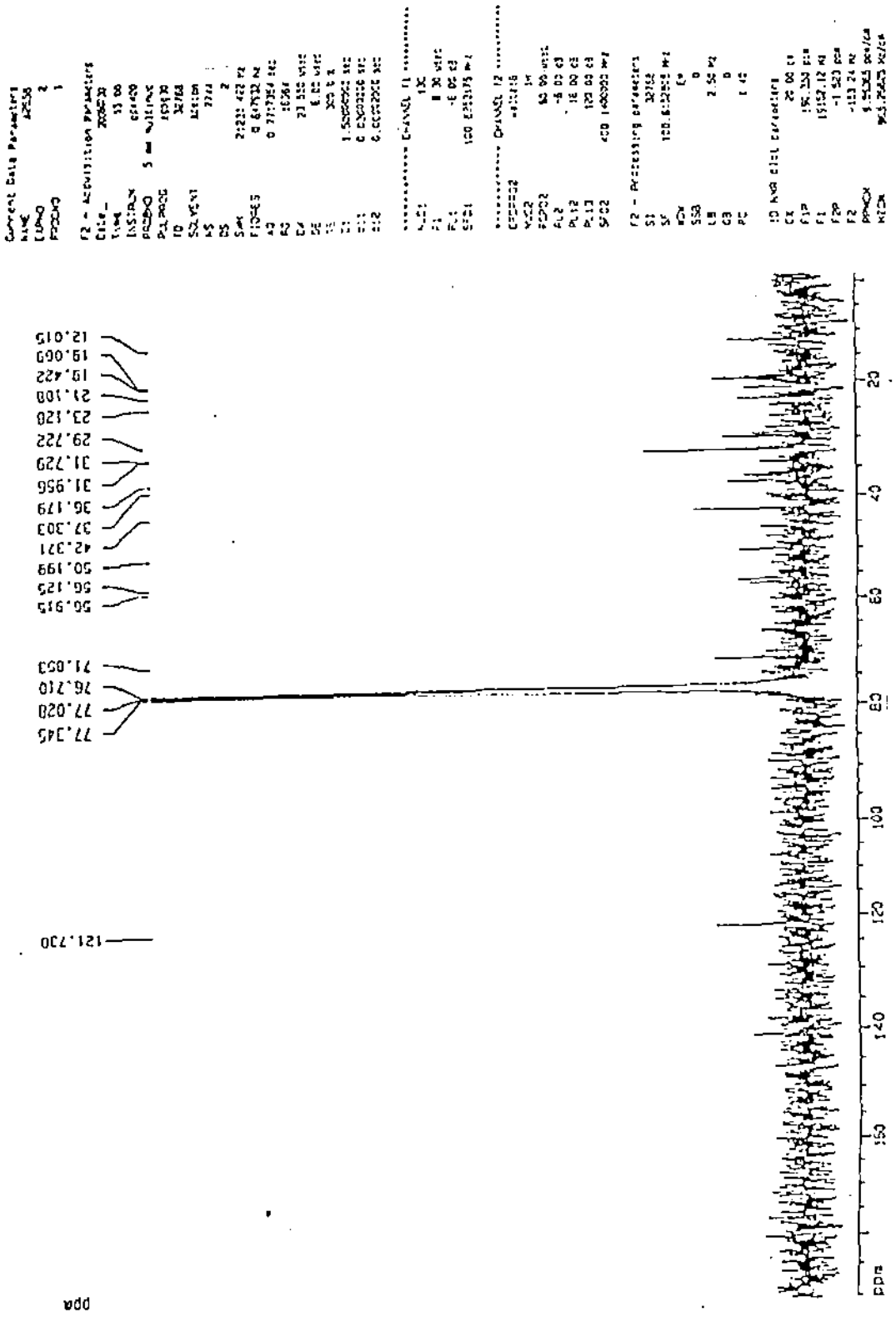


Figure F.29 : ^{13}C-NMR spectrum of compound SA-1

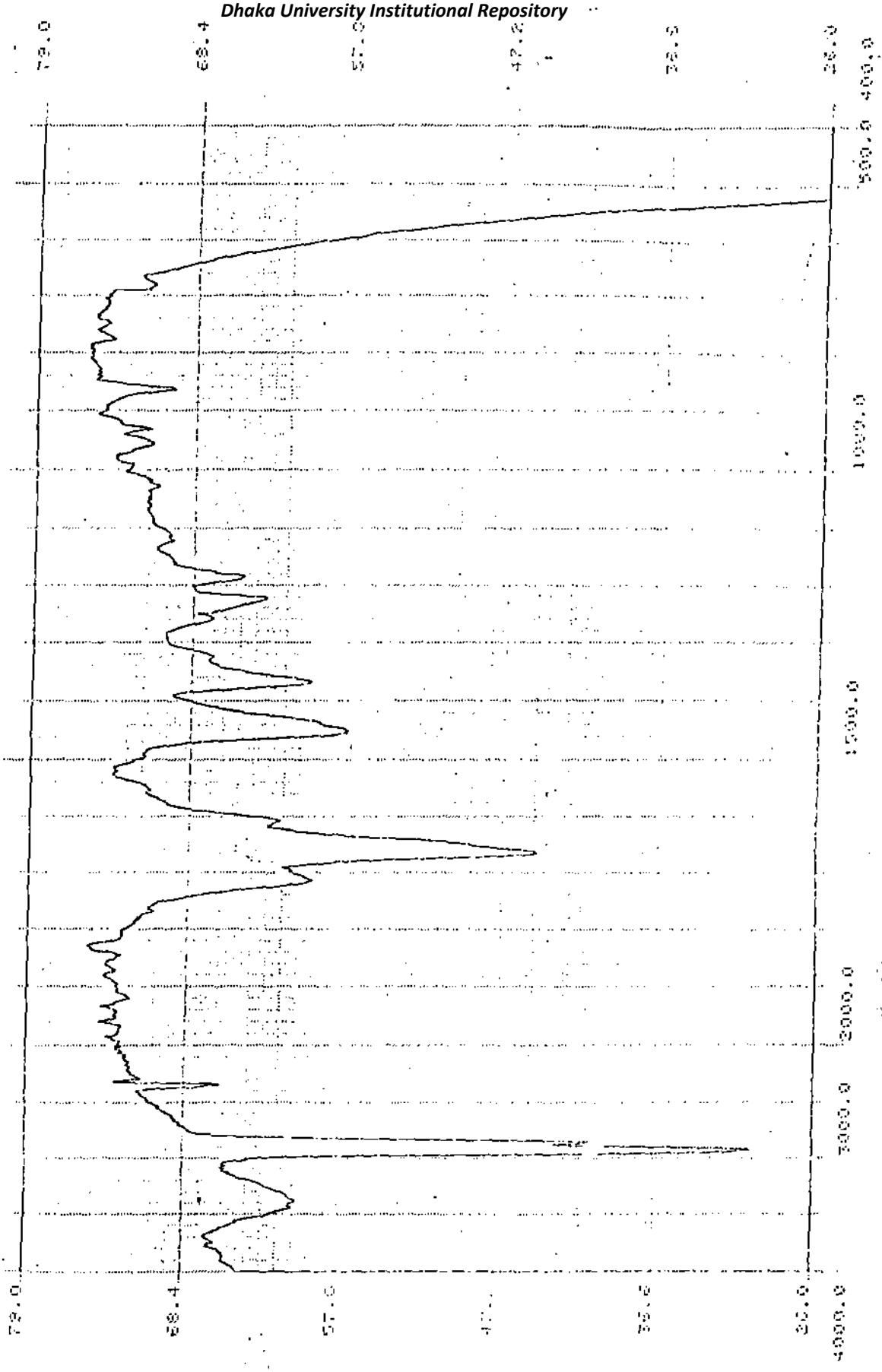


Figure F.30 : IR spectrum of compound SA-3

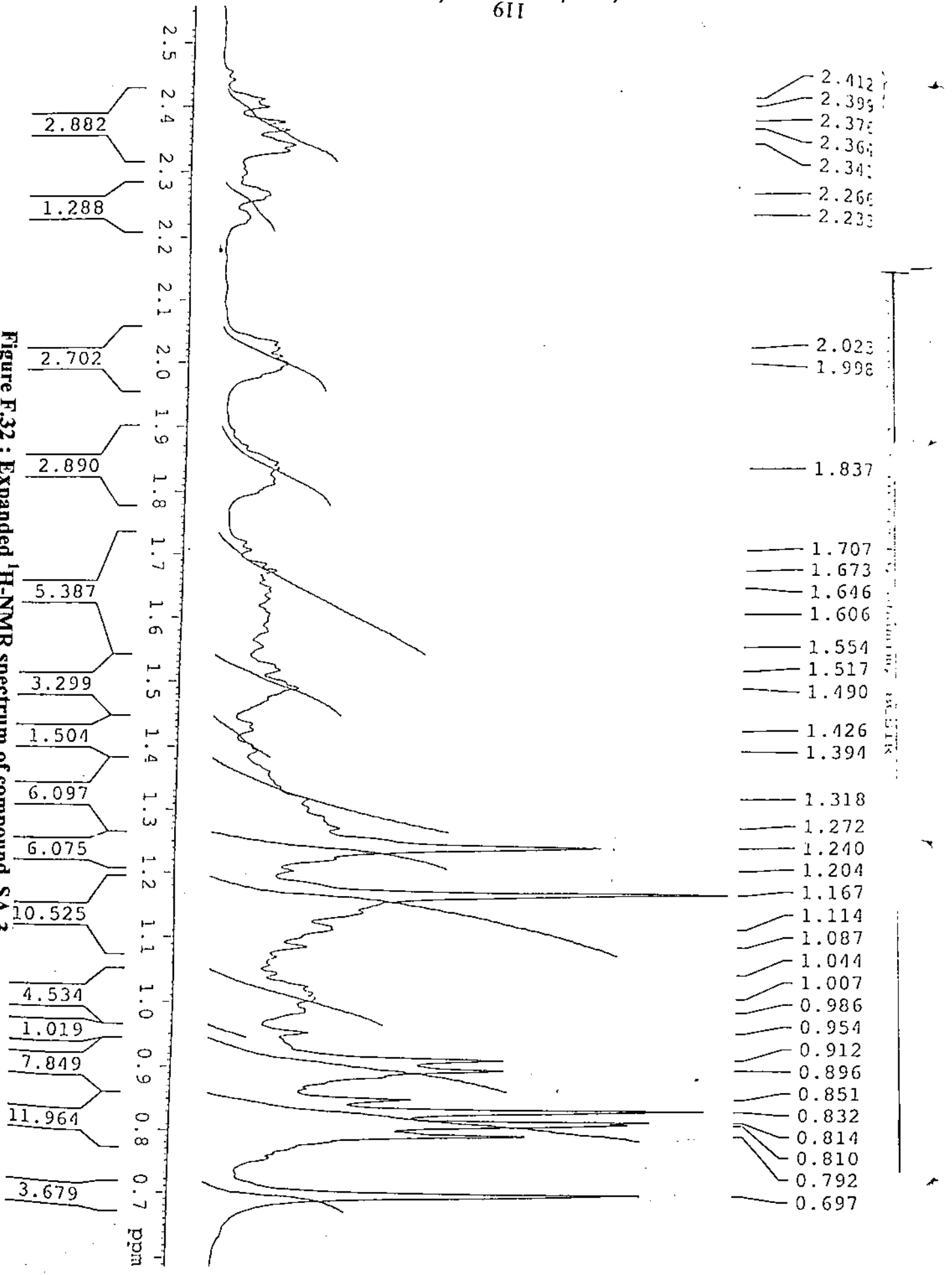


Figure F.32 : Expanded ^{1}H-NMR spectrum of compound SA-3

G. Results and Discussion

(*Enhydra fluctuans* Lour.)

G. Results and Discussion (*Enhydra fluctuans* Lour.)

G.1. Preliminary investigation of the plant material :

G.1.1. Plant material:

A species of the Compositae family, *Enhydra fluctuans* Lour., has been investigated in this work. The plant part used was the leaves and stem together.

G.1.2. Phytochemical screening:

Phytochemical screening for different phytochemical of *Enhydra fluctuans* Lour. was carried out (Sec. F.1.2) and some of phytochemicals were estimated (Sec. F1.3) and results are given in Table G.1.1 and Table G.1.2.

Table G.1.1: Qualitative analysis of the phytochemicals of *Enhydra fluctuans* Lour.:

Alkaloid	Tannin	Saponin	Flavonoid	Steroid	Terpenoid	Cardiac glycoside
+	+	+	+	+	+	–

Table G.1.2: Amount (mg per 100 g of dry powder basis) of crude alkaloid, flavonoid and saponin of *Enhydra fluctuans* Lour.:

Alkaloids (mg/ 100 g)	**Flavonoids (mg/ 100 g)**	**Saponin (mg/ 100 g)**
3.2	12.4	14.4

G.1.3 Extraction of the plant material :

Enhydra fluctuans Lour. was collected locally, dried and ground to a coarse powder. The powder sample (500.0 gm) was extracted with Soxhlet apparatus using petroleum ether. The residue obtained after extraction with petroleum ether was further extracted with ethyl acetate in the similar way (Sec. F.2.1 and F.2.2).

G.1.4 Isolation and characterization of compounds :

From the extractives pure compounds were isolated applying various chromatographic techniques (Sec. F.2.3 to F.2.3.4). The isolated pure compounds were then characterized using various spectroscopic techniques (sec. F.2.3.5).

G.2. Characterization of isolated compounds from *Enhydra fluctuans* Lour.

G.2.1. Characterization of isolated compound EF_3 as *ent*-kaur-16-en-19-oic acid :

The compound EF_3 was isolated from the fraction F_2 of petroleum ether extract of the plant *Enhydra fluctuans* Lour.. It was obtained as a solid having melting point 170-172°C. The TLC examination of this isolated compound showed brownish colored single spot on spraying with vanillin-sulphuric acid followed by heating in an oven at 110° C for about 10 minutes.

G.2.2. IR spectroscopic analysis :

The IR spectrum (Figure F.1) showed a very broad absorption band at 3450 cm^{-1} assignable to highly hydrogen bonded O-H stretching of carboxylic acid (-

COOH). The bands around 2900 cm^{-1} were due to the presence of C-H stretching (aliphatic). The small band at 3050 cm^{-1} was due to C-H stretching attached to olefenic bond. The band at 1680 cm^{-1} might be due to C=O stretching of carboxylic acid (-COOH). The band around 1650 cm^{-1} was suggestive of >C=C< group. The absorption band at 1460 cm^{-1} was indicative of $-CH_2-$ bending. The peaks around 1350 cm^{-1} was due to $-CH_3$ bending. The absorption band at 1210 cm^{-1} was due to C-O stretching. The peaks at 880 and 800 cm^{-1} were indicative of the presence of >C=C-H.

G.2.3. ^{1}H-NMR spectroscopic analysis :

The ^{1}H NMR (400 MHz in $CDCl_3$) spectra (Figure F.2, F.3 and F.4)of the isolated compound EF_3 had two signals at δ 0.94 and 1.23 ppm typical for the presence of methyl protons (H-8 and H-17). The signals at δ 4.72 and 4.78 ppm (one hydrogen each, broad singlet) were assigned to olefinic proton at H-17. The signal at δ 2.62 ppm was due to methine proton at H-13. The other signals of the spectrum between 1.41-2.16 ppm were due to the presence of different methylene and methine protons.The assignment of the ^{1}H-NMR signals of EF_3 is presented in Tabie G.2.1 and compared with the published data[60] of *ent*-kaur-16-en-19-oic acid. The assignment of the ^{1}H-NMR signals is shown in the structure below (Figure *G.1*).

Table G.2.1 : Comparative ^{1}H-NMR spectral data of isolated compound EF_3 with the published data[60] of *ent*-kaur-16-en-19-oic acid:

Protons	Chemical shift in ppm	
	δ value for EF_3	Published δ value[60] for *ent*-kaur-16-en-19-oic acid
H-13 (1H, br. s)	2.62	2.62
H-17 (2H, two br. s)	4.72 and 4.78	4.72 and 4.78
H-18 (1H, s)	1.23	1.23
H-20 (1H, s)	0.94	0.94
Methylene and methine proton	1.41-2.16	1.41-2.16

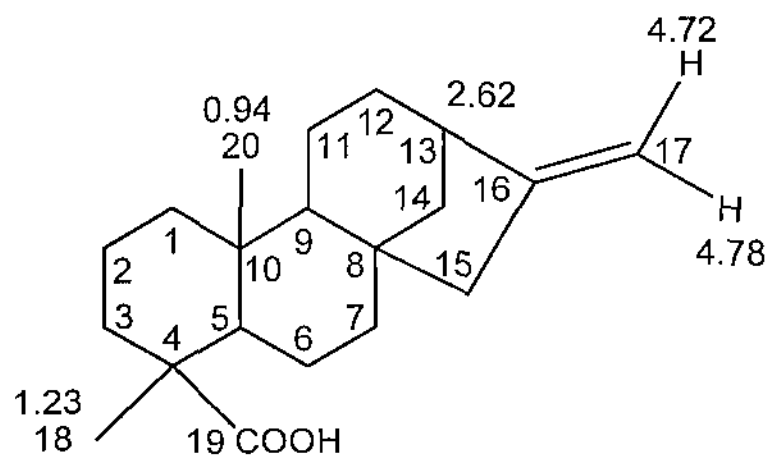


Figure G.1: Structure of the compound EF_3 showing ^{1}H signals

G.2.4. ^{13}C-NMR spectroscopic analysis:

The ^{13}C NMR spectra of **EF_3** (Figure F.5, F.6 and F.7) showed the presence of twenty carbons for a diterpene skeleton. The signals at δ 155.7 (C-16) and 103 (C-17) ppm were due to two olefinic carbon and signals δ 39.6 (C-10), 43.7 (C-4) and 44.2 ppm (C-8) were assignable to three quaternary carbons. The signals at δ 15.5 (C-20) and 28.9 ppm (C-18) were observed due to the presence of two methyl carbons. The signal at δ 185.0 ppm (C-19) was due to carboxylic acid carbon. The

assignment of the ^{13}C-NMR signals of EF_3 is presented in Table G.2.2 and compared with the published data[60] of *ent*-kaur-16-en-19-oic acid. The assignment of the ^{13}C-NMR signals is shown in the structure below (Figure G.2).

