Isolation of Secondary Metabolit es from Herbal Medicine and *Myristica fragrans* Houtt

Submitted by
Mohammad Ziaul Haque
Examination Roll No. 12
Registration No. 246/2001-2002

June, 2008.

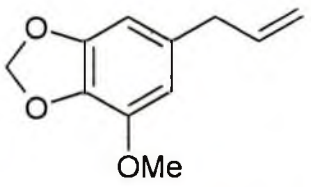
ORGANIC RESEARCH LABORATORY
DEPARTMENT OF CHEMISTRY
UNIVERSITY OF DHAKA
DHAKA-1000, BANGLADESH

DEDICATED TO MY PARENTS

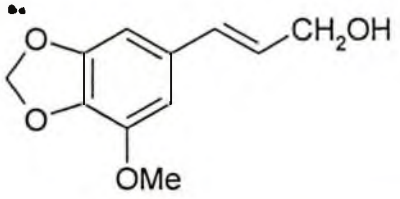
ABSTRACT

The thesis has two parts, isolation of secondary metabolites from (i) two herbal medicines, HM-01 and HM-02 (for secrecy code name were used in the thesis), and (ii) *Myristica fragrans Houtt* seeds. The medicine HM-01 is prescribed for the treatment of skin diseases such as acne, vulgaris, boils, skin rash, blemisher, urticarea, nose bleeding, constipation, indigestion and burning sensation during urination. It also relives general depression, improves complexion and helps to become slim and smart. The HM-02 medicine is also prescribed for similar complication. Major discrepancy of the medicines were (i) which part of the plants were used have not been cited, and (ii) how the aqueous extracts were made have not been mentioned. The present analysis showed that the herbal products are free from alcohol and adulteration with any modern drugs like steroids, alkaloids, etc. The herbal medicines were partitioned with organic solvents, n-hexane, dichloromethane and 1-butanol. Repeated column chromatography of the different partitioned fractions of the herbal medicines were carried out. From HM-01 benzoic acid (major constituent) and *p*-hydroxy methyl benzoate (minor) were isolated. These were characterized by their physical properties and UV, IR, ^{1}H- & ^{13}C-NMR spectroscopy including H-H COSY, HSQC and HMBC. From HM-02, only benzoic acid was isolated and characterized by its physical property and spectroscopic methods. There were also other signals in NMR spectra of the partitioned fractions but these were too small in comparison with benzoic acid. Benzoic acid found in the two herbal medicines as major constituent must not be from plant origin rather it was the sodium salt of benzoic acid which was added as preservative to prevent microbial growth. The amount of sodium benzoate was quantified by HPLC in HM-01 & HM-02 and found to be 12.99 mg/mL and 6.26 mg/mL, respectively which were very high in amount. Three heavy metals, arsenic (As), chromium (Cr) and lead (Pb) were quantified in HM-01 and HM-02 (0.16, 1.18, 10.5 & 0.09, 0.88, 4.55, mg/L, respectively) by Atomic Absorption Spectroscopy (AAS), which were also very high in amount and more than tolerance level.

The *Myristica fragrans Houtt* seed powder was defatted with pet-ether, and then extracted with 80% aqueous ethanol. The aqueous ethanol extract was divided into n-hexane, ethyl acetate and 1-butanol soluble parts. From the ethyl acetate soluble part, by repeated column chromatography two compounds were isolated. The compounds were characterized by spectroscopic methods, UV, IR, ^{1}H- & ^{13}C-NMR spectroscopy including H-H COSY, HSQC and HMBC as Myristicin, 6-allyl-4-methoxybenzo [d] [1,3] dioxal (**I**) and anthrascinol, (*E*)-3-(7-methoxy-benzol [1,3] dioxol-5-yl) prop-2-en-1-ol (**II**). Compound **II** was synthesized and marketed in USA as an antimicrobial drug.



6-Allyl-4-methoxybenzo [d] [1,3] dioxol



(*E*)-3-(7-Methoxy-benzo[1,3]dioxol-5-yl)-prop-2-en-1-ol

রসায়ন বিভাগ
ঢাকা বিশ্ববিদ্যালয়
ঢাকা-১০০০, বাংলাদেশ
ফোন : ৯৬৬১৯২০-৭৩/৭১৩০, ৭১৩১

DEPARTMENT OF CHEMISTRY
UNIVERSITY OF DHAKA
DHAKA-1000, BANGLADESH
Phone : 9661920-73/7130, 7131
E-mail : chem@udhaka.net

Dated 29/06/08

Certification

This to certify that Mohammad Ziaul Haque, M. Phil. student of the Department of Chemistry University of Dhaka has been jointly supervised by us and now he is submitting his thesis entitled "**Isolation of Secondary Metabolites from Herbal Medicine and *Myristica Fragrans Houtt*"** with our consent.

Professor Dr. Nilufar Nahar

Nilufar Nahar Ph.D.
Professor
Department of Chemistry
University of Dhaka
Dhaka-1000, Bangladesh
E-Mail : nilufarnahar@yahoo.com

Professor Dr. Habibul Bahar.

DR. MD. HABIBUL BAHAR
Professor
Department of Chemistry
University of Dhaka
Dhaka 1000, Bangladesh

PART A

CONTENTS

1.Introduction

2. Experimental

Schemes

List of Tables

List of Figures

PART B

1. Introduction

2. Experimental

d) ^{13}C NMR (100 MHz, $CDCl_3$) Spectroscopy

e) 2D NMR Spectroscopy

3. Results and discussion

Schemes

List o Tables

List of Figures

PART A

INTRODUCTION

PART A

1. INTRODUCTION

1.1. Herbal medicine[1,2]

Herbal medicine is the art and science of using herbs for promoting health and preventing and treating illness. The term herbs includes leaves, stems, flowers, fruits, seeds, roots, rhizomes and bark although in many traditions other naturally occurring substances including animal and mineral products are also used. There can be little doubt that the use of plants for healing purposes is the must ancient form of medicine known. Men and women led by instinct, taste and experience used plants for healing which was not part of their normal diet. Many well established medicines come from plants. For example, morphine comes from poppies, aspirin from willow bark, and digoxin from foxgloues, taxol from texous breviflora / yew tree.

Harbs had been used by all cultures throughout history. It was an integral part of the development of modern civilization, primitive man observed and appreciated the great diversity of plants, and the plants provided food, clothing shelter, and medicine. Much of the medicinal use of plants seems to have been developed through observations of wild animals, and by trial and error. As time passed on, each tribe added the medicinal power of herbs in their area to its knowledge base. They methodically collected information on herbs and developed well defined herbal pharmacopoeias. Indeed, well into the 20th century, much of the pharmacopoeias of scientific medicine was derived from the herbal lone of native people.

Many drugs commonly used today are of herbal origin. Indeed, about 25 percent of the prescription drugs dispensed in the United States contain at least one active ingredient derived from plan material. The use of 75% of the world population still relies primarily upon traditional healing practice most of which is herbal medicine. Some are made from plant extracts others are synthesized to mimic a natural plant compound.

1.2. History of herbal medicine [3]

Herbal medicine has a long and respected history. Herbal extracts is information about the origins of herbal medicine and evolved the very carefully research and benefit which we use herbs today.

Since the dawn of man, human relied upon plants and seeds as a vital source for both a mainstay of nutrition, as well as an important factor in the treatment of illness series of trials and errors over many generations, primitive people tasted and herbal combinations in an effort to cure disease and prolong life.

Archaeological digs have brought forth physical evidence of these early efforts to some dating back sixty thousand years to Neanderthal burial sites in Iraq, there sites going back many thousands of years in Egypt, Sumalia, India, China, and the separated by oceans, mountains and continents, many ancient societies methodizing independently developed closely related herbal species in almost identical remedy passed down by word of mouth and continued thÿÿugh ÿÿe age in herbal medicine worked. When a system of writing was developed, early written pharmacopoeia foundation of early herbal medicine and, more interestingly, the forerunner for example, the first century Greek physician wrote about benefits of white willow bark, which contains salicylic acid. This natural substance synthesised in 1838 and in 1899, the company introduced of the synthetic chemical compound similar to the active compound found in white willow brown "aspirin". What is more intriguing is the fact that this natural painkiller without any side effects typically associated with synthetic aspirin, it is believed that roughly twenty five percent of today's prescription drugs are said to be one of the ingredient derived from higher plants.