Table G.2.2 : Comparative ^{13}C-NMR spectral data of the isolated compound EF_3 and the reported value[60] of *ent*-kaur-16-en-19-oic acid

Carbon number	Types of carbon	Chemical shift in ppm	
		Observed δ value for EF_3	Reported δ value[60] for *ent*-kaur-16-en-19-oic acid
1	$-CH_2-$	40.7	40.7
2	$-CH_2-$	19.0	19.1
3	$-CH_2-$	37.7	37.7
4	>C<	43.7	43.2
5	>CH-	57.0	57.0
6	$-CH_2-$	21.8	21.8
7	$-CH_2-$	41.2	41.3
8	>C<	44.2	44.2
9	>CH-	55.1	55.1
10	>C<	39.6	39.7
11	$-CH_2-$	18.4	18.4
12	$-CH_2-$	33.1	33.1
13	>C<	43.8	43.8
14	$-CH_2-$	39.6	39.7
15	$-CH_2-$	48.9	48.9
16	>C=	155.7	155.8
17	$=CH_2$	103.0	103.0
18	$-CH_3$	28.9	28.9
19	-COOH	185.0	184.9
20	$-CH_3$	15.5	15.6

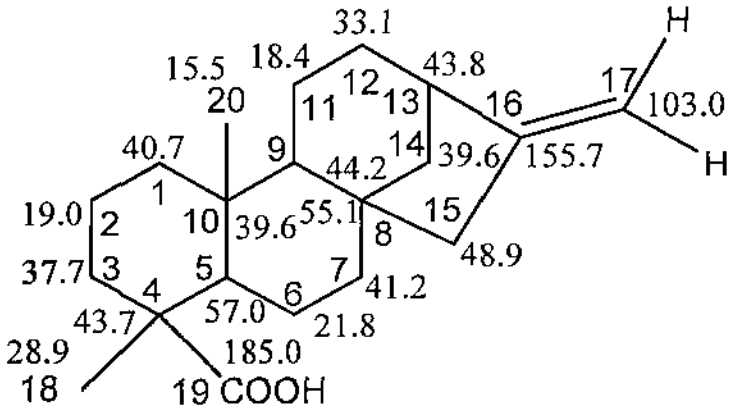


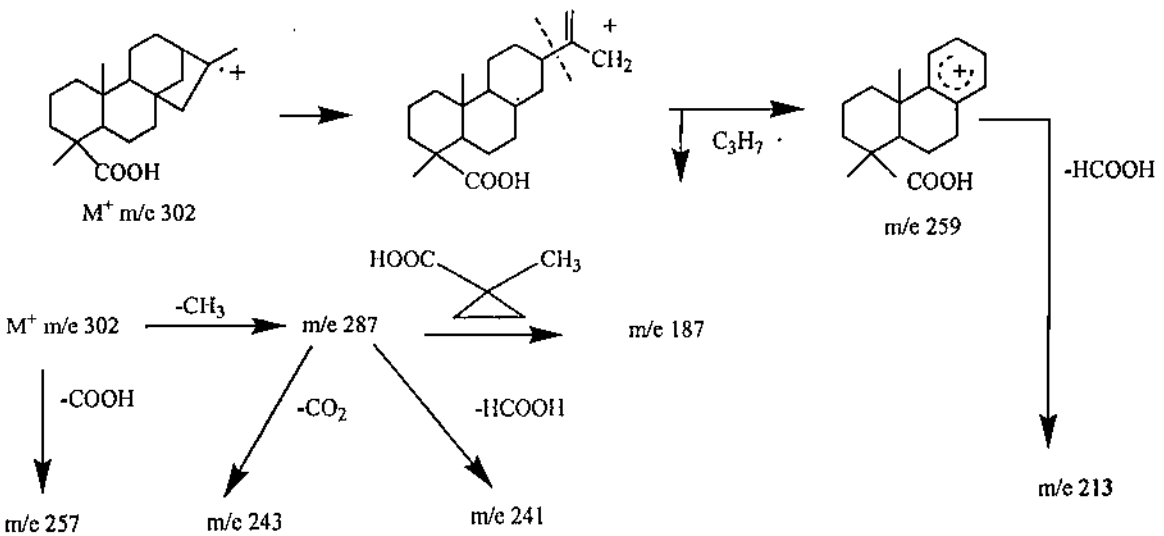
Figure G.2: Structure of the compound EF_3 showing ^{13}C signals

G.2.5. Mass spectroscopic analysis:

The mass spectrum of **EF_3** is presented in Figure F.8. The molecular ion peak obtained at m/z 302 corresponding to molecular formula $C_{20}H_{30}O_2$. The compound showed the expected loss of the C-10 angular methyl group (m/z 287) and C-4 carboxylic acid group (m/z 257). Thus the compound showed a peak at m/z 241 due to loss of HCOOH+Me from the molecular ion. The loss of C-10 methyl gr along with C2-C3-C4 fragment in ring A leading to the ion at m/z 187. Mass fragmentation pattern is shown in Scheme-G.1. Comparative mass spectral data of the isolated compound EF_3 and the reported value[37] of *ent*-kaur-16-en-19-oic acid is presented in Table G.2.3.

Table G.2.3 : Comparative mass spectral data of the isolated compound EF_3 and the reported value[37] of *ent*-kaur-16-en-19-oic acid:

Observed value ion peaks of EF_3 (m/z)	Reported value[37] ion peaks of *ent*-kaur-16-en-19-oic acid (m/z)	Tentative Assignment
302	302	$[M]^+$ ($C_{20}H_{30}O_2$)
287	287	$[M-CH_3]^+$
259	259	$[M-C_3H_7]^+$
257	257	$[M-COOH]^+$
243	243	$[M-CH_3-CO_2]^+$
241	241	$[M-CH_3-HCOOH]^+$
213	213	$[M-C_3H_7-HCOOH]^+$
187	187	$[M-C_3H_7-C_5H_8O_2]^+$



Scheme G.1: Scheme of Mass fragmentation pattern of *ent*-kaur-16-en-19-oic acid.

The structure of **EF_3** has been finally established by combining the IR, ^{1}H-NMR, ^{13}C-NMR and mass spectral data. All these spectral data were in good agreement with the reported spectral data of ***ent*-kaur-16-en-19-oic acid ($C_{20}H_{30}O_2$).**

On the basis of the forgoing discussion it could be concluded that the compound is ***ent*-kaur-16-en-19-oic acid** having the following structure:

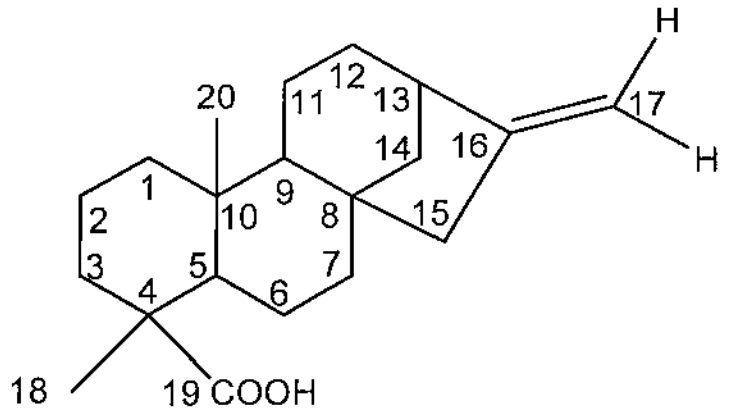


Figure G.3: Structure of EF_3 as *ent*-kaur-16-en-19-oic acid

The isolation of ***ent*-kaur-16-en-19-oic acid or *ent*-kaurenoic acid** from *Ehydra fluctuans* Lour. has already been reported[39] prior to this work. The compound *ent*-kaur-16-en-19-oic acid is a well known compound and its derivatives are widely occurred in the different species of plant of Compositae (Asteraceae) family.

G.2.6. Biological importance of the *ent*-kaurenoic acid isolated from the plant *Enhydra fluctuans* Lour. :

Kaurenes are intermediates in the biosynthesis of the gibberellins, which are plant growth hormones of several plants, some fungal metabolites and diterpene alkaloids. The biological activities of naturally occuring kaurene diterpene have been reported[61]. Antimicrobial, antiparasitic, insect antifeedant, anti-HIV and anti-inflammatory activities for different kaurene derivatives have been observed[62]. Interesting biological properties, including analgesic action[63] and antifungal effects[64] of *ent*-kaurenoic acid and its derivatives have been studied by various workers[65, 66, 67, 68].

G.2.7. *ent*-Kaurenoic acid biosynthesis in the plant:

Terpenoids are compounds made up of 5-carbon isoprenoid building blocks, joined head to tail. The basic biological isoprenoid unit is isopentenyl diphosphate (IPP). In the green parts of plants, IPP is synthesized in the stroma of plastids, using different precursors at each site. The plastidic pathway uses glyceraldehyde 3-phosphate and pyruvate, and the pathway is named for an important intermediate, methyl erythritol phosphate (MEP)[69].

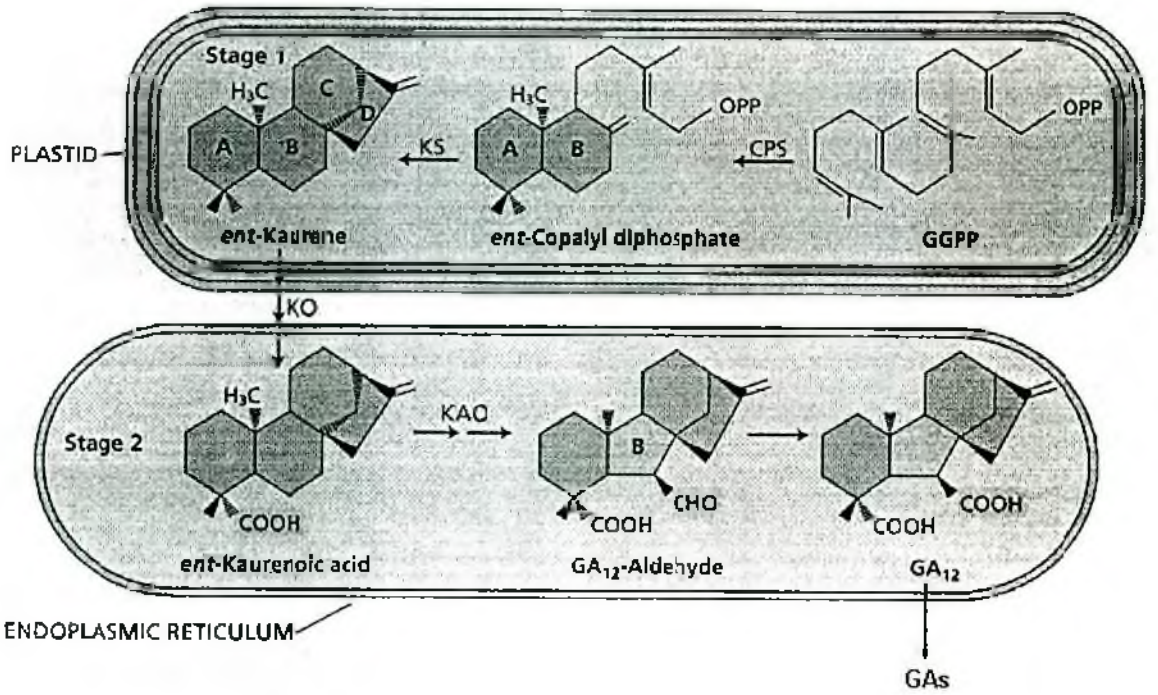


Figure G.4 : Biosynthesis of *ent*-Kaurenoic acid.

ent-Kaurenoic acid is diterpenoid that are formed from four biological isoprenoid units. They are assembled to give a 20-carbon linear molecule, geranylgeranyl diphosphate (GGPP). GGPP is a precursor of many diterpenoid compounds. There are two cyclization reactions that convert linear GGPP to *ent*-kaurene (enantiomeric form of kaurene). The formation of a bicyclic intermediate *ent*-copalyl-diphosphate (CPP) is catalyzed by *ent*-copalyl diphosphate synthase

(CPS), and the conversion of CPP to the tetracyclic *ent*-kaurene is catalysed by *ent*-kaurene synthase (KS). C-19 methyl (CH_3) group of kaurene is oxidized in three steps to give *ent*-kaurenoic acid (KA). These oxidations are catalyzed by *ent*-kaurene oxidase (KO). *ent*-kaurenoic acid is the precursor of many important secondary metabolite such as gibberellin (GA), GA_{12} and many different GAs[70].

G.3.1. Characterization of isolated compound EF_4 as stigmasta-5,22,25-trien-3β-ol :

The compound **EF_4** was isolated (Sec. F.2.3.4) as white needle like crystals (m.p-146°-148° C) from the petroleum ether extract of *Enhydra fluctuans* Lour.. The TLC examination of this isolated compound showed brown colored single spot in the iodine chamber. The spot turned into violet on spraying with vanillin-sulphuric acid followed by heating in an oven at 110° C for about 10 minutes.

The chloroform solution of the isolated compound (EF_4) showed the positive test for steroidal compound.

G.3.2. IR spectroscopic analysis:

IR spectrum (Figure F.9) showed a broad absorption band at 3450 cm^{-1} indicating the presence of assignable to hydroxyl group (-OH). A tiny absorption peak at 3050 cm^{-1} was indicative of >C=C-H skeleton. Two bands at 2920 and 2850 cm^{-1} were due to the presence of –C-H (aliphatic) stretching. The band around 1650 cm^{-1} was suggestive of >C=C< group. The absorption band at 1460 cm^{-1} was indicative of $–CH_2-$ bending. The peaks at 1370 and 1050 cm-1 were due to $–CH_3$ bending and C-O stretching respectively . The absorption band at 960 cm^{-1} was assigned for *trans* disubstituted double bond (-CH=CH-). The peaks at 880 and

800 were indicative of the presence of terminal methylene group (CH_2=C<) and trisubstituted double bond (>C=CH-) respectively.

G.3.3. ^{1}H-NMR spectroscopic analysis :

The ^{1}H-NMR (400 MHz in $CDCl_3$) spectra (Figure F.10, F.11 and F.12) of the isolated compound EF_4 had a sharp singlet at δ 0.68 ppm indicative of the three protons (H-18) of methyl group at C-13. The singlet at δ 1.00 ppm was indicative of the presence of three protons (H-19) of the methyl group at C-10.

The spectrum showed a doublet at δ 1.01 ppm due to the presence of methyl protons (H-21) at C-20, a broad singlet at δ 1.66 ppm was due to the presence of methyl protons (H-27) at C-25, a doublet of methyl proton at δ 4.68 ppm (H-26) at C-25 and a multiplet of methyl proton at 0.82 ppm (H-29) at C-28. The multiplet at 3.52 ppm was due to the presence of a methine proton (3α-H) at C-3 of a steroidal nucleus. The spectrum showed a multiplate as characteristic downfield signals at δ 5.24 and 5.17 ppm which were due to the presence of olefinic protons of H-22 and H-23 at C-22 and C-23. A doublet was also observed. at δ 5.34 ppm was due to the presence of an olefinic proton (H-6) at C-6 of a steroidal nucleus. A broad quatret at δ 2.41 ppm was due to the presence of proton (H-24) at the C-24 next to olefinic carbon at C-25. The other signals of the spectra (m, d, t, s) between δ 1.07-2.29 ppm were due to the presence of different methylene (-CH_2-) and methine (>CH-) protons. The assignment of the ^{1}H-NMR signals of EF_3 is presented in Table G.3.1 and along with the published data[71] of stigmasta-5,22,25-trien-3β-ol.

Table G.3.1 : Comparative ^{1}H-NMR spectral data of isolated compound EF_4 with the published data[71] of stigmasta-5,22,25-trien-3β-ol:

Protons	Chemical shift in ppm	
	δ value for EF_4	Published δ value[71] for stigmasta-5,22,25-trien-3β-ol
H-3α (1H, m)	3.52	3.52
H-6 (1H, d)	5.34	5.35 (br. d, J= 5.1 Hz)
H-18 (3H, s)	0.68	0.68
H-19 (3H, s)	1.00	1.00
H-21 (3H, d)	1.01	1.00 (d, J=6.5 Hz)
H-22 (1H, m)	5.24	5.24 (dd, J=15.3, 7.9 Hz)
H-23 (1H, m)	5.17	5.16 (dd, J=15.3, 6.4 Hz)
H-24 (1H, br. q)	2.41	2.42 (br. q, J=6.4 Hz)
H-26 (2H, br. s)	4.68	4.96
H-27 (3H, br. s)	1.66	1.64 (t, J=0.9 Hz)
H-29 (3H, m)	0.82	0.82 (t, J=7.3 Hz)
Methine and methylene protons (m, d, t, s)	1.07-2.29	-

On the basis of the ^{1}H-NMR assignment, the structure of the compound **EF_4** showing its ^{1}H-NMR signals may be given as (Figure G.5):

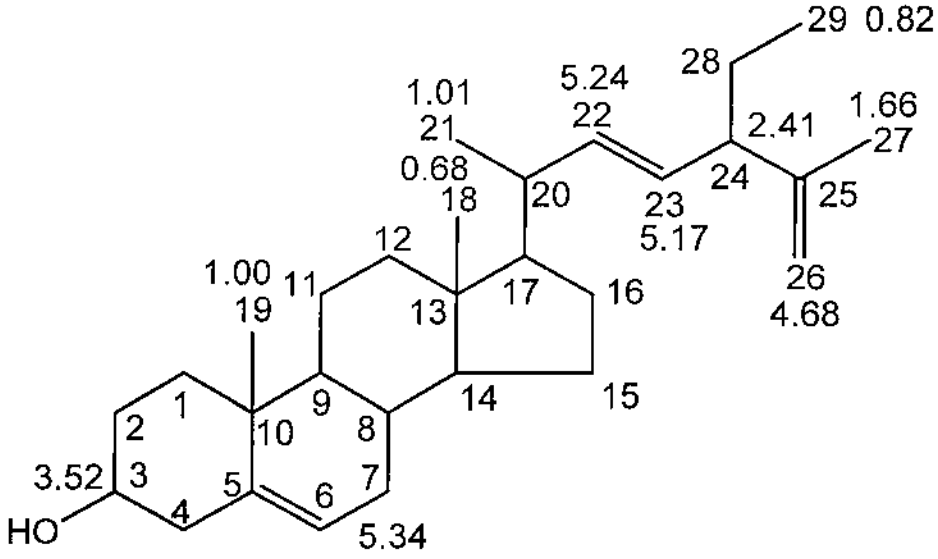


Figure G.5 : Structure of the compound EF_4 showing ^{1}H signals

G.3.4. ^{13}C-NMR spectroscopic analysis :

The ^{13}C NMR spectra (Figure F.13, F.14 and F.15) of isolated compound **EF_4** showed the presence of twenty-nine carbon signals. Among these twenty-nine carbon signals, five signals for methyl carbons, nine signals for methylene carbons, seven signals to methine carbons, two signals for quaternary carbons and six of them were for olefinic carbons.

The signals appeared at δ 12.0 (angular), 12.1, 19.4 (angular), 20.2 and 21.1 ppm were due to the five methyl carbons. The signals at δ 20.8, 24.3, 25.7, 28.7, 31.6, 31.9, 39.7 and 37.2 (double) ppm were due to nine methylene carbons. The signals at δ 31.9, 40.1, 50.1, 52.0, 55.9, 56.8 and 71.8 (alcoholic >C<u>H</u>-O-H) ppm were due to seven methine carbons. The quaternary two carbons resonated at 36.5 and 42.3 ppm. The signals appeared at δ 109.5 (terminal), 121.7, 130.0, 137.2, 140.7 and 148.6 ppm were due to six olefinic carbons. The assignment of the ^{13}C-NMR signals of **EF_4** is presented in Table G.3.2 and compared with the published data[71] of stigmasta-5,22,25-trien-3β-yl acetate.

Table G.3.2: Comparative ^{13}C-NMR spectral data of the isolated compound EF_4 and the reported value[71] of stigmasta-5,22,25-trien-3β-yl acetate:

Carbon number	Types of carbon	Chemical shift in ppm	
		Observed δ value for EF_4	Reported δ value[71] for stigmasta-5,22,25-trien-3β-yl acetate
1	$-CH_2-$	37.2	37.0
2	$-CH_2-$	31.6	27.8
3	>CH-O-H	71.8	74.0 (>CH-$OCOCH_3$)
4	$-CH_2-$	37.2	38.1
5	>C=	140.7	139.7
6	=CH-	121.7	122.6
7	$-CH_2-$	31.9	31.9
8	>CH-	31.9	31.9
9	>CH-	50.1	50.1
10	>C<	36.5	36.6
11	$-CH_2-$	20.8	21.0
12	$-CH_2-$	39.7	39.6
13	>C<	42.3	42.3
14	>CH-	56.8	56.8
15	$-CH_2-$	24.3	24.3
16	$-CH_2-$	28.7	28.7
17	>CH-	55.9	55.9
18	$-CH_3$	12.0	12.0
19	$-CH_3$	19.4	19.3
20	>CH-	40.1	40.2
21	$-CH_3$	21.1	21.4
22	=CH-	137.2	137.2
23	=CH-	130.0	130.1
24	>CH-	52.0	52.0
25	>C=	148.6	148.6
26	$=CH_2$	109.5	109.5
27	$-CH_3$	20.2	20.2
28	$-CH_2-$	25.7	25.7
29	$-CH_3$	12.1	12.1
	$-COCH_3$	-	20.8
	$-COCH_3$	-	170.5

The structure of **EF_4** (Figure G.6) showing its ^{13}C-NMR signals may be as given as

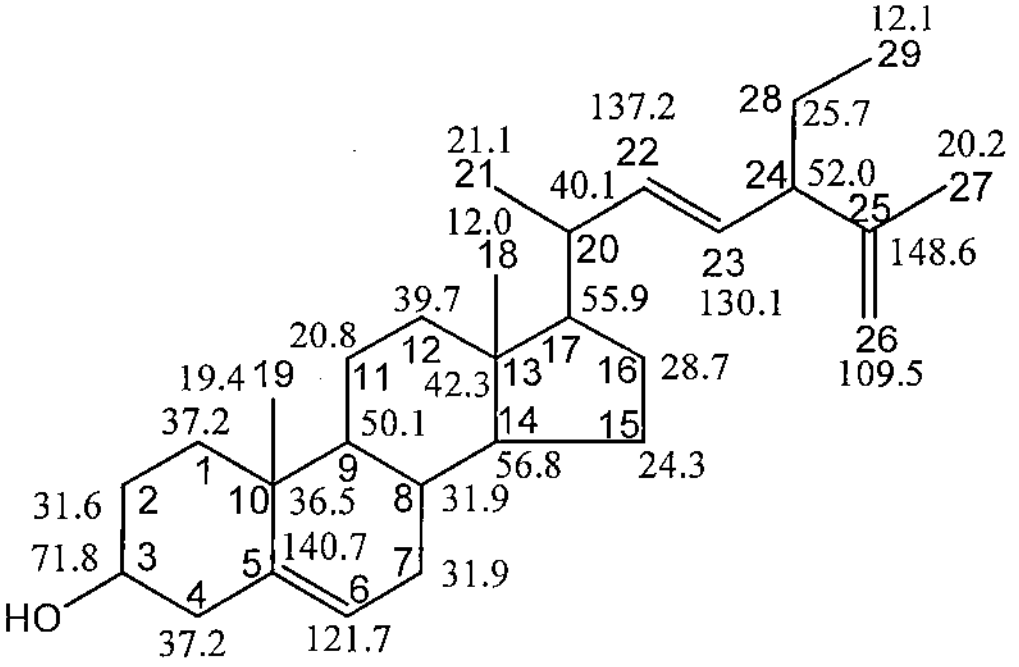


Figure G.6 : Structure of the compound EF_4 showing ^{13}C signals

The structure of **EF_4** was been finally established by combining the IR, ^{1}H-NMR, and ^{13}C-NMR spectral data. All these spectral data were in good agreement with the reported spectral data of **stigmasta-5,22,25-trien-3β-ol or (24S)-ethylcholesta-5,22(E),25-triene-3β-ol ($C_{29}H_{46}O$).**

Based on the IR, ^{1}H- and ^{13}C-NMR data and comparing these with those of reported values, the compound **EF_4** was found to be **stigmasta-5,22,25-trien-3β-ol** having the structure (Figure G.7).

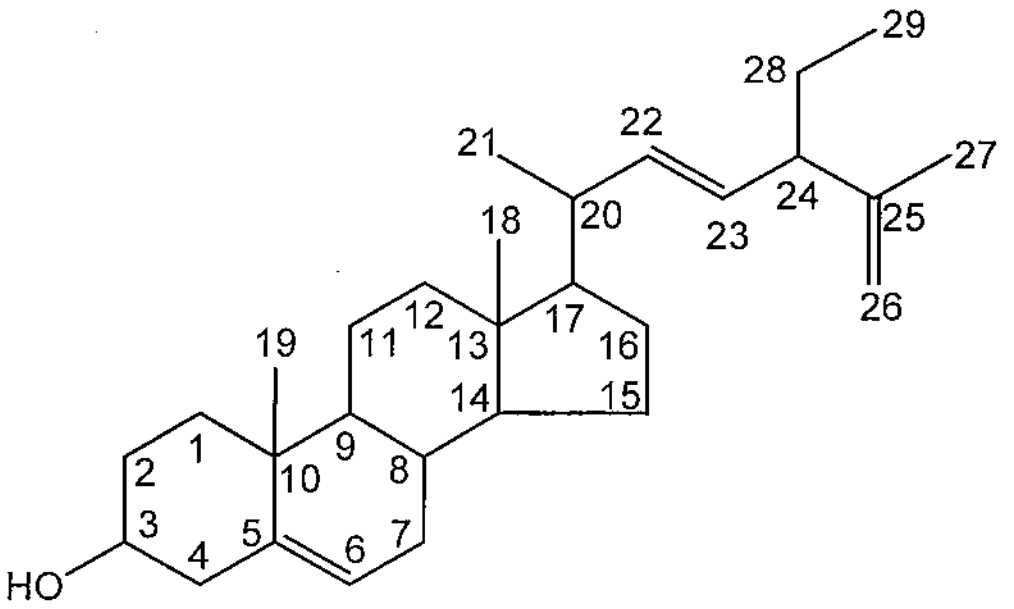


Figure G.7: Structure of EF_4 (stigmasta-5,22,25-trien-3β-ol)

Although this is a known compound and its isolation from *Enhydra fluctuans* Lour. has already been reported[37], but this finding is enriched with the modern techniques of isolation and characterization.

G.4.1. Characterization of isolated compound EFA as stigmasta-5,22,25-trien-3β-yl acetate :

The compound **EFA** was isolated as white needle like crystals (m.p- 142°-146° C) from the ethyl acetate extract of *Enhydra fluctuans* Lour.. The TLC examination of this isolated compound showed brown colored single spot in the iodine chamber. The spot turned into violet on spraying with vanillin-sulphuric acid followed by heating in an oven at 110° C for about 10 minutes.

The chloroform solution of the isolated compound **EFA** showed the positive test for steroidal compound.

G.4.2. IR spectroscopic analysis :

IR spectrum (Figure F.16) a tiny absorption peak at 3050 cm^{-1} was indicative of >C=C-H skeleton. Two bands at 2920 and 2850 cm^{-1} were due to the presence of --C-H (aliphatic) stretching. The strong and broad band at 1725 cm^{-1} was due to the presence of >C=O group of ester. The band around 1650 cm^{-1} was suggestive of >C=C< group. The absorption band at 1460 cm^{-1} was indicative of $-CH_2-$ bending. The peaks around 1370 cm^{-1} was due to $-CH_3$ bending. The strong absorption bands appeared at 1240 cm^{-1} and 1050 cm^{-1} was due to C-O stretching. The absorption band at 960 cm^{-1} was due to *trans* disubstituted double bond (-CH=CH-). The peak at 880 cm^{-1} was assignable to terminal methylene group (CH_2=C<) and at 800 cm^{-1} was indicative of the presence of trisubstituted double bond (>C=CH-).

G.4.3. ^{1}H-NMR spectroscopic analysis :

The ^{1}H-NMR (400 MHz in $CDCl_3$) spectra (Figure F.17, F.18 and F.19) of the isolated compound **EFA** had a sharp singlet at δ 0.68 ppm indicative of the three protons (H-18) of methyl group at C-13. The singlet at δ 1.00 ppm was indicative of the presence of three protons (H-19) of the methyl group at C-10.

The spectrum showed a doublet at δ 1.01 ppm due to the presence of methyl protons (H-21) at C-20, a broad singlet at δ 1.66 ppm was due to the presence of methyl protons (H-27) at C-25, a doublet of methyl proton at δ 4.68 ppm (H-26) at C-25 and a multiplet of methyl proton at 0.82 ppm (H-29) at C-28. The multiplet at 3.52 ppm was due to the presence of a methine proton (3α-H) at C-3 of a steroidal nucleus. The spectrum showed a multiplet as characteristic downfield signals at δ 5.24 and 5.17 ppm was due to the presence of olefinic protons H-22 and H-23 at C-22 and C-23 and a doublet at δ 5.34 ppm was due to the presence of

an olefinic proton (H-6) at C-6 of a steroidal nucleus. A broad quatret at δ 2.41 ppm was due to the presence of proton (H-24) at the C-24 next to olefinic carbon at C-25. The other signals of the spectra (m, d, t, s) between δ 1.07-2.29 ppm were due to the presence of different methylene (-CH_2-) and methine (>CH-) protons. A characteristic peak of the protons of the –OAc group was observed at 2.01 ppm.

Table G.4.1: Comparative ^{1}H-NMR spectral data of isolated compound EFA with the published data[71] of stigmasta-5,22,25-trien-3β-yl acetate:

Protons	Chemical shift in ppm	
	δ value for EFA	Published δ value[71] for stigmasta-5,22,25-trien-3β-yl acetate
H-3α (1H, m)	4.58	4.59
H-6 (1H, d)	5.36	5.36 (br. d, J= 4.6 Hz)
H-18 (3H, s)	0.68	0.68
H-19 (3H, s)	1.00	1.01
H-21 (3H, d)	0.99	1.00 (d, J=7.0 Hz)
H-22 (1H, m)	5.22	5.23 (dd, J=15.3, 8.2 Hz)
H-23 (1H, m)	5.17	5.17 (dd, J=15.3, 7.4 Hz)
H-24 (1H, br. q)	2.40	2.41 (br. q, J=7.4 Hz)
H-26 (2H, br. s)	4.68	4.68 and 4.69
H-27 (3H, br. s)	1.63	1.64 (br.s)
H-29 (3H, m)	0.82	0.82 (t, J=7.3 Hz)
OAc (3, 3H)	2.01	2.01
Methine and methylene protons (m, d, t, s)	1.12-1.85	-

The structure of **EFA** (Figure G.8) showing the ^{1}H-NMR signals may be represented as-

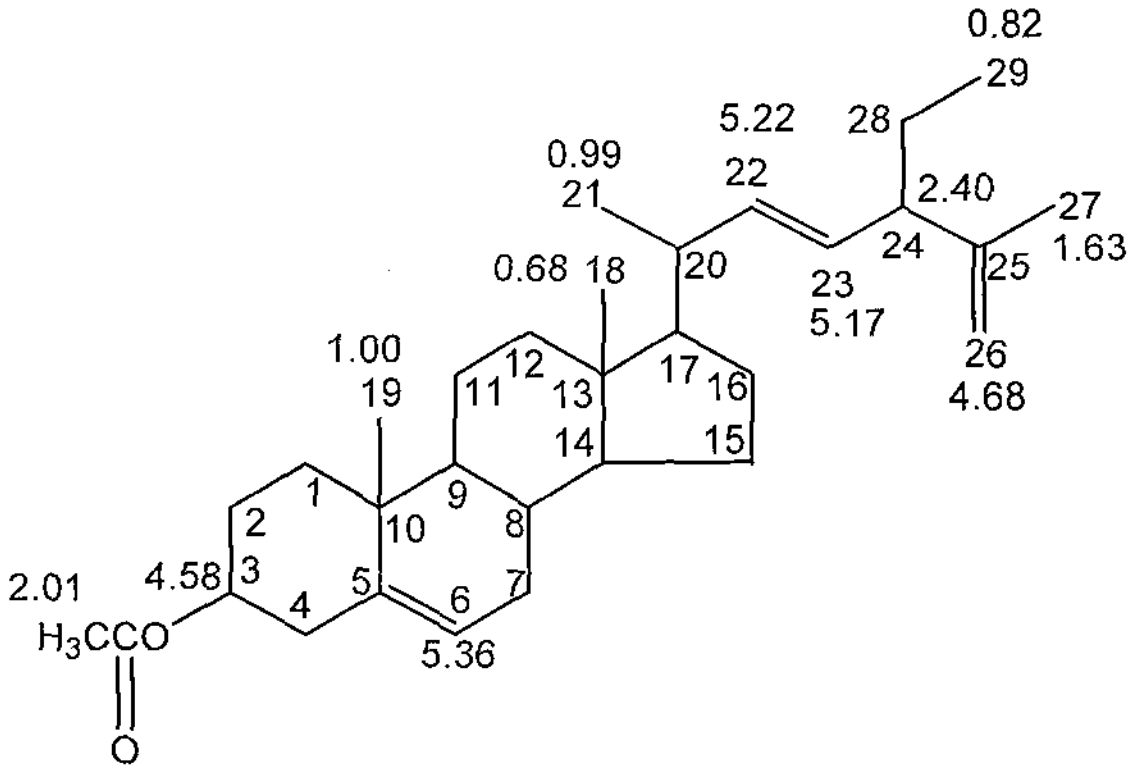


Figure G.8 : Structure of the compound EFA showing ^{1}H signals

G.4.4. ^{13}C-NMR spectroscopic analysis :

The ^{13}C NMR spectra (Figure F.20, F.21, F.22, F.23 and F.24) of isolated compound **EF_4** showed the presence of twenty-nine carbon signals. Among these twenty-nine carbon signals, five signals to methyl carbons, nine signals to methylene carbons, seven signals to methine carbons, two signals for quaternary carbons and six of them were assignable to olefinic carbons and two signals for acetate (-OAc) carbons.