In the Far East, the Emperor Shen Nong (ciraca 2697 B.C) of China is said to be the maker of earliest known Chinese pharmacopoeia, the pen Tsao (Canon of herbs). Among medicinal plants that he identified and recommended included the opium poppy, and Ma huang. He is said to have tested and tasted hundreds of herbs himself and the discovery of tea drinking. The Emperor thang Ti (dates vary from 800 B.C to believed to be author of the Nei ching(Yellow Emperors canon of internal) said to be the oldest medical textbook in the world and still used to this very day.

In Europe: Herbal medicine carried on the accumulation of knowledge from and herbalists of antiquity, and enhanced many of these remedies with the latest of the day. During the eighth century, Emperor Cha Holy Raman Empire encompassed half of present day Italy and Germany, parts of Spain, establishing a central government over Western Europe, prescribed official protection herbs to be grown within his domain.

The age of exploration, the discovery of new lands and the opening of commerce, many exotic plants and herbs were introduced into Europe from the Spanish conquistadors and other European explorers brought new plants and back to the old world and ushered in the "Golden Age of Herbals".

English herbals was Nicholas culpeper authored the English physician of 1652, and his updated English physician enlargest one of the best selling herbs of all time and contains descriptions of herbs, along recommendations for their use as remedies., well educated and versed in Latin and translated the Latin pharmacopoeia into everyday English, angering the medicine which had previously held a virtual monopoly on the medical knowledge.

During the first years of pharmaceutical chemistry, it seemed as if herbal medicine day. As twentieth century technology advanced, synthetic drugs had nearly been the mainstay of medicine the down of man. There are many physicians and new schools of medicine embracing the entire body and mind, not only with the latest medical breakthroughs, but also complementary and alternative natural treatments, which serve to broaden the experience.

1.3. Different types of herbal medicine[4]

Herbal medicine can be broadly classified into various basic systems. Ayurvedic herbalism, which is derived from Ayurveda. Traditional Chinese herbalism, which is part of traditional Unani oriental medicine. Unani medicine derived by the Hakims and western herbalism, which originally came from Greece and Rome to Europe and then spread to North and South America. Kampo herbalism, which is derived from Traditional Chinese herbalism but developed in Japan. Homophatic medicine is Unique from western medicine.

(a) Ayurvedic medicine [5, 6]

Ayurvedic medicine is a complete system of medicine introduced from India which has been used for thousand of years. As with traditional Chinese medicine, an Ayurvedic practitioner uses their own unique theories of diagnosis and treatment to heal disease and promote health and well being. Many herbs used within Ayurvedic medicine, such as Neem and Arjuna, are commonly found in India and are relatively unique to this system, where as more common herbs such as ginger may be used which are also used in other herbal systems. The herbs may be incorporated into meals, prepared or taken in a pill from.

b) Unani herbal medicine[5,6]

Unani system of medicine is one of the oldest systems of medicine in the world; it is still popular and practiced in Indian subcontinent and other parts of the world.

The term "UNANI" is derived from the world "UNAN" which means Greece in Arabic. Unani medicine was originated in Greece and its founder was great philosopher and physician, Hippocrates. He was the first unani physician who opened the education of medicine to all communities; it was first developed by the Greeks in 460 BC. It then spread throng the Roman empire by notable scientists such as Galen (201 AD) with the fall of the Roman empire came the decline in unani medicine. If re–emerged later in Iran, where Muslim physician translated the unani texts into Arabic. While in the middlest Muslim physician begin to refine and further develop unani medicine. India was then introduced to unani medicine in the 13th century through the numerous Muslim invasions. Today, unani medicine is practiced mainly in Indian sub-continent by Muslim physicians (called Hakim).

(c) Traditional Chinese medicine[5,6]

Chinese herbal medicine is a component of traditional Chinese medicine which includes the practice of acupuncture and Tunia. Herbal medicine has been used in China for centuries and is backed by a long and rich history of developments, use and research of Chinese herbal medicine is unique in that the diagnosis and treatments are based on the theories of tradition Chinese medicine. Besides discussing health issues, a practitioner of herbal medicine uses other signs and symptoms such as those found in tongue pulse to form a TCM diagnosis. The common cold, for example, may be diagnosed as "wind – cold invasion" and herbs which dispel wind and warm cold may be preserved, Chinese herbal medicine are usually raw and cooked into tea in a powder form and taken with hot water or in a pill form.

(d) Kampo herbal medicine[5,6]

Kampo herbal medicine is a system of using herbs based on the theory behind traditional Chinese medicine as they have been developed in Japan, as with Japanese acupuncture, the Japanese system of herbal medicine is a refined version of Chinese herbal medicine in that they have taken the time to look at a variety of theories and application of Chinese medicine and determined what works best clinically. Kampo practitioners use well defined formulae of herbs for conditions based on a persons Chinese medicine based diagnosis. This is in contrast to a practitioners of Chinese herbal medicine which may choose from a much wider range of herbs when developing a prescription. Kampo herbs are generally administered in a pill form.

(e) Western herbal medicine[7,8]

Western herbal medicine originally came from Greece and Romman and spread to North and South America. Western herbalism is now considered folk medicine is distinguished primarily by the use of herbs commonly found in the west *St. John's wont* (is a herb used for the treatment of mild to moderate depression), *Black Cohosh* (for female complaints "The Blues" Native Americans used this herb in the treatment of rheumatism as well as many female complaints, *Chamomile* (a herbs whose leaves, seeds, of flowers are used for flavoring food on in medicine) and *Feverfew* (a herb used for the prevention of migraine headache) are some example of herbs commonly used in western herbal medicine.

(f) Homeopathy

Homeopathy is a unique from of western medicine that is used around the world. The theories behind homeopathy are boned on the "law of similar" and a homeopathy practitioner uses theory unique to homeopathy to form a diagnosis and decide a course of treatment. Homeopathy attempts stimulate the body to heal itself by using small amounts of medicines (from plant, minerals, animals or chemicals) which in large amounts of might cause the condition for which are treated. The idea is loosely related to that behind vaccines.

1.3.1. Difference between unani and ayurvedic[7]

Unani medicine is very close to Ayurveda. Both are based on theory of the presence of the elements (a unani, they considered to be fire, water, earth and air) in the human body. (The elements attributed to the philosopher Empedocles, determined the way of thinking in medcinal Europe). According to followers of unani medicine these elements are present in different fluids and their balance leads to health and in balance leads to illness. Most medicines and remedis (often common herbs and foods) used in unani are also used in Ayurveda. While unani was influenced by Islam, Ayurveda is associated with vedic culture.

The base used in unani medicine is often honey. Honey is considered by some to have healing properties and hence is used in food and medicines practiced in the Islamic world. Real pearls and metal are also used in the making of unani medicine based on the kind of ailment it is aimed to heal.

Most of the herbal drugs are common in Ayurvedic and unani medicine in the ayurvedic system, names are in Sanskrit, where as in unani system, in Arabic or Persian. In fact both the systems have practically as one integrated system.