The signals appeared at δ 12.0 (angular), 12.1, 19.4 (angular), 20.2 and 21.1 ppm were due to the five methyl carbons. The signals at δ 20.8, 24.3, 25.7, 28.7, 31.6, 31.9, 36.5 and 37.2 (double) ppm were due to nine methylene carbons. The signals at δ 31.9, 40.1, 50.1, 52.0, 55.9, 56.8 and 74.0 (>CH-$OCOCH_3$) o seven methine carbons. The quaternary two carbons resonated at 36.5 and 42.3 ppm. The signals appeared at δ 109.5 (terminal), 121.7, 130.0, 137.2, 140.7 and 148.6 ppm were due

to six olefinic carbons. The signals at δ 20.8 and 170.5 ppm were appeared due to $CO\underline{C}H_3$ and -$\underline{C}OCH_3$ of acetate.

Table G.4.2: Comparative ^{13}C-NMR spectral data of the isolated compound EFA and the reported value[71] of stigmasta-5,22,25-trien-3β-yl acetate:

Carbon number	Types of carbon	Chemical shift in ppm	
		Observed δ value for EFA	Reported[71] δ value for stigmasta-5,22,25-trien-3β-yl acetate
1	$-CH_2-$	37.0	37.0
2	$-CH_2-$	27.8	27.8
3	$>CH-OCOCH_3$	74.0	74.0
4	$-CH_2-$	38.1	38.1
5	>C=	139.6	139.7
6	=CH-	122.6	122.6
7	$-CH_2-$	31.9	31.9
8	>CH-	31.9	31.9
9	>CH-	50.1	50.1
10	>C<	36.6	36.6
11	$-CH_2-$	21.0	21.0
12	$-CH_2-$	39.6	39.6
13	>C<	42.2	42.3
14	>CH-	56.8	56.8
15	$-CH_2-$	24.3	24.3
16	$-CH_2-$	28.6	28.7
17	>CH-	55.9	55.9
18	$-CH_3$	12.0	12.0
19	$-CH_3$	19.3	19.3
20	>CH-	40.1	40.2
21	$-CH_3$	21.4	21.4
22	=CH-	137.1	137.2
23	=CH-	130.0	130.1
24	>CH-	52.0	52.0
25	>C=	148.6	148.6
26	$=CH_2$	109.5	109.5
27	$-CH_3$	20.2	20.2
28	$-CH_2-$	25.7	25.7
29	$-CH_3$	12.1	12.1
	$-COCH_3$	20.8	20.8
	$-COCH_3$	170.5	170.5

Some unidentified peaks were observed in ^{1}H- and ^{13}C-NMR spectra. These may be due to trace of impurities and those were not considered in the characterization of **EFA**.

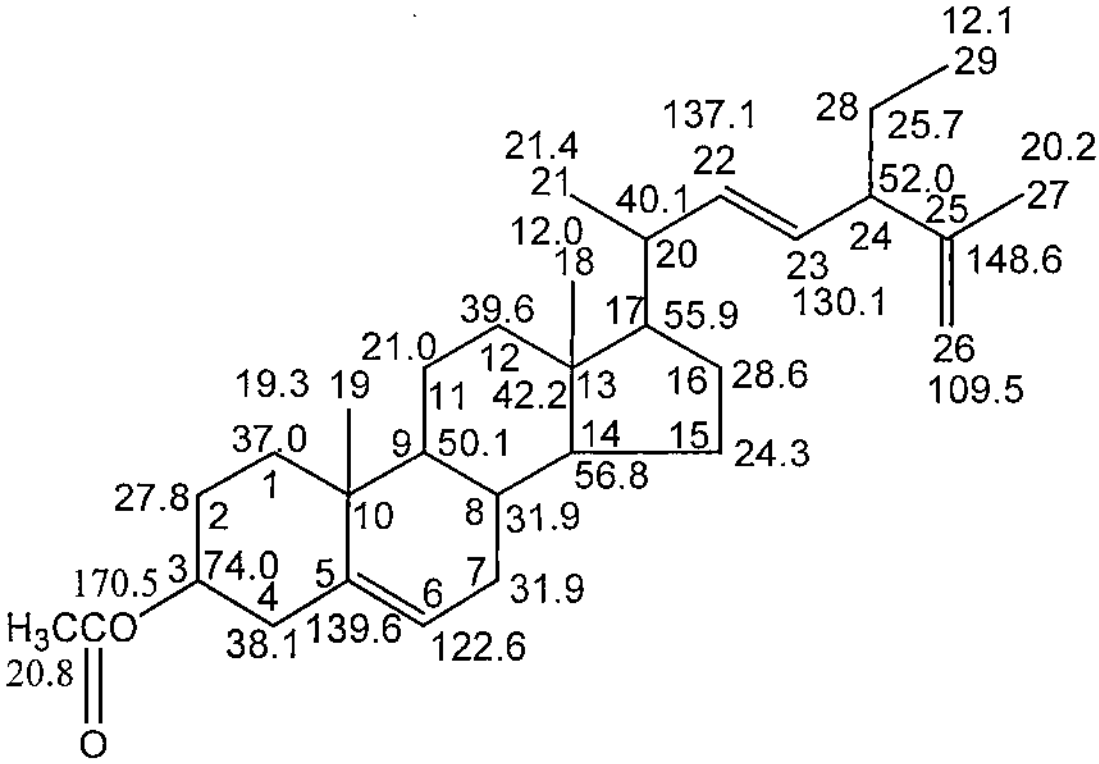


Figure G.9: Structure of the compound EFA showing ^{13}C signals

From the IR, ^{1}H-NMR, and ^{13}C-NMR spectral data and comparing these values with the reported values the compound **EFA** was identified as **stigmasta-5,22,25-trien-3β-yl acetate or (24S)-ethylcholesta-5,22(E),25-triene-3β-yl acetate ($C_{31}H_{48}O_2$).**

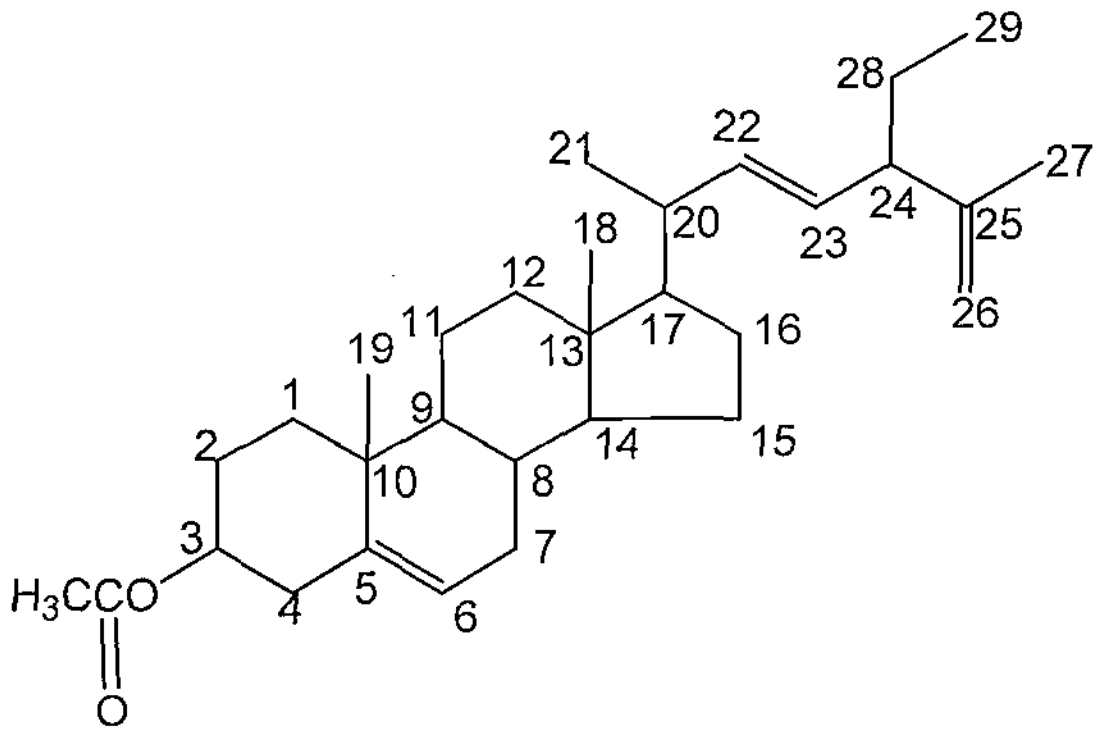


Figure G.10: Structure of EFA as stigmasta-5,22,25-trien-3β-yl acetate

Although the isolation of stigmasta-5,22,25-trien-3β-ol and stigmasta-5,22,25-trien-3β-yl stearate from *Ehydra fluctuans* Lour. have been reported[31] earlier, it appears from literature survey, the isolation of **stigmasta-5,22,25-trien-3β-yl acetate** from *Enhydra fluctuans* Lour. as a natural compound has not been reported prior to this work. Preparation of **stigmasta-5,22,25-trien-3β-yl acetate** by acetylation of stigmasta-5,22,25-trien-3β-ol has been reported. So isolation and identification of natural **stigmasta-5,22,25-trien-3β-yl acetate** from *Enhydra fluctuans* Lour. was reported here for the first time.

G.4.5. Biological importance of the sterols isolated from the plant *Enhydra fluctuans* Lour. :

Consumption of phytosterols has been associated with protection from cancer [72] and cardiovascular disease [73]. In the intestinal tract, phytosterols appear to displace cholesterol from the lipidic micellae, thus reducing the absorption of cholesterol [35,36,74]. Dietary phytosterols are cholesterol-lowering agents that interfere with the intestinal absorption of cholesterol. Their effects on cholesterol biosynthesis in human cells, particularly in the sterol-conversion pathway, have been reported [75]. Sterols containing a double bond at C-22 in the side chain (stigmasterol, brassicasterol and ergosterol) dramatically inhibited the activity of sterol Δ^{24}-reductase, as indicated by the decrease in radioactivity incorporation into cholesterol and the accumulation of its precursors (mainly desmosterol). Phytosterols with the saturated side chain (β-sitosterol and campesterol) were inactive in this regard. Since the plant *Enhydra fluctuans* Lour.contains stigmasterol[31], stigmast-5,22,25-trien-3β-ol[31] (EF_4), stigmasta-5,22,25-trien-3β-yl stearate[31] and stigmast-5,22,25-trien-3β-yl acetate (EFA), a group of Δ^{22}-unsaturated phytosterols, these might act as competitive inhibitor of sterol Δ^{24}-reductase and inhibit cholesterol biosynthesis in mammals.

H. Results and Discussion

(*Spilanthes acmella* Murr.)

H. Results and Discussion (*Spilanthes acmella* Murr.)

H.1 Preliminary investigation of the plant material

H.1.1 Plant material:

A species of the Compositae family, *Spilanthes acmella* Murr., has been investigated in this work. The powder of leaves of the plant was used for this investigation.

H.1.2. Phytochemical screening:

Phytochemical screening for different phytochemicals of *Spilanthes acmella* Murr. was carried out (Section: F.1.2) and some of phytochemicals were estimated (Section: F1.3) and results are given in Table H.1.1 and Table H.1.2.

Table H.1.1: Qualitative analysis of the phytochemicals of *Spilanthes acmella* Murr.:

Alkaloid	Tannin	Saponin	Flavonoid	Steroid	Terpenoid	Cardiac glycoside
+	+	+	+	+	+	--

Table H.1.2: Amount (mg per 100 g of dry powder basis) of crude alkaloid, flavonoid and saponin of *Spilanthes acmella* Murr.

Alkaloids (mg/ 100 g)	**Flavonoids (mg/ 100 g)**	**Saponin (mg/ 100 g)**
9.6	14.3	13.0

H.1.3 Extraction of the plant material :

Fresh leaves of *Spilanthes. acmella* Murr was collected, dried and ground to a coarse powder. The powder sample (*500.0* gm) was extracted with Soxhlet apparatus (Section: F.4) . using petroleum ether (40-60°C) followed by dichloromcthane for about 10 days and hence petroleum ether and dichloromethane extracts were obtained according to the scheme (Scheme-F.4.2).

H.1.4 Isolation and characterization of compounds:

The extractives were separately subjected to different chromatographic techniques to isolate pure compounds. A single compound SA-1 was obtained (Section: F.4.3.5) from petroleum ether extract and other two pure compounds SA-2 and SA-3 were obtained (Section: F.5.1 to F.5.9) from dichloromethane extracts. The isolated pure compounds were characterized by various spectroscopic techniques.

H.2 Characterization of isolated compounds from *Spilanthes acmella* Murr.

H.2.1 Characterization of SA-1 as stigmasterol :

SA-1 was obtained as white crystal from the column fraction of crude petroleum ether extract by elution with 2-3% methanol in CH_2Cl_2. Spraying the developed plate with vanillin-sulfuric acid spray reagent, followed by heating at 110°C for several minutes, gave a purple color. The compound was identified as stigmasterol by analysis ^{1}H-NMR and ^{13}C-NMR data.

H.2.2.IR spectroscopic study :

The IR spectrum (Figure F.25) showed an absorption band at 3450 cm^{-1} due to a hydroxyl group. The bands at 2920, 2850, 1455 and 1365 cm^{-1} were for C-H stretching and bending vibrations. The other bands at 1655 cm^{-1} for C=C stretching and 1040 cm^{-1} were due to C-O stretching vibration.

H.2.3 ^{1}H-NMR spectroscopic analysis :

The ^{1}H-NMR spectra (400 MHz, $CDCl_3$) of SA-1 (Table-H.2.1, Figure F.26, F.27 and F.28) showed multiplets at δ 3.51 and δ 5.35 typical for H-3 and H-6 of a steroidal nucleus.

The olefinic protons H-22 and H-23 appeared as characteristics downfield signal at δ 5.15 and δ 5.03 respectively in the ^{1}H-NMR spectrum. This two signals were observed as double doublet (J = 15.3 and 8.8 Hz and J = 15.3 and 8.6 Hz) which indicated coupling with the neighboring olefinic and methine protons.

The ^{1}H-NMR spectrum displayed two three-proton singlets at δ 1.0 and δ 0.69 indicating presence of to two tertiary methyl groups at C-19 and C-18 respectively.

The two doublets at δ 0.85 (J = 7.6 Hz) and 0.84 (J = 6.9 Hz) integrating for three protons may be due to the two methyl groups at C-26 and C-27. Another three-proton doublet at δ 0.91 (J = 6.4 Hz) could be attributed to the methyl group at C-21. These spectral features are characteristics of a steroidal carbon skeleton of stigmasterol. From the assignment of protons, the structure of SA-1 showing the ^{1}H-NMR signal may be represented as-

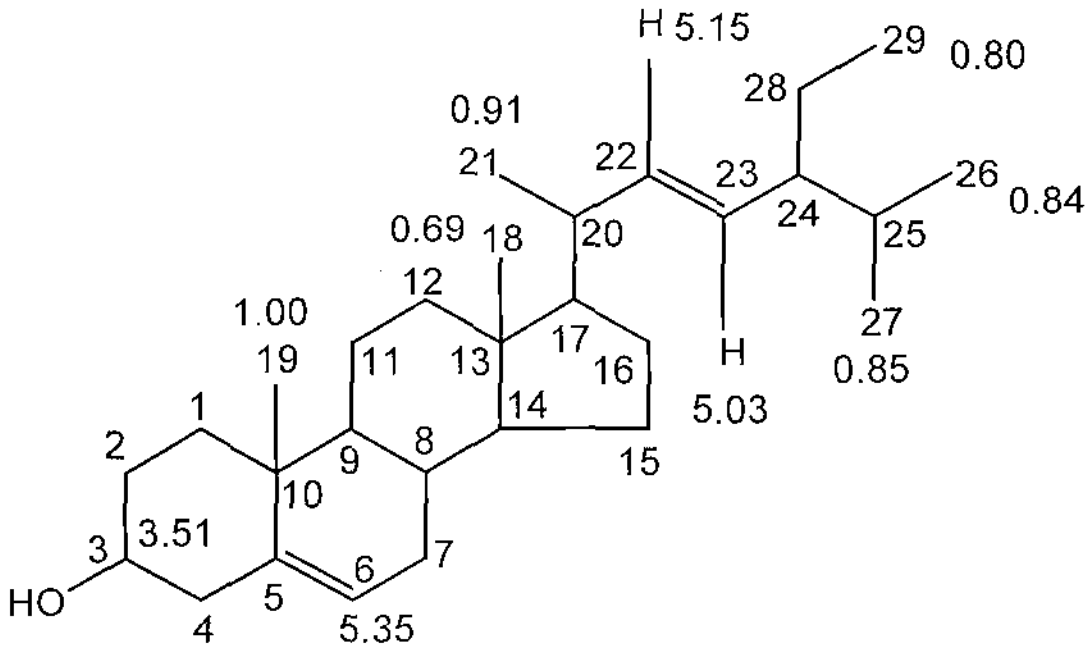


Figure.H.1: Structure of the compound SA-1 showing characteristic ^{1}H signal.

Table H.2.1 Comparative ^{1}H-NMR spectral data of isolated compound SA-1 with the published data[30a] of stigmasterol

Protons	Chemical shift in ppm	
	δ value for SA-1	Published δ value[30a] for stigmasterol
H-3 (1H, m)	3.51	3.52 (m)
H-6 (1H, d)	5.35	5.36 (d, J=5.1 Hz)
H-18 (3H, s)	0.69	0.69 (s)
H-19 (3H, s)	1.00	1.01(s)
H-21 (3H, d)	0.91 (J=6.4 Hz)	1.02 (d, J= 6.4 Hz)
H-22 (1H, dd)	5.15 (J= 15.3, 8.8 Hz)	5.16 (dd, J= 15.0, 8.4 Hz)
H-23 (1H, dd)	5.03 (J= 15.3, 8.6 Hz)	5.02 (dd, J= 15.0, 8.4 Hz)
H-26 (3H, d)	0.85 (J= 7.6 Hz)	0.85 (d, J= 8.0 Hz)
H-27 (3H, d)	0.84 (J= 6.4 Hz)	0.84 (d, J= 5.3 Hz)
H-29 (3H, t)	0.80 (J= 4.2 Hz)	0.80 (t, J= 6.0 Hz)

H.2.4 ^{13}C-NMR spectrum :

The ^{13}C NMR spectrum (Figure F.29, Table-H.2.2) revealed the presence of 29 carbons for a steroid skeleton. The signals at δ 141 (C-5), δ 42.4 (C-13) and δ 36.2 ppm (C-10) were assigned to three quaternary carbons respectively.

The signal at δ 71.8 ppm was assigned for methylene carbon C-3. The signals at δ 138.4 ppm and δ 129.4 ppm were for the C=C carbons of C-22 and C-23 and also the signal at δ 141.0 ppm and δ 121.7 ppm were typical for the C=C carbons of C-5 and C-6.

Table H.2.2 Comparative ^{13}C-NMR spectral data of the isolated compound SA-1 and the reported value[30b] of stigmasterol

Carbon number	Types of carbon	Chemical shift in ppm	
		Observed δ value for SA-1	Reported δ value[30b] for stigmastrerol
1	$-CH_2-$	37.3	37.4
2	$-CH_2-$	31.7	31.7
3	>CH-OH	71.8	71.8
4	$-CH_2-$	42.4	42.4
5	>C=	141.0	140.9
6	=CH-	121.7	121.7
7	$-CH_2-$	31.9	31.9
8	>CH-	31.9	31.9
9	>CH-	50.19	50.3
10	>C<	36.2	36.6
11	$-CH_2-$	21.1	21.1
12	$-CH_2-$		39.8
13	>C<	42.4	42.4
14	$-CH_2-$	56.9	57.0
15	$-CH_2-$	23.2	24.4
16	$-CH_2-$	29.7	28.9
17	-CH-	56.1	56.0
18	$-CH_3$	12.1	12.2
19	$-CH_3$	19.4	19.4
20	>CH-	--	40.5
21	$-CH_3$	21.1	21.1
22	=CH-	138.0	138.4
23	=CH-	128.5	129.4
24	>CH-	--	51.3
25	>CH-	31.9	31.9
26	$-CH_3$	19.0	19.0
27	$-CH_3$	21.3	21.1
28	$-CH_2-$	--	25.4
29	$-CH_3$	--	12.0

The structure of SA-1 showing its ^{13}C-NMR signals may be given as-

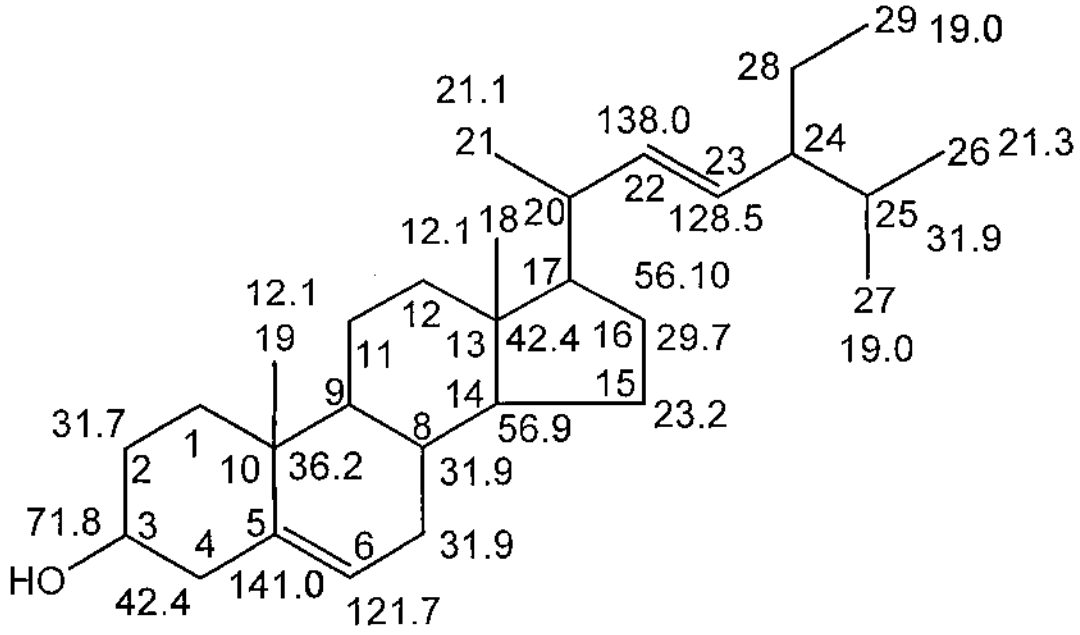


Fig.-H.2: Structure of the compound SA-1 showing ^{13}C signal.

Comparing ^{1}H- and ^{13}C-NMR spectral data of compound SA-1 with those of reported values, it was identified as stigmasterol having the following structure

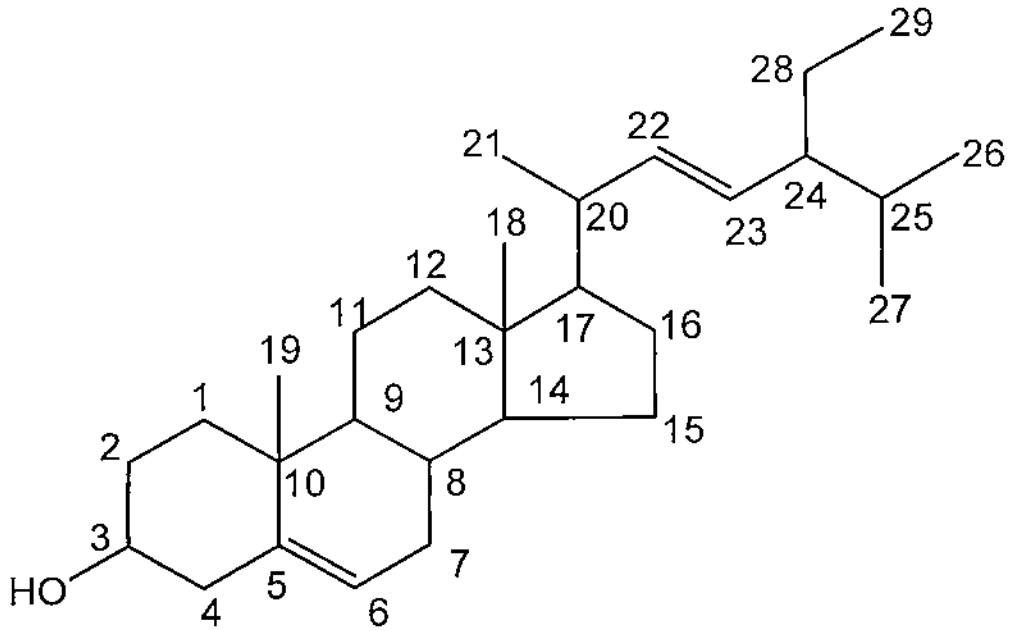


Fig.-H.3: Structure of SA-1 as stigmasterol

H.3.1 Characterization of SA-2 as stigmasterol :

This compound was isolated from dichloromethane extract of *Spilanthes acmella* Murr (Section: F.5.1 to F.5.6). This compound was found to be similar (Section: F.5.9.2.1) to compound SA-1 which was obtained from petroleum ether (40-60°C) extract and was identified as stigmasterol. So, in this investigation stigmasterol was obtained from both the petroleum ether extract and dichloromethane extract of the plant..

H.4.1 Characterization of SA-3 as sitosterone :

Compound SA-3 was isolated as white crystalline powder by sub column from the dichloromethane extract (Section: 5.9). It appeared as a dark quenching spot on the TLC plate under UV light at 254 nm. Spraying the developed plate with vanillin-sulfuric acid spray reagent, followed by heating at 110°C for several minutes, gave a purple color. It was found to be soluble in dichloromethane and chloroform (R_f = 0.45; Toluene : ethyl acetate= 95 :5).

H.4.2 IR spectroscopic study :

The IR spectrum showed (Figure F.30) an absorption band at 3450 cm^{-1} due to a hydroxyl group. The bands at 2920, 2850, 1455 and 1365 cm^{-1} were for C-H stretching and bending vibrations. The band at 1655 cm^{-1} appeared due to >C=O conjugated with C=C stretching and the band at 1040cm^{-1} was due to C-O stretching vibration.

H.4.3 ^{1}H-NMR spectroscopic analysis :

The ^{1}H NMR spectra (400 MHz, $CDCl_3$) of SA-3 (Table-H.4.1, Figure F.31 and F.32) showed one one-proton singlet at δ 5.71.This significant downfield signal of the olefinic proton for SA-3 (δ 5.71) typical for H-4 of a steroidal nucleus containing a ketone group at C-3 position. The ^{1}H-NMR spectrum also displayed two three-proton singlets at δ 1.16 and δ 0.69 was assignable for the methyl group at C-19 and C-18 respectively. Two three-proton doublets at δ 0.84 (J =7.6 Hz) and 0.80 (J = 7.2 Hz) could be assigned to the two methyl groups at C-26 and C-27 respectively and another three-proton doublet at δ 0.90 (J = 6.4 Hz) could be attributable for C-21. These ^{1}H-NMR spectral features are characteristics of steroidal carbon skeleton of sitosterone. The compound was identified as sitosterone by comparing the ^{1}H-NMR data (Table-H.4.1) with those published for the compound[32, 38].

Table H.4.1 Comparative ^{1}H-NMR spectral data of isolated compound SA-3 with the published data[32, 38] of sitosterone

Protons	Chemical shift in ppm	
	δ value for SA-3	Published δ value[32, 38] for sitosterone
H-4 (1H, s)	5.71	5.71(1H,s)
H-18 (3H, s)	0.69	0.70 (3H,s)
H-19 (3H, s)	1.16	1.17 (3H,s)
H-21 (3H, d)	0.90 (J= 6.4 Hz)	0.91(3H,d, J= 6.8 Hz)
H-26 (3H, d)	0.84 (J = 7.6 Hz)	0.81(3H,d, J= 7.6 Hz)
H-27 (3H, d)	0.80 (J = 7.2 Hz)	0.85 (3H,d, J= 7.6 Hz)
H-29 (3H, br.s)	0.79	
Methylene & methine proton	1.24-2.4(s,d,m)	

The ^{13}C-NMR spectral analysis of the compound SA-3 was not possible due to lack of this sample. Therefore, on the basis of the chemical tests and IR and ^{1}H-NMR spectral data the compound **SA-3** was identified tentatively as **sitosterone** and the following structure was proposed.

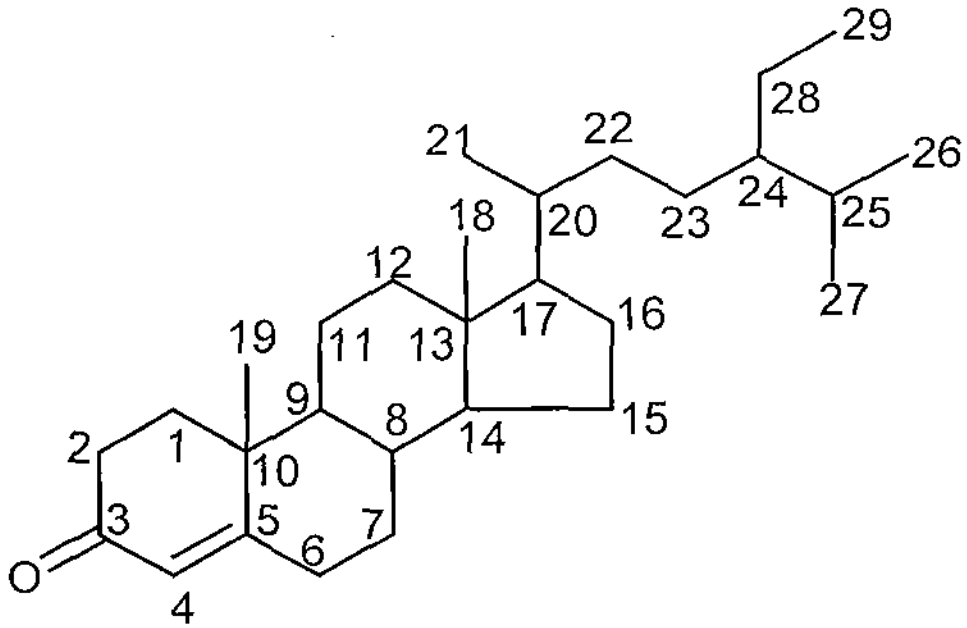


Fig.-H.4: Structure of SA-3

CHAPTER TWO

Fatty acid analysis

2. Fatty acid analysis

2.1. Introduction :

The fatty acid series was so named because some of the higher members, particularly palmitic and stearic acids occur in natural fats[76] Fats are glyceryl esters of fatty acids. Since glycerol is a trihydroxylic alcohol and fatty acids are monobasic, a normal glyceride is a triglyceride and on hydrolysis yields three molecules of fatty acids and a molecule of glycerol.

$$C_3H_5(C_{18}H_{35}O_2)_3 + 3H_2O = C_3H_5(OH)_3 + 3C_{18}H_{36}O_2$$

Stearin Glycerol Stearic acid

The great numbers of the naturally occurring fatty acid belong to a few homologous series. The general formula of the fatty acids is $C_nH_{2n}O_2$ may represent the series to which stearic acid belongs. As, however, their functional group is the carboxyl group, — COOH, they are more conveniently expressed as $C_nH_{2n+1}COOH$, since these show the nature of the functional group. Nearly all the naturally occurring fatty acids have even number of carbon atoms in the molecule. The members of greatest importance are shown in Table 2.1.1.

Table 2.1.1: Some common fatty acids found in plant and animal kingdom

Common name	Carbon atoms	Double Bonds	IUPAC name	Source
Butyric acid	4	0	Butanoic acid	Butter fat
Caproic acid	6	0	Hexanoic acid	Butter fat
Caprylic acid	8	0	Octanoic acid	Coconut oil
Capric acid	10	0	Decanoic acid	Coconut oil
Lauric acid	12	0	Dodecanoic acid	Coconut oil
Myristic acid	14	0	Tetradecanoic acid	Palm kernel oil
Palmitic acid	16	0	Hexadecanoic acid	Palm oil
Palmitoleic acid	16	1	9-hexadecenoic acid	Animal fats
Stearic acid	18	0	Octadecanoic acid	Animal fats
Oleic acid	18	1	9-octadecenoic acid	Olive oil
Vaccenic acid	18	1	11-octadecenoic acid	Butterfat
Linoleic acid	18	2	9,12-octadecadienoic acid	Grape seed oil
Alpha-linolenic acid (ALA)	18	3	9,12,15-octadecatrenoic acid	Flaxseed
Gamma-linolenic acid (GLA)	18	3	6,9,12-octadecatrienoic acid	Borage oil
Arachidic acid	20	0	Eicosanoic acid	Peanut oil, fish oil
Gadoleic acid	20	1	9-eicosanoic acid	Fish oil
Arachidonic acid	20	4	5,8,11,14-eicosatetracnoic acid	Liver fats
EPA	20	5	5,8,11,14,17-eicosapentaenoic acid	Fish oil
Behenic acid	22	0	Docosanoic acid	Rapeseed oil
Erucic acid	22	1	13-docosenoic acid	Rapeseed oil
DHA	22	6	4,7,10,13,16,19-docosahexaenoic acid	Fish oil
Lignoceric acid	24	0	Tetracosanoic acid	Small amounts in most fats

The fatty acids in plants are generally straight-chain compounds, ranging from three to eighteen carbons. Fatty acids containing an even number of carbon atoms are present in substantial amount because they are built up of two carbon atoms at a time, from acetic acid units. Fatty acids may be saturated or unsaturated; the unsaturated acids can also exist in both *cis* and *trans*- forms.