1.3.2. Difference between herbal medicine and conventional medicine

The primary focus of the herbalists is to treat people on individuals irrespective of the disease or condition they have and to stimulate their innate healing power through the use of such interventions as herbs, diet and lifestyle. The primary focus of conventional physicians is to attack disease using strong chemicals that are difficult for the body to process, or through the removal of organs. Not only does this ignore the unique makeup of the individual, but many patients under conventional care suffer from side effects that are as bad as the condition being treated, the philosophical difference between herbalists and conventional physicians has profound significance

1.4. The importance of herbal medicine[9]

The medical establishment has become a threat to health wrote Ivan illich, an eminent researcher, in his book limits to medicine, these words grimly describe the state in which modern medicine find itself today. The modern allopathic system of medicine stakes no claim to its ability fully cure several different to treat chronic disease. There is a distinct possibility that the curative properties required to treat them successfully may lie within the den of herbal medicinal systems. Certain herbs including Echinacea, as tragalus, and garlic, have been found to have remarkable immunological effects in both the lab and clinic. The herbs support the body's own process to stay at the peak of vitality and prevent development of disease. Keeping in mind that herbs are not "magic bullets" designed to combat a specific disease. Rather than fighting an illness directly, herbs act to strengthen the functioning of the body so that it can repair the problem. We "catch" a cold or flu when condition in the body is opportune for a virus to take cold. Many of us view illness as an inconvenience to be suppressed or combated as soon as possible. We just want to take a pill to make it go away.

The herbs described here have gentle, yet profound effects upon the body. They are natural sources of vitamins, minerals, and other substances that the body uses to nourish and strengthen resistance to illness as well as to create an environment for healing in allopathy, there is no medicine to cure for treating liver disorder satisfactory. It is very encouraging to remember here that herbal medicine does have liver protective liver curative properties. There are many herbal medicines which are effective for many incurable diseases.

1.5. Global promotion of herbal medicine[10]

Medicinal herbs are moving from to mainstream use with a greater number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals. Recently, considerable attention has been paid to utilise eco-friendly and biofriendly plant based products for the prevention and cure of different human diseases. Considering the adverse effects of synthetic drugs, the western population is looking for natural remedies, which are safe and effective. It is documented that 80% of the world's population has faith in traditional medicine, particularly plant drugs for their primary healthcare.

1.5.1. Traditional medicine is going to be popular day by day[4,7,10]

Traditional medicine (TM) and what is called complementary alternative medicine (CAM) are primary sources of health care for millions of people about 80 percent of the world depend on traditional techniques alone for treating and curing illness. In many parts of Africa, Asia and Latin America, TM is the only health care many people receive at the same time; the user of alternative medicine is growing in Australia, Europe and North America Now- a days traditional medicines are taken along with modern medicine which is called integrated medicine. Integrated medicines are prescribed in many countries (Germany, France, UK. etc) in Europe and in India.

1.5.2. Users of herbal medicine[11]

Herbal medicine is the oldest form of healthcare known to mankind. The world health organization (WHO) estimates that 4 billion people (80% of the world population) presently use herbal medicine for some aspect of primary healthcare. Herbal medicine is a medicine and a common element in ayurvedic, homeopathic, naturopthic, traditional oriental, and Native American Indian medicine. WHO have noted that 119 plant-derived pharmaceutical medicines, about 75% percent are used in modern medicine an that correlated directly with their traditional uses as plant medicines by native cultures. Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal value.

In China: Traditional herbal preparation accounts for 30% - 50% of the total medicinal consumption. In Ghana, Mali, Nigeria and Zambia, the first line of treatment for 60% of children with high fever resulting from malaria is the use of herbal medicines at home.

In Europe: North America and others industrialised regions, over 50% of the population have used traditional complementary medicine (TCM) or Traditional alternative medicine (TAM) at least once. In San Fransisco, London and South Africa, 75% of peoples living with HIV/ AIDS use TCM / TAM.Seventy percent of the populations in Canada have used complementary medicinal least once.

In Germany: 90% of the population have used a natural remedy at some point in their life. People in the United States are continually gaining interest in herbs because of an increasing number of success stories. One example is the use of St. John's wort to treat forms of depression. People have used this drug to avoid using prozac, which produces unwanted side effects certain ayurvedic herbs are commonly used to help those with condition including diabetes and high cholesterol. Herbs such as (ginkgo) and Ginsing continue to increase in popularity because of their helpful effects.

1.5.3. Priorities for promoting the use of traditional medicine

Overall one third of the population in developing countries lack access to essential medicines. The western population is looking for natural remedies which are safe and effective. The provision of safe and effective TM/CAM therapies could become a critical tool to increase access to health care it is generally estimated that over 6000 plants in India and other parts of the world are used as traditional herbal medicine representing about 75% of the medicinal needs of the third world countries. While the China, the Democratic People Republic of Korea, and Vietnam have fully integrated traditional medicine into their health care system, many countries are yet to collect and integrate standardised evidence on this type of health care.

1.6. The most debatable argument in the present scenario about safety and side effect of herbal medicine[12]

Medical science says- it is highly impossible for any medicine to have effects without side effects, that if herbs are claimed to be free from side effects they are probably not effective either. This is rational view, within its own terms. Any intervention at one site is always like to lead to reactions at other sites, either because of functional or structural connection or because of similarities in sensitivity. On the other hand, there is a persistent popular belief that herbs are safe. Probably the main reason why patients first turn to herbs is they assume that they are free from side effects, "not likely drugs". Many herbalists emphasize the same point. They refer to the uninterrupted use of the most established remedies by millions of people since prehistory. They also talk of herbs being used for different reasons than conventional drugs, promoting healing responses rather than targeting pathology or symptoms, as a whole herbs being a complex package among the active constituents. All these logical exceptions neither compensate nor draw the veil over the truth "safety"; though it is much transparent and delicate matter but considerably debatable issue at the present time. Then question arise how can we resolve the matter. Simple, as concerns safety what is needed today to develop awareness by pooling information regarding the "safety of herbs".

Despite popularity and apparent neutrality, herbal medicine is still in its early stages and shouldn't be substituted for traditional medicine yet. Long-term effects of this type of medicine have not yet discovered. Herbal medicine should not replace pharmaceutical medicine and should definitely not be used to try and cure serious health problems. One of the drawbacks is that preparation process is sometimes very time-consuming, not to mention cooking odors, which are, off-times quite unpleasant. On the other hand documentation of the herb to used in preparation of herbal medicine, process of the herb (Storages herb best in extraction), safety of the medicine (Toxicity) and efficacy and dose of the medicine (Standardization) are big issues arises by the Western communications. A General strategy is being found out for the herbal medicine.

1.7. General strategy for preparation of the herbal drug for the commercial market[13]

1.7.1. Collection

Collection of herbal drugs from cultivated plants always ensures true natural source and a reliable product. Many drugs are collected from wild plants. While pine, podophyllum, ginseng and many other drugs were collected in the Blue Ridge. Mountain region of USA by the Native American Ashville, North Carolina is one of the important collection areas, the proper time of harvesting or collecting is particularly important because the nature and quantity of constituents vary greatly in some species according to the season. The most advantageous collection time is when the part of plant that constituents the drug is highest in the content of active principles and when the material will to give the maximum quality and appearance.

1.7. 2. Harvesting

Some drugs may be collected by hand labor and some are by the mechanical devices. With some drugs, where the skillful selection of plant parts is an important factor.

1.7. 3. Drying

By drying the plant material, one can remove sufficient moisture to ensure good keeping qualities and to prevent molding. The action of enzymes, the action of bacteria and chemical and other possible changes. Proper and successful drying involves two main principles; control of temperature and regulation of airflow. For some natural product, such as vanilla, process of fermentation or sweating is necessary to bring about changes in the constituents; such drugs require special drying processes usually called curing.

1.7. 4. Garbling

Garbling is the final step in the preparation of a crude drug. Garbling consists of the removal of extraneous matter, such as others parts of the plant, dirt and added adulterants, this step is done to some extant during collection, but should be carried out after the drug is derived and before it is packed. Although garbling may be done by mechanical means in some cases, it is usually semi-skilled operation.