Fatty acids are insoluble in water and are extracted from plant tissues by organic solvents of low polarity like petroleum ether or chloroform. They are generally found in ester linkage but are also found free and in loose molecular

complexes with protein. Although alkaloids, terpenoids, steroids, flavones and their glycosides, phenols, and phenolic acids constitute a major portion of non-carbohydrate materials of a plant, fatty acids are always present in varying amounts in all plant materials.

These fatty materials may influence the handling of the plant tissues as well as any chemical treatment done on it. Therefore, the study of fatty acids, the major constituent of all fatty matters is important.

2.2. Gas Chromatography (GC) :

Chromatography[77] is a physical process of separation in which the components to be separated are distributed between two phases a stationary phase having large surface area (for example a porous solid) and a mobile phase (a gas or liquid) moving in contact with the stationary phase which is generally coated on a porous solid phase. Different forms of chromatography are named according to the physical state of mobile phase employed; further subdivision is done according to the type of interaction between the solute and stationary phase. Now each type of chromatography can be subjected to three forms of developments: Elution, Frontal analysis or Displacement. In elution technique a carrier fluid (eluent) which is relatively inert towards the stationary phase, is kept flowing continuously and components of sample, though mutually resolved, are in mixture with the carrier fluid. Actually it is this technique that is generally employed in gas chromatography (GC) is fundamentally a separation technique but it provides identification of a compound and with due calibration permits quantitative estimation as well. Today it is used by analytical chemists in almost every branch of science as a powerful analytical tool. It finds application in various fields such as gases and pollutants, petroleum products, oils and fats, foods and flavours, drugs, beverages, elemental organic analysis and a number of varied materials.

2.3. The principle advantages of GC :

The principal advantages of GC to an analyst are

i. The technique has strong separation power and even quite complex mixtures can be resolved into constituents.
ii. It is a micro method and only a few mg samples are enough for analysis; sensitivity of the method is very high.
iii. Speed of analysis is quite fast.
iv. Gives good precision and accuracy.
v. It involves relatively simple instrumentation; operation of a gas chromatograph and related calculation do not require highly skilled personnel and thus the technique is very suitable for routine analysis.
vi. The cost of equipment is relatively low and life is generally long.

Thus in large number of instances GC is employed as the only method able to provide the desired results, in other cases it forms better alternative test method.

Any sample that can be vaporized (or the components could assume a vapor pressure of at least few mm of Hg) without thermal decomposition at the operating temperature, could be analyzed by GC. At present due to various reasons, including difficult instrumentation and lack of high temperatures materials, the separating temperature is generally limited to about 450°C. Samples that cannot be vaporized are converted into volatile derivatives (for example, fatty acids converted into methyl esters) and then subjected to GC analysis.

2.4. Principle of GC separations :

When some gas or vapour comes in contact with an absorbent, a certain amount of it gets absorbed on the solid surface; according to the well known laws of

Fraundilich ($x/m = KC^{1/n}$) (x is the mass of the vapour adsorbed in mass 'm' of (dissolved) in the bulk liquid; which follows the partition law of Henry ($x/m=kC$). Now both the sorption phenomena are selective and there are different k-values (distribution co-efficient), in general, for different vapor sorbent pairs; for the same sorbent, different vapors will have different K-values, i.e. varying affinities.

Gas chromatographic separation is accomplished in a tubular column made of glass, metal or Teflon. The column is filled with a sorbent as the stationary phase, adsorbents are packed as such in the form of fine size graded powder whereas liquids are either coated as fine film on the column wall of first coated over an inert porous support such as fine powder and then packed into the column. A gas such as hydrogen, nitrogen, helium etc. serving as mobile phase, flows continuously through the column. It is called the carrier gas and serves to transport the sample components in the column. The sample is introduced as a sharp plug of vapour at the carrier gas entrance end of the column. Different components of the sample are absorbed on the stationary phase to different extents depending upon their distribution co-efficient. But immediately there after, the portion of each component in the gas phase is swept further by the carrier gas and so a fraction of the sorbed amount also desorbs out to maintain the k-value; at the same time, out of the swept amount some portion will go into sorbent at the next point in the column, again to maintain the k-value. This goes on successfully and continuously and as a whole the band for each component move further in the column and having the shape of, more or less Gaussian Distribution (peak shape). Now components having varying affinities for the sorbent (stationary phase) will held up in the latter to different degrees and consequently will move along the column with different speeds; if a column of sufficient length is chosen, these components will emerge out of the column at different intervals. This is how the separation of components is achieved in GC.

2.5. Procedure of Fatty acid analysis[78]:

2.5.1. Analysis of fatty acids:

Petroleum ether extract (2.25 g) of *Enhydra fluctuans* Lour. was dissolved in petroleum ether (50 ml) and extracted with 5% sodium bicarbonate solution (25 ml ×2).The mixture was taken in a seperatory funnel and shaken vigorously and allowed to stand for overnight. Two layers were obtained. The lower layer (aqueous) was separated and taken for the analysis of free fatty acid (FFA). The upper layer was separated and taken for the analysis of bound fatty acid (BFA). Similar procedure was followed for *Spilanthes acmella* Murr.

2.5.2. Isolation of FFA:

The lower part was acidified (pH 2.5) by 2M sulphuric acid. The mixture was then extracted with petroleum ether (b. p 40-60°C) (25 ml x 3). The pet ether fraction was dried over anhydrous sodium sulphate, filtered and the filtrate was evaporated to dryness.

Now the saponified materials obtained from the petroleum ether (b. p 40-60°C) extract was taken in a pear shaped flask and 2 ml of borontrifluoride-methanol (BF_3-MeOH) complex was added and the mixture was refluxed in a boiling water bath for 6 to 10 min. The mixture was then evaporated in a rotary vacuum evaporator to dryness and transferred in a small separatory funnel containing a little water (6 ml). The mixture was shaken vigorously and then extracted with hexane. The aqueous layer was discarded. The hexane part containing the methyl esters of fatty acids was made free from water by adding anhydrous sodium sulphate. The solution was filtered and the filtrate was concentrated for the analysis of free fatty acids by GLC (Shimadzu 9A, Column-BP-50, Detector-FID, 170°C-1 min/4°C-270°C-30 min).The results are given in Table 2.6.1.

2.5.3. Isolation of BFA:

The upper part was taken in a pear shaped flask; methanolic sodium hydroxide (0.5M, 10 ml) was added to it and shaken well. The mixture was refluxed for 30 min in a boiling water bath. Then the mixture was evaporated to dryness by means of a rotary vacuum evaporator. A little water was added to the mixture and transferred to a seperatory funnel to settle down. The non-saponified materials were separated from the saponified portion (aqueous layer) by extraction with hexane.

The aqueous layer containing fatty acids (as salts) was acidified by adding sulphuric acid and to pH 2.5. The mixture was extracted with hexane. The hexane part was taken in a conical flask and made from water by adding anhydrous sodium sulphate and then filtered. The filtrate contained saponified materials.

Now the saponified materials obtained from the pet ether extract was taken in a pear shaped flask and 2 ml of borontrifluoride-methanol (BF_3-MeOH) complex was added and the mixture was refluxed on a boiling water bath for 6 to 10 mins. The mixture was then evaporated in a rotary vacuum evaporator to dryness and transferred in a small separatory funnel containing a little water (6 ml). The mixture was shaken vigorously and then extracted with hexane. The aqueous layer was discarded. The hexane part containing the methyl esters of fatty acids was made free from water by adding anhydrous sodium sulphate. The solution was filtered and the filtrate was concentratcd for the analysis of bound fatty acids by GLC (Shimadzu 9A, Column-BP-50, Detector-FID, 170°C-1 min/4°C-270°C-30 min). The results are given in Table-2.6.2.

2.6. Results:

Table 2.6.1: Relative percentages of free fatty acids and bound fatty acids in petroleum ether extract of *Enhydra fluctuans* Lour.:

	Free fatty acid	Bound fatty acid
Fatty acid	**Relative percentage (%)**	**Relative percentage (%)**
Caprylic	1.39	-
Palmitic acid	49.50	38.49
Stearic acid	14.78	17.74
Arachidic acid	9.87	13.13
Behinic	15.74	19.94
Lignoceric acid	8.72	10.70

Table 2.6.2: Relative percentages of free fatty acids and bound fatty acids in petroleum ether extract of *Spilanthes acmella* Murr :

	Free fatty acid	Bound fatty acid
Fatty acid	**Relative percentage (%)**	**Relative percentage (%)**
Laurie acid	4.13	-
Palmitic acid	46.54	47.93
Oleic acid	18.87	10.28
Stearic acid	12.79	13.32
Arachidic acid	4.13	12.48
Behinic acid	7.44	9.39
Lignoceric acid	6.11	6.59

2.7. Discussion:

Fat is important for biological systems and some fats are needed to eat in daily diet. Dietary fats are classified by their structure. Different types of fats react differently in the metabolic processes. Saturated fats (found mostly in animals) increase blood cholesterol, which is a risk factor in coronary heart disease. Unsaturated fats (found mostly in plant and marine foods) tend to lower blood cholesterol. From Table-2.6.1 and Table-2.6.2, it appears that palmitic is the highest in both the plant *Enhydra fluctuans* Lour and *Spilanthes acmella* Murr., but it is also evident that the proportion of unsaturated fatty acids as

both free and bound fatty acids are quite significant. This indicates that the oils of these plants may be important for edible purposes and hence these plants may play important role in our metabolic system.

CHAPTER THREE

Ascorbic acid analysis

3. Ascorbic acid analysis

3.1 Introduction:

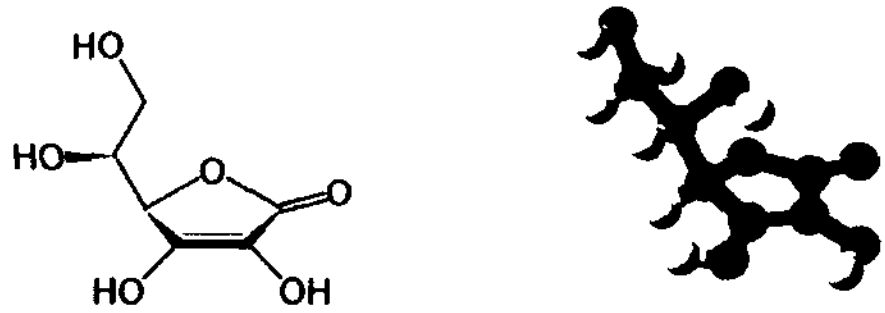


Figure 3.1: Vitamin C

Vitamins[79] are defined as an organic compound that are required for normal growth and maintenance of health and must be supplied in the diet in small amounts since as a rule they are not synthesized in the body. Deficiency causes specific diseases, which are prevented only by restoring the vitamins to the diet. There are two types of vitamins, lipid-soluble vitamins (vitamin A, vitamin D, vitamin E, vitamin K) and water-soluble vitamins (vitamin C, vitamin B_1, vitamin B_2, vitamin B_6, vitamin B_{12}, vitamin H).

Vitamin C (Ascorbic acid) is one of the important vitamins for human beings. Almost[80] 90% of dietary vitamin C comes from foods of plant origin. Vitamin C can be found in fruits (especially citrus) and vegetables, including green and red peppers, tomatoes, potatoes, and green, leafy varieties (e.g. spinach and collard greens).

Vitamin C (Ascorbic acid) has many important functions[80] such as it acts as a reducing agent in a number of hydroxylation reactions in the body such as proline 4-hydroxylase, which is essential for collagen synthesis. Vitamin C is a water-soluble antioxidant and can quench both reactive oxygen species and

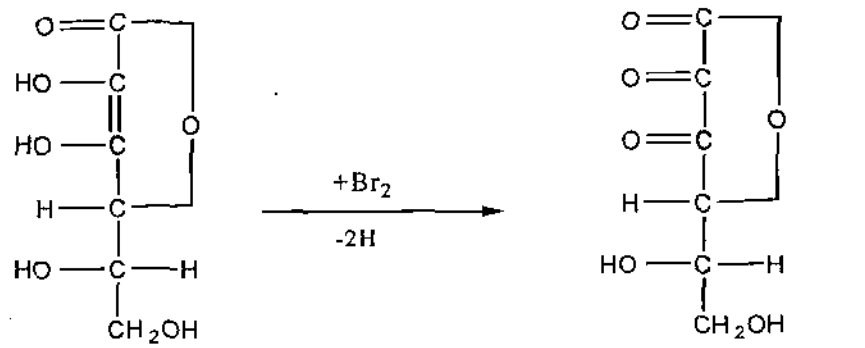
reactive nitrogen species. This antioxidant activity diminishes lipid peroxidation, oxidative DNA damage and oxidative protein damage. Oxidation of low density lipoprotein (LDL) has been suggested to be a factor causing atherosclerosis. Vitamin C prevents LDL from being oxidized. Vitamin C increases high density lipoprotein (HDL) cholesterol, probably resulting in decreased risk of atherosclerosis. Vitamin C can prevent the formation of carcinogens as nitrosamines in foods and in the gastrointestinal tract. Vitamin C is required for the enzyme reaction that converts tryptophan to 5-hydroxytryptophan, a precursor of neurotransmitter serotonin. Vitamin C has many biological activities in addition to antioxidant activity (antihypertensive antiviral).

Vitamin C deficiency[79] causes scurvy include skin changes, fragility of blood capillaries, gum decay, tooth loss, gangrene and bone fracture, many of which can be attributed to deficient collagen synthesis.

Recommended dietary allowance of adult male is 90 mg/day and adult female is 75 mg/day.

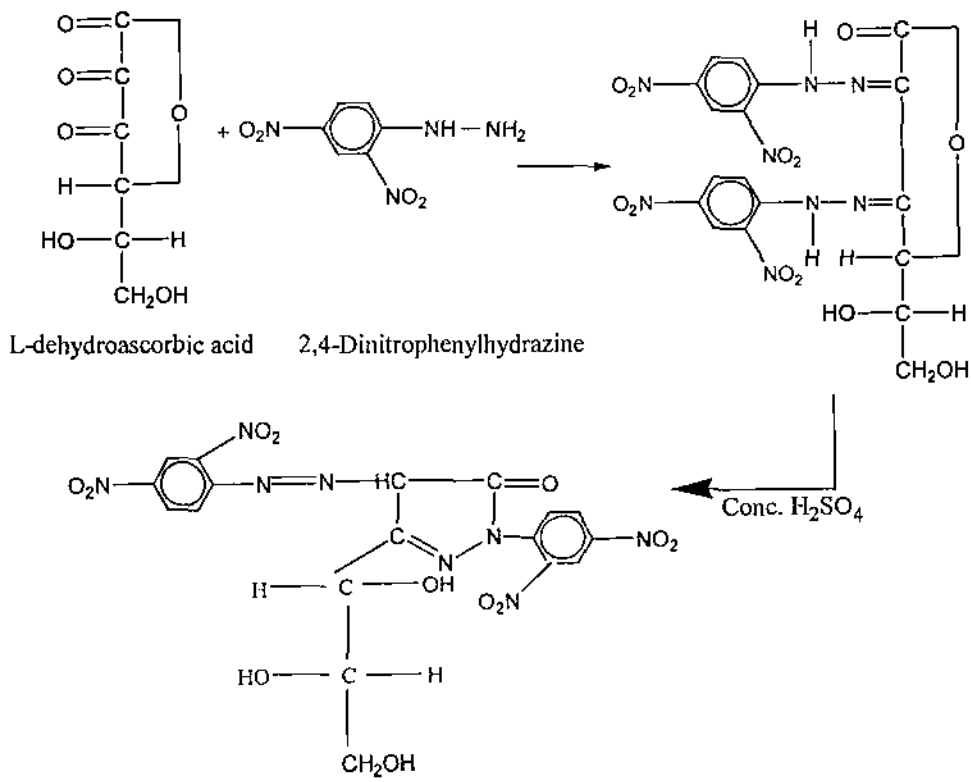
3.2 Principle:

The 2,4-dinitrophenylhydrazine method (DNPH)[81] is a well-established method for simultaneous determination of the ascorbic acid (vitamin C) content in food samples. This method employs the coupling reaction of 2,4-dinitrophenylhydrazine dye with vitamin C, oxidized earlier with bromine water, followed by spectrophotometric determination. During the present investigation this method was employed.



L-ascorbic acid L-dehydroascorbic acid

Figure 3.2: Conversion of ascorbic to dehydroascorbic acid.