1.7. 5. Packaging, storage and preservations

The packaging of drugs depends on their final disposition Leaf and herb material is usually baled with power balers into solid compact man that is sown into a burlap cover. Drugs that are likely to deteriorate from absorbed moisture are packed in moisture- proof cans, proper storage and preservation are important factors in maintaining a high degree of quality of the drug. Drugs should not be stored in wooden boxes or in drawers and never in paper bags. Its deterioration hastened bal odors are communicated from one drug to another, attacks by insects are facilited and distraction by mice and rats may occur. Adding to the container a few drops of chloroform or carbon tetrachloride from time to time may control insect damage. High temperature accelerates all chemical reaction. So drugs must always be stored at a standard low temperature as possible.

1.8. Assessment of safety of herbal medicine[14]

Safeties of a medicine concern the following two things:

1. **Toxicity**
2. **Side effects.**

1.8.1. Toxicity of herbal medicine

The safety and quality of herbal medicine are a major concern for health authorities, pharmaceutical industries and the public due to the increase of traditional medicines by consumers in the global market. Potentially hazardous contaminants and residues in herbal medicines are:

Physicochemical and environmental contaminants and residues, such as:

Arsenic and toxic metals

i) Radioactive substances
ii) Mycotoxins and other endotoxins
iii) Pesticide residues
iv) Processing residues and solvents
v) Persistent organic pollutants (POPs) and other contaminants
vi) Microbial contaminants and parasites
vii) Determination of Aflatoxins which test for the presence of aflatoxins B1, B2, G 1, and G2 contaminants in material of plant origin.

1.8.1.1. Guidelines for toxicity investigation of herbal medicines

These guidelines are intended to indicate the standard methods of non-clinical toxicological studies refitted to assessing the safety of herbal medicines. Not all tests are necessarily required for each herbal medicines intended for human study.

Acute toxicity test: Animal species

Some regulatory agencies require that at least two species be used, one of them to be selected from rodents and the other from non-rodents. And at least one of the species, males and females should be used.

1.8.1.2 Long-term toxicity test: Animal species

Many regulatory agencies require that at least two species be used, one a rodent and the other a non-rodent. And the same number of male and female animals should be used.

Number of animals

In cases of rodents, each group should consist of at least ten males and ten females. In the case of non-rodents, each group should consist of at least three males and three females. When interim examinations are scheduled, the number of animals should be increased accordingly.

1.8.1.3. Observations and examinations

Observations and examinations should be performed on the following items (from i to

i) ***General signs, body weight and food and water intake***

For all experimental animals, the general signs should be observed daily and body weight and food intake should be measured periodically. If useful, water intake should also be determined. The frequency of measurements should normally be as follows: Body weight: before the start of drug administration, at least once a week for the first three months of administration and at least once every four weeks thereafter. Food intake: before the start of drug administration, at least once a week for the first three months of administration and at least once every four weeks thereafter. If the test substance is administered mixed in the food, the intake should be measured once a week.

ii) ***Hematological examination***

For rodents, blood samples should be taken before autopsy. For non-rodents, blood samples should be taken before the start of drug administration, at least once during the administration period (for studies of longer than one month), and before autopsy. For both hematological and blood chemistry examinations, it is desirable to include as many parameters as possible.

iii) ***Renal and hepatic function tests***

Since the liver and kidneys are the usual organs of metabolism and excretion, potentially toxic agents easily affect them; their functions should be monitored in long-term toxicity studies. For rodents, a fixed number of animals from each group should be selected and urinalysis should be performed before the start of drug administration, and at least once during the administration period.

iv) *Other function tests*

If appropriate, ECG and visual, auditory tests should be perfumed. For rodents, ophthalmologic examination should be performed on a fixed number of animals from each group at least once during the administration period.

v) *Recovery from toxicity*

In order to investigate the recovery from toxic changes, animals that are allowed to live for varying lengths of time after sensation of the period of administration of the test substance, should be examined.

1.8.1.4. Test of other ingredients present in herbal medicines

i) Microbial Contamination

Aerobic bacteria and fungi are normally present in plant material and may increase due tofaulty growing, harvesting, storage or processing. Herbal ingredients, particularly those with high starch content may be prone to increased microbial growth. It is not uncommon for herbal ingredients to have aerobic bacteria present at 102-108 colony forming units per gramme. Pathogenic organisms including *Enterobacter, Enterococcus, Clostridium, Pseudomonas, Shigella* and *Streptococcus* have been shown to contaminate herbal ingredients. The European Pharmacopoeia (Ph Eur) gives non-mandatory guidance on acceptable limits.

ii) Pesticides

Herbal ingredients, particularly those grown as cultivated crops, may be contaminated by DDT or other chlorinated hydrocarbons, organophosphates, carbonates or polychlorinated biphenyls. Limit tests are necessary for acceptable levels of pesticide contamination of herbal ingredients. The Ph Eur includes details of test methods together with mandatory limits for 34 potential pesticide residues.

iii) Fumigants

Ethylene oxide, methyl bromide and phosphine have been used to control pests which contaminate herbal ingredients. The use of ethylene oxide as a fumigant with herbal drugs is no longer permitted in Europe due to concerns about carcinogenic residues. There are concerns, however, that products imported from outside the ED may have been treated with this fumigant.

iv) Toxic Metals

Lead, cadmium, mercury, thallium and arsenic have been shown to be contaminants of some herbal ingredients. Limit tests for such toxic metals may be needed for certain herbal ingredients.

v) Other Contaminants

Tests to limit other contaminates such as endotoxins, mycotoxins and radionuclide may need to be considered to ensure suitable quality for medicinal purposes.

1.8.1.5. Importance of efficacy trial on herbal medicine[15]

Herbal drug has been used since ancient times as medicines for the treatment of a range of diseases. Most of the world population, which lives in developing countries, depends essentially on medicinal plants for primary health care because of poverty and lack of access to modern medicine. Over the past decade, the phytomedicine market has grown at an expressive rate worldwide and used as a common form of alternative therapy in all over the world. Herbal medicinal preparation is a traditional part of developing countries; currently western world had demonstrated renewed interest for the development of standardized phytotherapeutic agents. Drug efficacy, safety and quality are the major issues for standardization of herbal drugs and this information are essential for assessing "risk-to-benefit" ratio of the product. Herbal medicine with well established history of human use or traditional practices is in safe corner but insufficient data exist for most of the herbal medicine to guarantee their quality, efficacy, and safety. Though safety considered being the first requirement, the efficacy remains a

major issue in the scientific evaluation of herbal medicine. Depending on the very nature of medicine, efficacy trial are designed. For pharmacopeal drugs with unbroken tradition of use, the WHO requires a different level of efficacy and safety trial. On the other hand, for non-pharmacopeal formulations, full fledge efficacy trials are required which meets all the requirements of ethical and clinical criteria. This presentation will focus on recent development in efficacy trials, based on modern scientific tools and

1.8.1.6. Safety of Herbal Medicine[16]

With the paucity of hard facts, the issue of herbal safety is a matter of contention. The following are the arguments marshaled by the doubters, particularly those who feel their duty to public safety is to assume the worst-case scenarios.

The doubts

i) Herbal remedies are complex mixtures, about whose effects on the body little is known even in their isolated state, let alone when mixed in infinitely variable ways.

ii) Chemical complexity can work in both directions, buffering against and towards potentiating of adverse effects.

iii) Traditional use likely to have spotted only acute and relatively frequent adverse reactions; chronic delayed or infrequent reactions would probably not have been associated with the herb.

iv) Traditional reputations are in any case highly unreliable in their transmission.

v) It is possible to exceed modern standards of risk, say 1:1000 and still statistically mean that very few working practitioners (herbalist) will see the adverse events in their lifetime.

vi) There are particularly heavy bases in the way of reporting adverse effects of herbal remedies at the present time; therefore the current state of information almost certainly understands the risk.