L-dehydroascorbic acid 2,4-Dinitrophenylhydrazine

Figure 3.3: Reaction of L-dehydroascorbic acid with DNPH and H_2SO_4

3.3 Experimental:

5% *meta*-Phosphoric acid - 10% Acetic acid solution:

50 g of solid *meta*-phosphoric acid (E. Merck) was dissolved in a mixture of 40 ml glacial acetic acid (BDH) and 450 ml of distilled water in a 500 ml volumetric flask.

10% Thiourea solution:

1 g of thiourea (BDH) was dissolved in 9 ml of distilled water.

Bromine water solution:

1.0 ml liquid bromine was added to 100 ml water to make a homogenous solution.

2,4-dinitrophenylhydrazine solution:

2 g of 2, 4-dinitrophenylhydrazine (BDH) was dissolved in 80 ml of distilled water and 14.67 ml of concentrated sulphuric acid (BDH, specific gravity 1.84). Then the solution was filtered and stored.

Standard vitamin C (ascorbic acid) solution:

50 mg standard crystalline ascorbic acid (BDH) was dissolved in 100 ml 5% *meta*-phosphoric acid - 10% acetic acid solution.

3.4 Sample preparation:

1.600 g of sample (fresh leaves of *Enhydra fluctuans* Lour.) was blended and was homogenized with about 25 ml of 5% *meta*-phosphoric acid-10% acetic acid solution. It was quantitatively transferred to a 50 ml volumetric flask was shaken gently until a homogeneous dispersion was obtained. Then it was diluted up to the mark with 5% *meta*-phosphoric acid-10% acetic acid solution.

The solution was then filtered and the clear filtrate was collected for the determination of vitamin C in the sample.

Table 3.1.1: Preparation of the standard, blank and sample solution.

Test tube	Standard blank and sample	Standard Ascorbic Acid (ml)	Sample (ml)	Distilled H_2O (ml)	Br_2 solution (drops)	10% Thiourea (drops)	DNPH (ml)
1	Standard 1 (5 ppm)	4	--	--	3 to 4	2 to 3	1
2	Standard 2 (10 ppm)	4	--	--	3 to 4	2 to 3	1
3	Standard 3 (15 ppm)	4	--	--	3 to 4	2 to 3	1
4	Standard 4 (20 ppm)	4	--	-- .	3 to 4	2 to 3	1
5	Standard 5 (25 ppm)	4	--	--	3 to 4	2 to 3	1
6	Blank	0	--	4	3 to 4	2 to 3	1
7	Sample	--	4		3 to 4	2 to 3	1

3.5 Estimation of Vitamin C:

Few drops (3 to 4) of bromine water were added to 4 ml of the filtered sample solution until the solution became coloured (to confirm the completion of the oxidation of ascorbic acid to dehydroascorbic acid).Then 2 to 3 drops of thiourea solution was added to it to remove the excess bromine and to get a clear solution.

The standard solution of ascorbic acid (2.5 ppm, 5 ppm, 10 ppm, 20 ppm and 40 ppm) were prepared from 500 ppm stock solution of ascorbic acid by proper dilution. Then 1 ml of 2,4-dinitrophenylhydrazine was mixed thoroughly with

all standards and also with the oxidized ascorbic acid. For completion of the reaction all the standards and the samples were kept at 37°C for 3 hours in a water bath (thermostatic). After the incubation, the solutions were cooled in an ice bath and were treated with 5 ml of 85% H_2SO_4 with constant stirring and a colored solution was obtained. The absorbances of these solutions were measured at 522 nm against a reference solution (prepared identically without ascorbic acid solution). For obtaining a calibration curve the absorbance of the standards were plotted against their corresponding concentrations. The concentrations of the sample solution as well as the content of vitamin C of the sample were calculated from the calibration curve for the corresponding absorbance of the sample (converted to coloured compound).

Tabie 3.1.2 : Standard calibration table of Vitamin C (Ascorbic acid)

Sl. No	Ascorbic acid	Br_2–H_2O (Drops)	Thiourea (Drops)	2,4-DNP (ml)	85% H_2SO_4 (ml)	Absorbance (at 510 nm)
1	Std -1 (2.5 ppm)	2	1	1	5	0.112
2	Std- 2 (5 ppm)	2	1	1	5	0.229
3	Std-3 (10 ppm)	2	1	1	5	0.453
4	Std- 4 (20 ppm)	2	1	1	5	0.902
5	Std-5 (40 ppm)	2	1	1	5	1.742

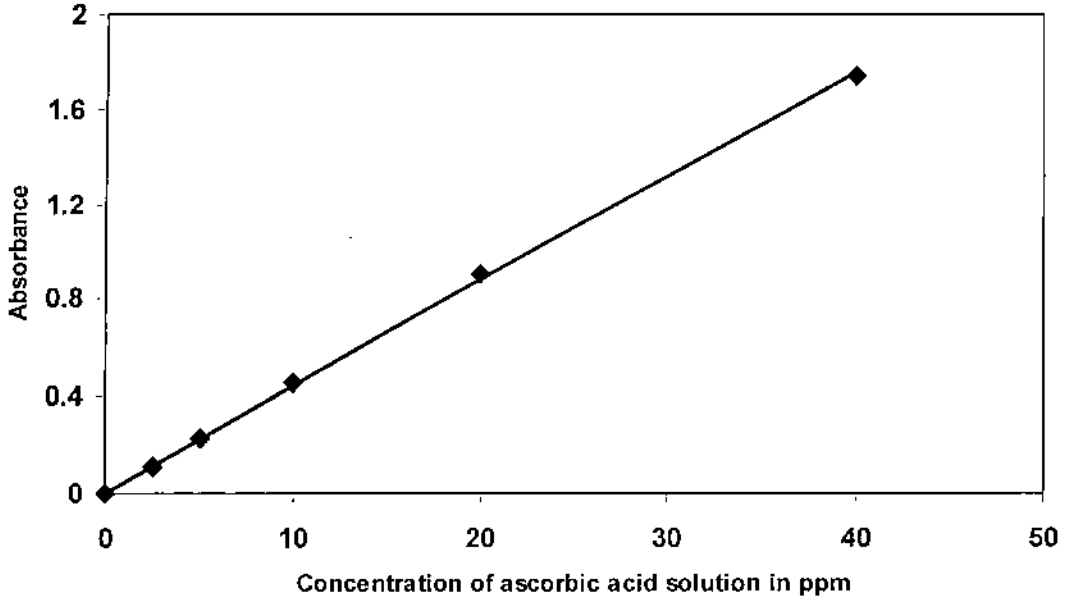


Figure 3.4: Plot of absorbance of the standards against concentration of ascorbic acid solution.

Three samples of *Enhydra fluctuans* Lour. had been taken for the determination of ascorbic acid (Vitamin C) content. Average value of absorbance is presented in Table-3.1. From the calibration curve the following results are obtained.

Table 3.1.3 : Absorbance data for *Enhydra fluctuans* Lour.

Serial No.	Absorbance (at 510 nm)	Average absorbance
1	0.324	0.323
2	0.322	
3	0.323	

3.6 Results :

Average value of the absorbances for *Enhydra fluctuans* Lour. was plotted on the calibration curve for standard ascobic acid (Fig.-3.4). The concentration of ascorbic acid in the sample of *Enhydra fluctuans* Lour. was calculated. From the final calculation, the estimated value of ascorbic acid (Vitamin C) in *Enhydra fluctuans* Lour. was found to be 31.64 mg in100 g of fresh leaves.

3.7. Discussion :

Ascorbic acid of *Enhydra fluctuans* Lour.was extracted with meta phosphoric acid and it was estimated following the standard procedure (section 3.3, 3.4, 3.5). A standard calibration curve (Table 3.1.2 and Fig.-3.4) was prepared, and the amount of ascorbic acid in the sample was calculated from the calibration curve (Fig.-3.4) using the formula $A = \varepsilon cl$ (where A= absorbance, c= concentration and l = length of the cell and ε= absorption co-efficient). The concentration of ascorbic acid was found to be 31.64 mg in 100 g of fresh *Enhydra fluctuans* Lour. leaves. The result showed that the leaves of *Enhydra fluctuans* Lour. is a good source of ascorbic acid (vitamin C). About 200 g of these leaves might fulfill our daily requirement of ascorbic acid. In term of nutritional status, these leaves are beneficial for our health.

CHAPTER FOUR

Ash and iron content analysis

4.A Ash content analysis

4.A.1 Introduction:

The "*ash content*" is a measure of the total amount of minerals present within a food, Ash is the inorganic residue remaining after the water and organic matter have been removed by heating in the presence of oxidizing agents, which provides a measure of the total amount of minerals within a food.

4.A.2 Procedure :

A weighed quantity of sample was taken in a crucible. The contents were heated carefully to the ignition point and allowed to burn spontaneously. When the burning was completed, the crucible heated at a dull-red heat in a muffle-furnace at 600°C for three hours. The crucible was then cooled and weighed with accuracy[82] until a constant weight is obtained. The results are calculated on dry powder basis and are given in Table 4.3.1.

4.A.3 Results :

The results of the determination of the amount of ash content of *Spilanthes acmella* Murr. and *Enhydra fluctuans* Lour. have been calculated and given in the following Table 4.3.1.

Table 4.3.1.The ash content of *Spilanthes acmella* Murr and *Enhydra fluctuans* Lour:

Sl. no.	Name of the samples	Weight of empty crucible (g)	Weight of crucible + sample (g)	Weight of sample M_1 (g)	Weight of Crucible + Ash (g)	Weight of Ash M_2 (g)	Percentage (%)
1.	*Enhydra fluctuans*	27.8431	30.0278	2.1847	28.1693	0.3262	14.93
2.	*Spilanthes acmella*	27.8431	29.8567	2.0136	28.1966	0.3535	16.55

4.A.4. Discussion :

The amount of ash contents of the two plants *Spilanthes acmella* Murr. and *Enhydra fluctuans* Lour. were about 16.55 and 14.93 %. The results show that the ash content of *Spilanthes acmella* Murr is higher than that of *Enhydra fluctuans* Lour (Table 4.3.1.).

4.B. Iron content analysis

4.B.1 Introduction:

Iron is a trace element which is needed by the body for the formation of blood. The human body normally contains 3-4 g of iron, more than half of which is in the form of hemoglobin, the red pigment in blood. Hemoglobin transports oxygen from the lungs to the tissues. Iron is a constituent of a number of enzymes.

Iron deficiency is believed to be fairly common in the general population. Symptoms of iron deficiency anemia include tiredness and breathlessness especially on physical exertion, giddiness, palpitations, headache and poor concentration.

Iron Requirements
The US Recommended Dietary Allowances-
Males
9 to 13 years: 8 mg/day; 14 to 18 years: 11 mg/day; 19 and older: 8 mg/day.
Females
9 to 13 years: 8 mg/day; 14 to 18 years: 15 mg/day; 19 to 50 years: 18 mg/day; 51 and older: 8 mg/day.

Good plant sources of iron include dried fruits, whole grains (including whole meal bread), nuts, green leafy vegetables, seeds and pulses. Other foods rich in iron but which are usually eaten in smaller amounts includes soya flour, parsley, watercress, black molasses and edible seaweeds. The use of ironware when cooking foods also contributes to dietary intake.

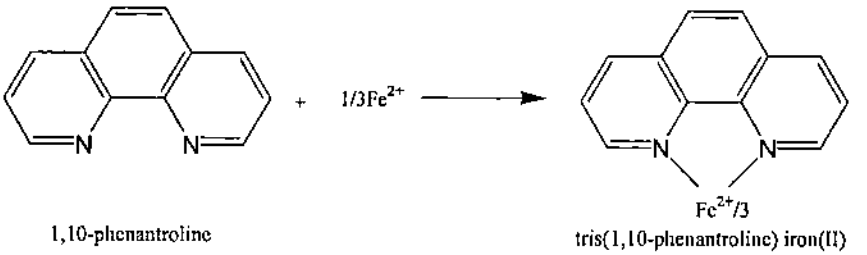
4.B.2 Principle :

Iron in the residue (ash) remaining after the destruction of the organic matter of leaves, steams and roots is dissolved in H_2SO_4. This solution is transferred to a volumetric flask and diluted with distilled water.

The concentration of iron in that solution is determined spectrophotometrically as the color complex which is formed when iron (II) reacts with 1,10-phenanthroline. The absorbance of the colored complex of iron(II) with 1,10-phenanthroline, $Fe(C_{12}H_8N_2)_3^{2+}$, is measured with a spectrophotometer[83]. Hydroxylamine (as the hydrochloride salt to increase solubility) is added to reduce any Fe^{3+} to Fe^{2+} and to maintain it in that state.

Equation:

$$4Fe^{3+} + 2NH_2OH \longrightarrow 4Fe^{2+} + N_2O + 4H^+ + H_2O$$



1,10-phenantroline

tris(1,10-phenantroline) iron(II)

4.B.3 Procedure

About 2.1847 g and 2.0136 g dry powders (leaves and steams) of *Enhydra fluctuans* Lour and *Spilanthes acmella* Murr., respectively, were transferred to in each of the crucible. The crucibles with materials were put on the burner and the materials were burnt into ash. Each of the ashes of *Enhydra fluctuans* Lour and *Spilanthes acmella* Murr were dissolved separately, in conc. H_2SO_4 and each were transferred into 100 ml volumetric flask. Distilled water was added

to dilute the solution to 100 ml. These sample solutions were used to determine the concentration of iron.

Standard iron(II) solution (10 ppm) was prepared using ferrous ammonium sulphate, $Fe(NH_4)_2(SO_4)_2.6H_2O$. Into a series of 100 ml volumetric flasks, 2.5, 5.0, 10.0, 20.0 and 40.0 ml of the standard iron solution was added. Into another 100-ml volumetric flask 50 ml distilled water was placed for a blank. The unknown sample was furnished in another 100 ml volumetric flask. To each of the flasks (including the unknown) 1.0 ml of the hydroxylammonium chloride solution (10% aq. solution) and 5.0 ml of the 1,10-phenanthroline solution (0.1% aq. solution) was added. Each solution was buffered by the addition of 8.0 ml of the sodium acetate solution (10% aq. solution) to produce the red color of ferrous 1,10-phenanthroline (the iron(II)-phenanthroline complex formed at p^H 2 to 9). The sodium acetate neutralizes the acid present and adjusts the p^H to a value at which the complex formed. At least 15 minutes was allowed after adding the reagents before making absorbance measurements so that the color of the complex was fully developed [84,85].

Each solution was diluted to exactly 100 ml. The standards were corresponding to 0.25, 0.5, 1.0, 2.0 and 4.0 ppm iron, respectively. The blank solution was used as the reference solution. The absorbance against the wave length was plotted and the wavelength of absorption maximum was selected (510 nm). A data calibration curve was prepared by measuring the absorbance of each of the standard solutions at the wavelength of maximum absorbance. Similarly the absorbance of the sample solution was measured. The absorbance of different standards was plotted against concentration in ppm (Figure 4.B). From this plot using the absorbance of the sample solution, the final concentration of iron(II) in the sample solution was determined and the amount of iron in sample was reported.

Table-4.B.1: The absorbance of each of the standard solutions at the wavelength of the maximum absorbance (at 510 nm).

No. of standard iron(II) solution	Concentration of standard iron(II) solution (ppm)	Absorbance
Strandard-1	0.25	0.054
Strandard-2	0.50	0.109
Strandard-3	1.00	0.212
Strandard-4	2.00	0.421
Strandard-5	4.00	0.845
Stabdard-6	Blank	0.000

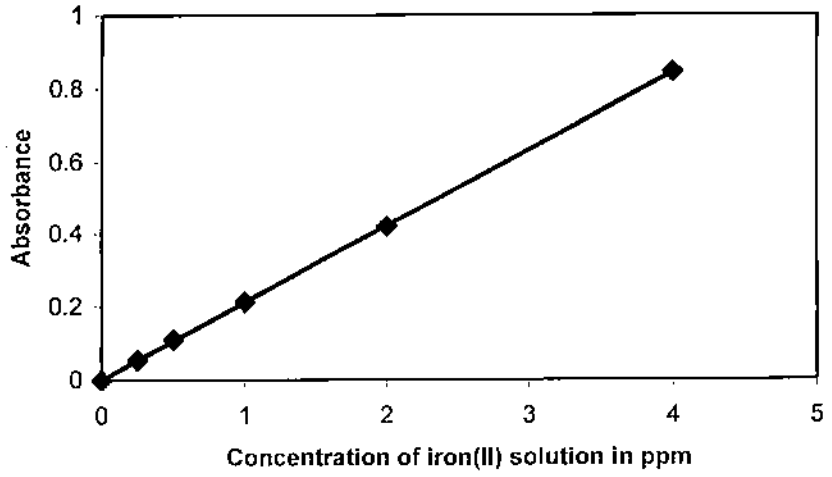


Fig.-4.B.1: Calibration curve: Plot of absorbance against concentration of standard iron (II) solution

Table4.B.2: The concentrations of iron in the dried leaves and stems.

Sample-1(*Enhydra fluctuans* Lour), the weight taken = 2.1847 g

Sample-2(*Spilanthes acmella* Murr), the weight taken = 2.1360 g

Sample No.	Absorbance at 510 nm (Average value)	Concentration of samples (ppm)	Amount of iron (mg) in 100 ml solution	The amount of iron (mg/100g)
Sample-1	0.032	0.1524	0.0152	0.6976
Sample-2	0.063	0.2524	0.0252	1.1816

4.B.4 Results:

Sample-1 (*Enhydra fluctuans* Lour) contained 0.6976 mg iron/100 g dry powder.