1.9. Standardization of Herbal Medicine[17]

Standardization of herbs

i) Cultivation and collection of plant materials

Cultivation

Cultivation allows producers to have more control over quality and purity than dose, collecting plants from the wild, cultivars (cultivated varieties) of a number of medicinal plant species have Gbeen developed to produce high yields of the desired constituents, some plants that are grown commercially for medicinal purposes are propagated vegetative,

Some medicinal plants are grown from selectively bred hybrid seeds, while others are varieties of plants that are unchanged from their natural form,

As for any agricultural crop, producers of medicinal plants must provide plants with adequate moisture and nutrients and must control pests and diseases pesticides must be used cautiously to reduce the risk of harmful residues on plants,

Most herbs are at their peak flavor just before flowering, so this is a good time to collect them for drying and storage. Cut off the herbs early in the morning just after the dew has dried. Cut annuals off at ground level, and perennials about one-third down the main stem, including the side branches.

Sometimes harvesting involves picking leaves and flowers manually, In the future, tissue culture may be used for producing plant material.

ii) Methodologies of Standardization of herbal medicine[17]

Herbal medicine by its very nature makes standardization a complex and perhaps even an unfathomable issue. Herbal medicines contain more often than not material from many different plants and are usually the products of complex preparative processes. The plants, which are raw material for the herbal drugs, may have slightly different constituents when collected from different localities, environments or climate conditions.

Although they can consist of many thousand of chemical constituents whose individual or combined effects on human physiology are unknown, it must be remembered that they form the backbone of indigenous medical systems providing treatment to want a quarter of the worlds population. Herbal cures are becoming increasingly popular even in the west, the world wide herbal market being estimated to be worth 60 billion US dollars. There is therefore a growing demand for the identification of herbal drugs of demonstrated efficacy and the availability of such drugs in standardized formulations. The future development of herbal medicine is very much dependent on success in this area. The WHO has formulated guidelines for quality standardized herbal formulations which identifies the need for quality control of raw materials, the preparation process and the finished product and assessment of shelf life, safety and efficacy of the herbal drug. In Germany most of the herbal drugs are registered as conventional drugs and meet the same stringent criteria of quality, efficacy and safety as synthetic drugs. Chromatographic (TLC, HPTLC, HPLC and GC) and spectroscopic (UV, fluorometry, GC-MS) analysis for active principle or major constituent (chemical marker) content have been used in developing protocols for the standardizations of herbal drugs. It has been suggested that DNA fingerprinting could be developed into a powerful tool for this purpose. The importance of quality control of the raw materials and throughout the entire process leading to the finished product should be emphasized. Modern biotechnology offers quicker methods for the determination of the mechanism of action and the true active

constituent of herbal drugs that could form the basis of the chemical marker to be used in standardization. In many cases the activity is derived from several compounds showing synergistic pharmacological effects and this may require the adoption of more than one chemical marker in the standardization process. A successful programmed for the standardization of herbal drugs therefore requires not only the setting up of well equipped

quality control laboratories but also the initiation of extensive research programmed aimed at developing protocols for the standardization of herbal drugs.

1.10. Extraction

Quality Manufacturing

The most fundamental concept is that herbs require different methods. The most common extraction methods are discussed below.

Extraction

Extraction is a process whereby the desired constituents of a plant are removed using a solvent. The following section describes several methods used for preparing extracts, including organic solvent extraction, supercritical gas extraction, and steam distillation.

Organic Solvent Extraction

Organic solvent extraction is one process for separating the desired substance from plant material. As was previously mentioned, dried plants are usually used for extraction, although fresh plants are sometimes used. The plants are first ground and then thoroughly mixed with a solvent such as hexane, benzene, or toluene inside a tank. The choice of solvent depends on several factors including the characteristics of the constituents being extracted, cost, and environmental issues. If product will contain trace amounts of residual solvent, a nontoxic solvent must be use solvent dissolves the desired substances of the plant, it is called "miscella." The miscella is separated from the plant material. There are a number of techniques for solvent extraction which include maceration, percolation, and countercurrent extraction. The following is a brief description of each.

Maceration

Maceration simply refers to soaking the cut and milled herb in a suitable solvent (the solvent is known as the 'menstruum', often a combination of water, ethanol or glycerol) for a specified time after which the herb is drained and pressed. This method is mainly used for extraction of water-soluble constituents. The cough -soothing mucilage in marshmallow is thus well extracted in a combination of water and glycerol. For most other constituents, maceration is an inefficient extraction method, often only extracting less than 70% of active constituents.

Products: Types of Extracts

Liquid Extracts

There are essentially three different types of liquid extracts.

Non-Standardized

These extracts are known as tinctures, liquid extracts or fluid extracts. The solvent is usually a mixture of ethanol and water, but may also include glycerol and other solvents. Ethanol and water are considered the most effective solvents. The drug-solvent ratio varies from 1: 1, 1 :2, 1:5 or weaker. As the level of active constituents in the raw material varies from batch to batch, the level in the finished product will also vary. Quality control, however, can still be applied by using HPLC to identify the level of active constituents or marker compounds, although these levels may not be stated on the label.

Standardization of Finished Herbal Products

Chemical and biological techniques could be used to establish the methods for identification and standardization. Each herbal medicine and its products contain a complex mixture of natural products. When the active constituents are known, quality control and assessment should be based on these compounds. In many cases, however, the active constituents of herb medicines are not yet clearly known. One or two chemical markers from plants were usually chosen for quantitative determination. Fingerprinting was proved to be one of the powerful techniques for identification and consistency assessment of herb materials botanical and product. The fingerprinting of HM is generally referred to as chemical fingerprinting established by chromatographic and spectroscopic methods. Chromatography used for fingerprinting includes HPLC, thin layer chromatography (TLC), gas chromatography (GC), and capillary electrophoresis (CE). Spectroscopy such as infrared spectroscopy (IR), ultraviolet spectrometry (UV), nuclear magnetic resonance (NMR) and mass Spectrometry (MS) can also be applied for fingerprinting. The combination of chromatography with Spectroscopy LC - MS/ MS, LC - NMR,' LC - NMR MS, can provide additional structure information on constituents of mixture and may be more effective when identification of TM is necessary to quality control.

1.11. Efficacy of herbal medicine[18]

It is important for herbal medicines and particularly for those made from mixture of herbal products that the requirements for proof of efficacy, including the documentation required to support the indicated claims, should depend on the nature and level of the indications. For the treatment of main or disorders. For non specific indications or for prophylactic uses, less stringent requirements may be adequate to prove efficacy, especially when the extent of traditional use and the experience with a particular herbal medicine and supportive pharmacological data are taken in to account. The level of the evidence and the grading of recommendations must correspond to the nature of the illness to be treated on the nature of the physical or mental function to be influenced and regulated. Definitions of levels of evidence and the grading of recommendations from the USA agency for health care policy and Research may be used for guidance. many other national documents, such as the Australian Guidelines for levels and kinds of evidence to seaport claims for the therapeutic goods could also be used for reference. The therapeutic alternatives available with in the community and the risks of the herbal medicine have to be taken in to account. It should be noted that is the case of herbal medicine mode from herb matures, a therapeutic or scientific rationale meets exist for the presence of each herb in the mixture.

Many kinds of traditional procedure based therapists such as acupuncture and manual therapies, have already been widely used in health care systems in a number of countries. However, there is an increasing demand to study and evaluation the efficacy of these therapies.

The efficacy of most forms of traditional procedure based therapies depends heavily upon the proficiency of the practitioners, including their skills and experiences this may partly explain the many disparity or inconsistency of results reported by different authors, even though the method of the studies were equally sound. Non-specific effects of the therapy also contribute to efficacy. But these are difficult to measure or quantify. Therefore, clinical trials and other the evaluation of the efficacy of traditional procedure based therapies.

1.12. Herbal Good Manufacturing Process (GMP) [19]

All herbal medicine by law is made according to the code of pharmaceutical GMP. Ethically, this is a fail- safe system of quality assurances and quality control, which defines a number of procedures and observations, but in practice, herbal manufacturing under GMP is more complex than conventional drugs. The practice of GMP in a well-regulated environment should eliminate the possibility of these types of contaminations. There is no absolute safety in life. Every human activity is risky and accidents will happen, even to herbalist. Some plants are likely to dangerous when consumed; as a toxicity concern, because herbs are bound to interact with the body in ways that are not always convenient and not predictable. So, long –term consumptions is not courage full .It is very peculiar, however, considering the vast usage around the world of plants as medicines; a few problems have been laid at the door of herbal medicine/ total herbs, only because there is little epidemiological or clinical evidence for proving that this is harmful activity. It is possible that the safety record for herbalism is better than for any other widespread human activity. The problem with legislator medical professionals and public guardians (consumers) is that they inevitably find themselves balancing risk with benefit, but no risky venture at all. If they can see a public benefit as well as a demand for an activity they will

1.13. Objective of the present study

Herbal medicine is traditional medicine and it has been using since ancient time as medicines for the treatment of a wide range of disease. Most of the world population, which lives in developing countries, depends mainly on herbal for primarily health care due to poverty and lack of knowledge to modern medicine. Over the past decade, the phytomedicine market has grown at an expressive rate worldwide and used as a common form of alternative theory in all over the world. Herbal medicine preparation is a traditional part of developing countries, currently western world had demonstrated renewed interest for the development of standardized phototherapeutic agents, medicine efficacy, safety and quality are the major issues for the standardized of herbal medicine and this information is essential for assessing " risk to benefit " ratio of the product. Herbal medicine with well established history of human use but insufficient data exist for

most of herbal medicine to guarantee their quality, efficacy and safety, herbal medicine is widely used in our country, but very little is known about their safety, efficacy and actual constituents in this work, it was therefore considered important to perform to some chemical work on a wide used of herbal product. The study was taken with great interest on herbal medicine of local company to isolate and characterized chemical constituents present in the two herbal medicines HM-01 and HM-02 (For secrecy actual name of the has not been mentioned in any part of the thesis).It was also aimed to determine HPLC profiles of the two medicines so that the profile can be used in quality control of two medicines.

PART A

EXPERIMENTAL

2. EXPERIMENTAL

2.1. General Methods

2.1.1. Solvents and Chemicals

Analytical and laboratory reagent grade (e.g., SIGMA, E. Merck or BDH) solvents and chemicals were used in most of the experiments. Commercial grade of Hexane, DCM, ethyl acetate, methanol, butanol, acetone and chloroform were distilled in glass distillation set before use.

2.1.2. Evaporation

All evaporations were carried out under reduced pressure using rotary vacuum evaporator at bath temperature not exceeding 40°C. Small volume of non aqueous solvents was concentrated by flushing with nitrogen at room temperature. The residual solvent in the extract and compounds were removed under high vacuum or by freeze-drying.

2.1.3. Freeze-drying

All freeze-drying were carried out with a Varian 801 models LY-3 TT and HETOSIC 52 (Hetolab equipment, Denmark) freeze-dryer. Before freeze-drying organic solvents were completely removed by evaporation and by using a vacuum pump aqueous fractions were first frozen in round bottom flasks in a methanol freezer (HETROFIG, CB5, Hetsbrikero, Denmark) at 30^0C to 40^0C and finally the materials were subjected to freeze-drying operation.

2.1.4. Chromatographic methods

2.1.4.1. Thin layer chromatography (TLC)

Percolated TLC plates: 0.2 mm thin coating of silica gel on aluminum sheets, were used for TLC.

2.1.4.2. Applications of the samples and development of the TLC plate

Capillary tubes were used for application of the samples on TLC plates they were developed in different solvent systems by the ascending method in a glass Jars or tanks. After development, the plates were taken out from the tank and dried in air. The plates were developed by one of the following methods to detect the positions of the spots.

2.1.4.3. Location of spots

For the location of separated components, the plate were examined by one the following methods.

UV Light

The TLC plates were examined under UV Light wave lengths, 250 nm and 350 nm.

Spray reagent

The plates were sprayed with 1% vanillin Sulphuric acid reagent followed by heating an oven at 120^0C for 15 minutes.

Iodine vapor

The TLC palate were placed inside a closed iodine chamber and kept for a few minutes.

2.1.4.4. Preparation of vanillin- sulphuric acid reagent (1%)

To prepare vanilllin-sulphuric acid reagent 1.0 g of vanillin was dissolve in 100 ml of concentrated sulphuric acid, the prepared solution was then kept in a glass made sprayer.

2.1.4.5. Preparation of ceric sulphate-sulphuric acid reagent (1%)

1.0g of ceric sulphate was dissolved in 100 ml of 5N sulphuric acid to prepare the reagents. The prepared solution was kept in a glass sprayer.

2.1.4.6. Solvent systems

Some of the solvent were used for TLC in this work are given below:

Binary solvent systems were used for the less polar fractions and compounds e.g.,

i). Hexane: dichloromethane (in different ratio)

ii). Hexane: ethyl acetate (in different ratio)

iii). Dichloromethane: methanol (in different ratio)

iv). Ethyl acetate: methanol (in different ratio)

The polar fractions and compounds, trinary solvent systems were used, e.g.,

v). Chloroform: methanol: water (in different ratio)

2.2. Column chromatography

Preparation of silica gel flash column

Glass columns of different size were used for chromatographic separations. Column grade silica gel G-60 (230-400 mesh, particle size 0.04-0.06 mm, Art. 7734, ASTM, Mesh) was used for stationary phase. To prepare a particular column, the required amount of silica gel was swelled into a selected solvent (e.g., hexane, Dichloromethane, Methanol, chloroform, ethyl acetate or a mixture of different solvents in different ratio) for a while and then poured into the column with continuous flow of the solvent for homogeneous packing, the column was equilibrated with three volume of solvent Separation was performed by gravitational flow with solvent of increasing polarity.

2.2.1. Application of the sample into the column

The sample to be chromatographed was introduced as pre adsorbed on the packing material. For pre-absorption, the sample was dissolved in a particular solvent or a mixture of solvents and silica get (sample: gel, 1:2-3 w/w) was added to the sample solution. The solvent was then removed by evaporation; the dried material was ground thoroughly in a mortar to make it a fine powder, which was then applied carefully to the top of the column.

2.2.2. Fraction and monitoring procedure

After application of the sample, the column was eluted with equilibrating solvent and the polarity of the mobile phase was increased gradually by adding more polar solvent .The eluted sample was collected in conical flasks or test tubes and using TLC profiles monitor fractions. Fractions were combined on the basis of their R_f values.

2.2.3. Melting point apparatus

Melting point were determined by using an electro thermal point apparatus (Melting-temp, OGAWA SEIKI Co. Japan)

2.2.4. HPLC Analysis

HPLC separations were performed on supelco discovery reversed phase c-18 colunm (25 cm x 4.6 mm i.d. particle size 5 μm) using a Shimadzu SCI-10A *VP* machine equipped with a variable wavelength UV–Visible detector (SPD-10A *VP*) the sample were injected manually by using a 100 μL sample loop. HPLC working conditions were binary gradient wavelength was set up at 230 nm, eluent was Acetonitrile: Water (50:50) and flow rate 1 mL/min and injection volume was 100 μL.

2.2.5. Spectroscopic methods

2.2.5.1. Ultraviolet (UV) spectroscopy

Shimadzu UV 160-A recoding spectrometer was used for UV spectrum. The samples were dissolved in solvents and the solutions were taken in (1cm x 1cm) quartz cell for recoding the spectrum main bands (λmax) were recoded as wavelength.

2.2.5.2. Infrared (IR) spectroscopy

A Shimadzu IR 470-spetrometer was used to record the IR spectrum. The IR spectra were recorded making KBr pellet. Before recording IR the compound was dried in freeze- drier to remove moisture completely to avoid undesired -OH peaks.