Sample-2 (*Spilanthes acmella* Murr) contained 1.1816 mg iron/100 g dry powder.

4.B.5. Discussion :

Both the plants *Enhydra fluctuans* Lour and *Spilanthes acmella* Murr contain a considerable amount of iron which may be the sources of dietary iron. It is reported (Section 3.6) that these plants also have a good amount of ascorbic acid. Absorption of iron by our body is enhanced when plant sources of iron are taken as food containing vitamin C. The combined effect of iron and ascorbic acid plays an important role in our metabolic system.

CHAPTER FIVE

Antimicrobial Activity

5. Antimicrobial Activity

5. Introduction:

Human diseases of bacterial origin are varied and many. In the humid tropic land of Bangladesh, Cholera, typhoid, dysentery, septicaemia and diphtheria are only a few of the killer diseases reigning during the last quarter of the 20^{th} century. Plants and plant material have been tried to relieve human ailments even from the prehistoric era. Still there is a desperate need for the study on the antibacterial activities of plants to control the common diseases.

5.1 Herbs as antimicrobial agents :

Anti-microbial activity of herb extracts was evaluated with antibiotic susceptible and resistant microorganisms. Many herbs have been used because of their antimicrobial trait, which are due to compounds synthesized in the secondary metabolism of herb. Their active substances, for example, phenolic compounds that are part of essential oils.

5.2 .Materials :

The herbs were collected and separated from unwanted materials. The herbs were washed with water to remove dust, mud and then dried at 50°C overnight. Dried herbs were then ground to powder in a grinding machine.

Extraction: Dry powders of the herbs were extracted successively with chloroform, ethyl acetate, methanol and water.

5.2.1 Extraction of the components soluble in chloroform :

The powder (10 g) was taken in a 150 ml conical flask. Chloroform (40 ml) was added to the powder and was kept at 25°C for 12 hours. The suspension was filtered successively through a piece of sterile cotton fabric and a filter paper (Whatman No. 41). The filtrate was then evaporated by vacuum dryer at 40°C overnight to get the chloroform extract.

5.2.2 Extraction of the components soluble in ethyl acetate :

The solid residue left after chloroform extract was dried at 40°C overnight to remove residual chloroform. The solid residual powder was added to ethyl acetate (40ml) and kept at 25°C for 12 hours, and ethyl acetate extract was recovered by the same procedure like chloroform extract.

5.2.3 Extraction of the components soluble in methanol and water :

The solid residue left after ethyl acetate extract was dried at 40°C overnight to remove residual ethyl acetate. The solid residual powder was added to 75% methanol (40 ml) and kept at 25°C for 12 hours. The suspension was separated successively through centrifugation at 12000 rpm, for 5 minutes. The filtrate was then dried overnight in a beaker at 50°C to get the (75%) methanol extract. The solid residue was dried at 40°C overnight to remove residual methanol (75%). The filtrate was then evaporated by vacuum dryer at 40°C for 6 hours to get the methanol and water extract. Then 40ml distilled water was added to the powder containing methanol and water extracts and suspension was collected. Then centrifugation was done again at 12000 rpm for 5 minutes, filtrate containing water soluble compounds was collected, dried in vacuum dryer at 40°C for 6 hours and finally the powder containing water soluble part was obtained as the water extract. The solid residue was dried in a vacuum dryer,

and methanol extract was collected. The chloroform, ethyl acetate, methanol and water extracts were taken for the analysis of their antibacterial activity.

5.3 Assay of antimicrobial activity :[86]

5.3.1. Antibacterial activity of chloroform, ethyl acetate and methanol extracts:

The chloroform, ethyl acetate and methanol extracts were tested for antibacterial activity by customized Kirby-Bauer disc diffusion. Paper discs were soaked with chloroform (150 µL) extract at concentration (50 mg/mL). Paper discs were also soaked with ethyl acetate (150 µL) and methanol extract separately at concentration (50 mg/mL). 18 hours old bacterial cultures of interest were prepared in Nutrient Broth. Mueller-Hinton agar plates were seeded with the suspensions by sterile cotton buds to yield lawns of the bacteria. The discs were applied on the surface of the plates aseptically using sterile forceps. The discs were pressed gently against the agar by the blunt end of the forceps. The plates were put in incubation in inverted position after 30 minutes. The plates were examined for zone of inhibition after 24 hours incubation at 37°C.

5.4 Antibacterial activity of water extract :

The antibacterial activity of water extract was assessed by Agar-diffusion method. Solution of water soluble extract was prepared in sterile distilled water at concentration of 50 mg/mL.

18 hours old bacterial cultures of interest were prepared in nutrient Broth. Mueller-Hinton Agar plates were seeded with suspensions by cotton buds to yield lawns of bacteria. Wells were made in the agar plates aseptically by sterile borer. Solutions of the extracts in water were dispensed into the wells at 60µL/well. The plates were put in incubation after 30 minutes. The plates were

examined for zone of inhibition after 24 hours incubation at 37°C.The zone diameters were examined under magnifying glass and were measured by a millimeter scale. In every case of positive and negative controls were used. Negative control contained no water extract.

5.5 Observation and Results :

5.5.1 Preparation of different solvent extracts :

The dried powder of *Enhydra fluctuans* Lour and *Spilanthes acmella* Murr plant was extracted with chloroform, ethyl acetate, methanol and water serially to yield crude organic compounds. The extracts were then dried in a rotary vacuum evaporator at below 40°C. The different extracts obtained from different herbs were given in

Table-5.1.1 : Yields of different herb extracts in different solvents :

Sample	Solvent	Yield dry extract
Enhydra fluctuans Lour.	Chloroform	127mg
	Ethyl acetate	362 mg
	Methanol	267 mg
	Water	345 mg
Spilanthes acmella Murr.	Chloroform	245 mg
	Ethyl acetate	189 mg
	Methanol	343 mg
	Water	375 mg

5.5.2. Testing the different extracts of different herbs for antimicrobial activity against pathogen of interest:

The crude chloroform, ethyl acetate, methanol and water extract of different herbs were tested for antibacterial activity against 18 hours old nutrient broth culture of different pathogenic bacteria lawned on Mueller-Hinton agar plates. The plates were observed for zones of inhibition after 24 hours incubation at 37°C.

5.5.3 Test with Chloroform, ethyl acetate and methanol extract :

Each of different herb extracts (150 μL) at concentration (50 mg/ml) was impregnated into paper disc and dried at 40°C. The discs were then applied on Mueller-Hinton agar plates which lawned by different bacteria. Mueller-Hinton agar plate was incubated at 37°C. Formation of clear zones was observed.

5.5.4 Test with water extract :

The water extracts were tested on different bacteria by agar diffusion method by cutting wells on MHA plates and incubated overnight at 37°C. Clear zones were observed.

5.5.5 Antibacterial activity of different extracts of *Enhydra fluctuans* Lour

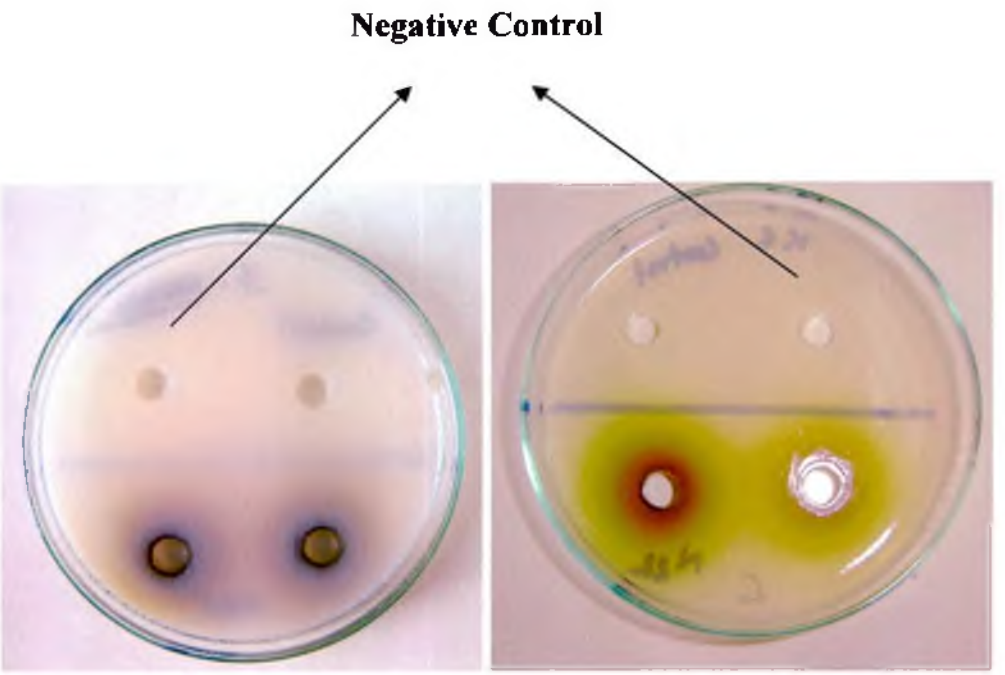
Antibacterial activity of different extracts of *Enhydra fluctuans* Lour on different bacteria was studied (in the following Table-5.1.2)

Table-5.1.2 : Antibacterial activity of different extracts of *Enhydra fluctuans* Lour :

Bacteria	Zone of inhibition in millimeters			
	Chloroform extract disc 150µL of 50mg/mL	Ethyl acetate extract disc 150µL of 50mg/mL	Methanol extract disc 150µL of 50 mg/mL	Water extract in well 60µL of 50mg/mL
Escherichia coli (ATCC 25922)	No zone	No zone	No zone	No zone
Shigella dysenteriae (ATCC 25226)	No zone	No zone	No zone	No zone
Vibrio cholerae 01 (ATCC 9458)	No zone	No zone	No zone	**14 mm**
Salmonella typhi (ATCC 27785)	No zone	No zone	No zone	No zone
Staphylococcus aureus (ATCC 9458)	**10 mm**	**15 mm**	No zone	**12 mm**

It appears from the table that the water extracts of *Enhydra fluctuans* Lour showed antimicrobial activity against *V. cholerae* and *S. aureus* (Figure 5.1) and the chloroform and ethyl acetate extracts were was also active against *S. aureus* (Figure 5.2, 5.3). Other extracts were not found to be active against the bacteria tested.

Control extract at 50 mg/ml concentration except negative control.



S. aureus *Vibrio cholerae*

Figure 5.1: Antibacterial activity of water extract of *Enhydra fluctuans* Lour on *S. aureus* and *V. cholerae.* (each well contain 60µl of extract at 50mg/ml concentration except negative control)

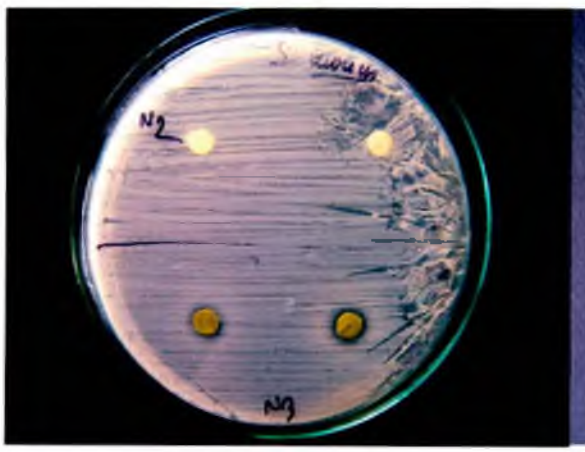


Figure 5.2: Antibacterial activity of activity of chloroform extract of *Enhydra fluctuans* Lour on *S. aureus.*

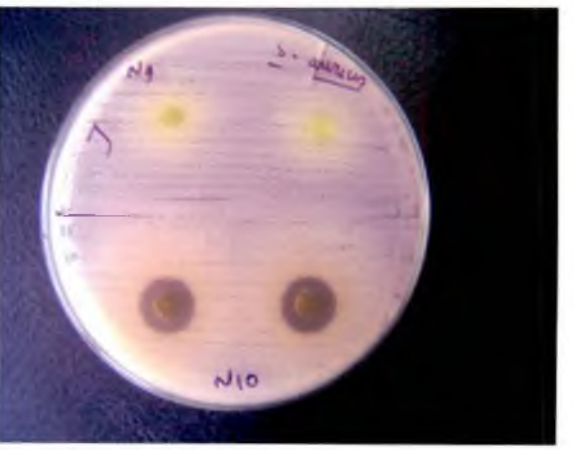


Figure 5.3: Antibacterial ethyl acetate extract of *Enhydra fluctuans* Lour on *S. aureus*

5.5.6 Antibacterial activity of different extracts of *Spilanthes acmella* Murr :

Antibacterial activity of different extracts of *Spilanthes acmella* Murr on different bacteria was studied (in the following Table-5.1.3).

Table-5.1.3: Antibacterial activity of different extracts of *Spilanthes acmella* Murr :

Bacteria	Zone of inhibition in millimeters			
	Chloroform extract disc 150µL of 50mg/mL	Ethyl acetate extract disc 150µL of 50mg/mL	Methanol extract disc 150µL of 50mg/mL	Water extracts in well 60µL of 50mg/mL
Escherichia coli(ATCC 25922)	No zone	No zone	No zone	No zone
shigella dysenteriae (ATCC 25226)	No zone	No zone	No zone	No zone
Vibrio cholerae 01 ATCC 9458	No zone	No zone	No zone	**13 mm**
Salmonella typhi ATCC 27785	No zone	No zone	No zone	No zone
Staphylococcus aureus(ATCC 9458)	No zone	**10 mm**	No zone	No zone

The antibacterial activity of different solvent extracts of *Spilanthes acmella* Murr. The table indicated that the water extract of *Spilanthes acmella* Murr. was active against *V. cholerae* (Figure 5.5), and the ethyl acetate extract was active against *S. aureus* (Figure 5.4). Other extracts were not active against the bacteria tested.

Figure 5. 4: Antibacterial activity of ethyl acetate extract of *Spilanthes aemella* Murr *against Stapylococcus aureus.*

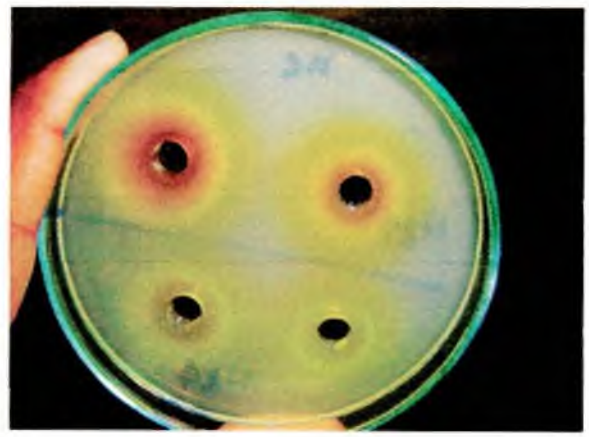


Figure 5.5: Antibacterial activity of water extract of *Spilanthus aemella* Murr

5.6 Discussion :

Bangladesh is a developing country and most of the people of our country are poor. They are unable to take costly drug as a medicine. So use of herb and herbal product as a drug is most effective and useful for them

In this study, the chloroform, ethyl acetate, methanol and water extracts of two herbs *Enhydra fluctuans* Lour, *Spilanthes acmella* Murr, were collected, which are soluble in chloroform, ethyl acetate, methanol and water. Five bacteria (*Salmonella typhi* ATCC 9458, *Staphylococcus aureus* ATCC 25923, *Vibrio cholera* O1classical ATCC 9458, *Shigella dysenteriae* type-1 ATCC 25226, *Escherichia coli* ATCC 25922) were used to observe the antibacterial activity of these herb extracts.

The chloroform extract of *Enhydra fluctuans* Lour showed antibacterial activity against only *Staphylococcus aureus*. The ethyl acetate extract of *Enhydra fluctuans* Lour, *Spilanthes acmella* Murr showed antibacterial activity against only *Staphylococcus aureus*. But no antimicrobial activity was observed against other bacteria tested.

In case of methanol extract, no clear zone was found against any bacteria which indicate no antibacterial activity of methanol extracts.

The water extract of *Enhydra fluctuans* Lour showed activity against two organisms *V. cholerae* and *Staphylococcus aureus*. Clear zones on Mueller-Hinton agar plate were found against *V. cholerae* for *Spilanthes acmella* Murr

In the whole study, it was found antibacterial activity against only two bacteria *Staphylococcus aureus* and *V. cholerae*.

The result showed that extracts of *Enhydra fluctuans* Lour have good antibacterial activity against both *Staphylococcus aureus* and *V. cholerae*. *Staphylococcus aureus* is a common food poisoning organism and also implicated in pus causing wounds, so the extract could be used as an alternative in the treatment of wound infection. *V. cholerae* causes cholera disease by producing toxin which is also inhibited. So it could be used by the local people in remote areas. In the experimental condition low activity samples was observed. This may be due to incomplete diffusion of organic solvent extracts through the agar medium. Samples of higher concentration may be required for producing large zone of inhibition or it may not have good antimicrobial activity.

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