2.2.5.3. Nuclear magnetic resonance (NMR) spectroscopy

The ^{1}H- NMR and ^{13}C-NMR spectra of pure compounds were recorded by using a 400 MHz NMR spectrometer (Bruker DP×400). For NMR spectra the samples were dissolved in $CDCl_3$ and solvent pick was used as standard reference. The DEPT experiment of ^{13}C spectra were obtained by varying the pulse width (θ) at 135 and two dimensional NMR (H-H COSY, HMBC, HSQC) spectra were obtained suing standard pulse sequences.

2.3. Preparation of Dragendorff's reagent

This reagent was prepared by mixing equal parts (v/v) of 1.7% bismuth subnitrate dissolved in 20% acetic acid in water and a 40% aqueous of potassium iodide alkaloids gave positive color reaction (usually orange red color) with this spray reagent instantly.

2.4. Atomic Absorption Spectrophotometer (AAS-640 F; Shimadzu)

A Shimadzu (AAS-F) Atomic Absorption Spectrophotometer was used to determination of Quantitative elementary analysis of heavy metals. The sample was dissolved in $CDCl_3$. The solvent was used as standard references.

2.5. Material

Two herbal medicines, HM-01 and HM-02 were bought from the local herbal medicinal Shop. The medicines are being used for skin diseases such as acne vulgaris, boils, blemisher, and urticarea check nose bleeding etc. These are in liquid form, one bottle contained100 mL and 450 mL each bottle cost Tk: 20 and Tk: 80 only. 450 mL of ten (10) bottles each medicine was purchased for the analysis.

2.6. Analysis of Herbal medicine of HM-01.

Physical characteristics of the sample

The liquid medicine is greenish in color. It is sour and bitter in taste.

i) Flavor: No smell of fermentation
No smell of Alcohol
Gives flavor of plan material.

The composition of the medicine as described by the company is given bellow.

ii) Plants with amount are used to prepare the medicine

Name of the Plants.	**Amount of plants in each 5ml (as aqueous extract)**
Cassia angustifoli	– 17.00 mg
Rheum emodi	– 13.00 mg
Cassia Occidentalis	– 12.50 mg
Ocimum sanctum	– 2.50 mg
Ipomoca turpethum	– 2.00 mg
Rosa damasceua	– 2.00 mg
Sphaeranthus indicus	– 2.00 mg
Gentiana Kurroo	- 2.00 mg
Oldenlandia corymbosa	– 2.00 mg
Clitoria termatea	– 2.00 mg
Artemisia absinthium	– 2.00 mg
Nymphae alba	– 1.25 mg
Dalbergia sissoo	– 1.25 mg
Pterocarpus santalinus	- 1.25 mg
Tinospora cordifolia	– 1.25 mg
Terminalia chebula	– 1.25 mg
Zingiber zerumbet	– 1.25 mg
Swertia chirata	– 1.25 mg
Andrographis Paniculata	– 1.25 mg
Bautinia Varieguta	– 1.25 mg
Azadirachta indica	– 1.25 mg
Curcuma longa	– 1.25 mg

iii) pH of the medicine was determined and found to be 6.90.

iv) Boiling point of the liquid sample was determined and found to be 119 -121^{0}C.

IV) Determination of Dry mass:

Procedure: HM-01(20 mL) liquid sample was taken into a 100 mL round bottom flask. Then the sample was evaporated by a rotatory vacuum evaporator at bath temperature below 40^{0}C into small volume (5 mL), the concentrated sample was dried by a freeze-dryer. The total dry mass of HM-01 was found to be 1.849g (9.25%).

v) Ash content

Ash content of HM-01

The freeze dried sample (HM-01; 1.052g) was ignited about 2-3 hours in direct flame until the fume disappeared and then it was burned at 750^{0}C about 4-5 hours. It was repeatedly burnt and cooled until and constant weight was obtained (0.0698g, 6.63%).

vi) Charcoal Treatment

10 mL of the sample HM-01 was taken in a beaker and diluted into 30 mL with water. The sample solution color was brown. Then small amount of conditioned charcoal was added to it. Then the beaker was shacked for a few minutes. After that it was filtered. Filtrate was colorless. Then it was concentrated by vacuum rotavapor. The concentrated sample was 10 mL.

Test for steroid

1. Salkowsti Test

Small amount of charcoal treated colorless liquid was mixed with CH_3OH, then a few drops of conc. H_2SO_4 was added to it followed by a few drops of Ac_2O. No characteristic color of steroid was observed.

2. Test for alkaloid

About 0.5 mL charcoal treated HM-01 was mixed with 5 mL 1% HCl then it was filtrated. To the 1 mL filtrate 3 drops Dragendorff's reagent was added. No characteri stic color of alkaloid was observed.

vii) Determination of Heavy metals HM-01

HM-01 (3.696 g) sample was supplied to the BCSIR analytical Research laboratory of BCSIR, Dhaka to determine the Quantitative elementary analysis of heavy metals. The results are given in the Table No 1.

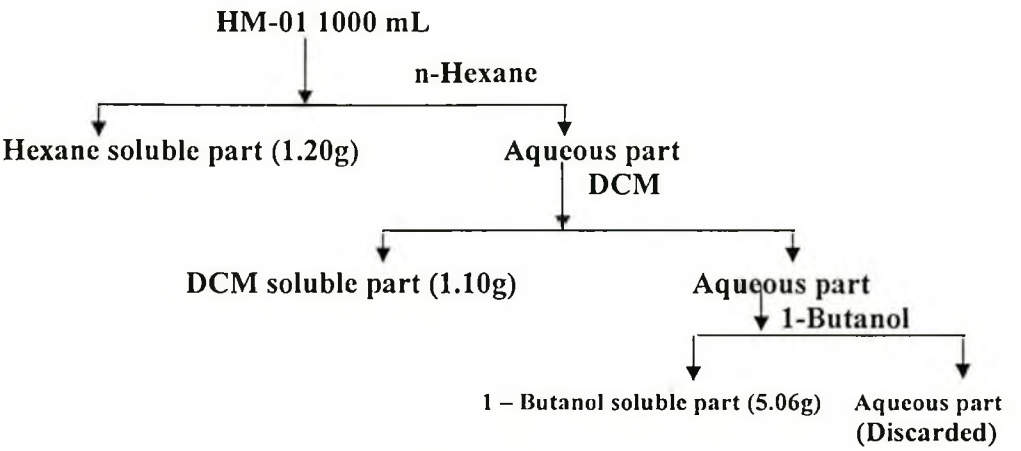
Table-1 Presence of heavy metal in HM-01

Sample Name	Elements	Concentration mg/L
HM-01	Pb	10.5
	As	0.16
	Cr	1.18

2.6.1. Extraction of HM-01

2.6.2. Extraction (Scheme 1) of HM-01 with organic solvent

1000 mL aqueous HM-01 was taken into a separating funnel and it was partitioned with 1000 mL hexane three times, at room temperature. The hexane soluble part and aqueous part was then separated. The hexane soluble part was evaporated to dryness by a rotary vacuum evaporator and finally dried by high vacuum freeze-dryer to give 1.20 g of solid material. The aqueous part was further partitioned with 1000 mL DCM three times. The DCM-soluble part was evaporated to dryness to get 1.10 g of DCM Extract. Then the remaining aqueous part was partitioned with 1000 mL 1-butanol three times. The 1-butanol soluble part and the aqueous part were evaporated separately to dryness finally dried by high vacuum freeze-dryer. The extraction scheme is shown in next page.



Extraction Scheme 1

2.6.3. Analysis of hexane soluble part

Hexane soluble part gave white crystalline solid. It was re-crystallised and nice crystals were obtained. Melting point of the crystals was found to be 110-120^{0}C.

2.6.4. Fractionation of DCM extract by column chromatography

The DCM extract was fractionated by silica gel column chromatography. A glass column (50 cm × 3 cm) was packed with 40 g of column grade silica gel in as column equilibrating solvent (column volume 250 ml). The DCM extract (1.10g) was dissolve in DCM with added MeOH and column grade silica gel (5.0g) was added to the solution .It was then evaporated to dryness. The dried sample was applied carefully on the top of the column bed. After application of sample solvent, it was eluted hexane, DCM and finally MeOH with increasing polarity. The eluted samples were monitored by TLC and were combined on the basis of their R_f values in TLC. Finally 4 fractions were obtained .The amount of each fraction, the corresponding solvent polarity and TLC pattern are given in Table 2 and 3.

Table 2 Fractions of DCM extract by column chromatography.

Hexane	DCM	MeOH	Volume of solvent	Number of test tube
100	0	0	300	1-17
60	40	0	200	18-28
50	50	0	200	29-40
40	60	0	200	41-51
20	80	0	200	52-60
0	95	5	300	61-77
0	90	10	400	78-103
0	70	30	400	104-131
0	60	40	500	132-164
0	50	50	500	165-193
0	0	100	200	194-221

Table 3 Different fraction of DCM soluble part obtained by column Chromatography

Fraction Number	Test tube Number	Amount (g)	TLC pattern of the sample
ZF-1	18-60	0.325	Single spots
ZF-2	61-131	0.120	Two spots with tailing
ZF-3	132-193	0.200	Mixture of spots
ZF-4	194-221	0.150	Mixture of spots

2.6.5. Isolation of compound ZF-1.

The fraction ZF-1 (0.325g) was a white solid material. It was soluble in DCM. This fraction was purified by washing with hexane and a white solid material obtained. It was crystallized from hexane and it was labeled ZF-1 (0.250 g).

2.6.5.1. Characterizations of the compound

2.6.5.2. Characterization of the compound ZF-1

2.6.5.3. Physical and chemical characteristics of the compound:

The compound ZF-1was a colorless crystals materials. It was readily soluble in DCM. It melting point was found to be 120^0C.

2.6.5.4. Characterization of the compound ZF-1 by spectroscopic method

a) Ultra violet spectroscopy

In UV spectrum of the compound (ZF-1) (Fig. No-1) the absorption maxima, λmax at 782, 644, 633 and 221 nm.

b) Infrared (IR) spectroscopy

In IR spectrum of the compound (ZF-1) (Fig. No-2) the absorption frequencies at 3010, 2000, 1750, 1510, 1410, 1360, 1015, 905, 810, 760, 640 cm^{-1}.

c) ^{1}H NMR (400MHz) spectroscopy:

^{1}H NMR (400 MHz, $CDCL_3$) spectral data of the compound ZF-1 and their assignment are given the Table 4 and spectra are presented in (Fig 3-4).

a) Table 4 ^{1}H-NMR spectra data of compound ZF-1

δ values in ppm	Integration	Splitting pattern	Coupling constant (*J* in Hz)
8.13	2H	Doublet	*ortho* proton,7.69
7.62	1H	Triplet	*para* proton, 1.05
7.48	2H	Triplet	*meta* proton, 2.82

d) ^{13}C NMR (100MHz) spectroscopy

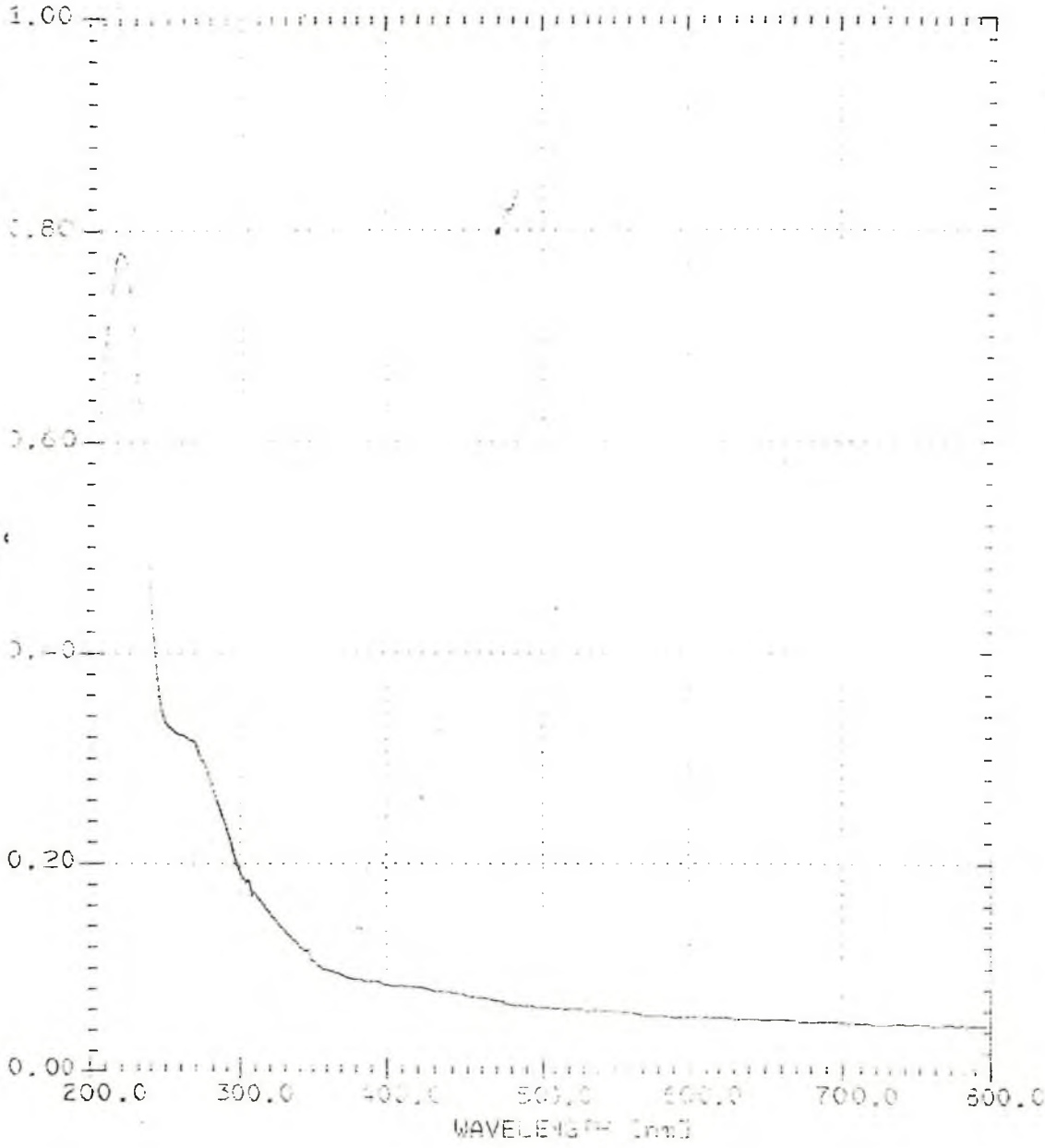
^{13}C NMR (100 MHz, $CDCl_3$) Spectrum (Fig-5-7) of compound (ZF-1) was recorded, the spectral data and their assignment are presented in the Table -5.

Table 5 ^{13}C NMR spectra data of compound ZF-1

Carbon No	Chemical Shift in ppm	Types of corbon
C-1	129.3	Quaternary carbon
C-2	133.2	Aromatic Carbon
C-3	128.4	Aromatic Carbon
C-4	130.2	Aromatic Carbon
C-5	128.4	Aromatic Carbon
C-6	133.2	Aromatic Carbon
-COO-	172.2	Carboxyl carbon

Table-6 DEPT Spectral data of the compound (ZF-1)

Carbon No	Chemical Shift in ppm	Types of corbon
C-2	133.2	=CH
C-3	128.4	=CH
C-4	130.2	=CH
C-5	128.4	=CH
C-6	133.2	=CH



*** PEAK-PICK ***

No.	-- PEAK -- λ	ABS	-- VALLEY -- λ	ABS
1	644.0	0.052	762.0	0.044
2	221.0	0.779	635.0	0.051

Figure No. -1 Ultraviolet Spectrum of Compound ZF-1

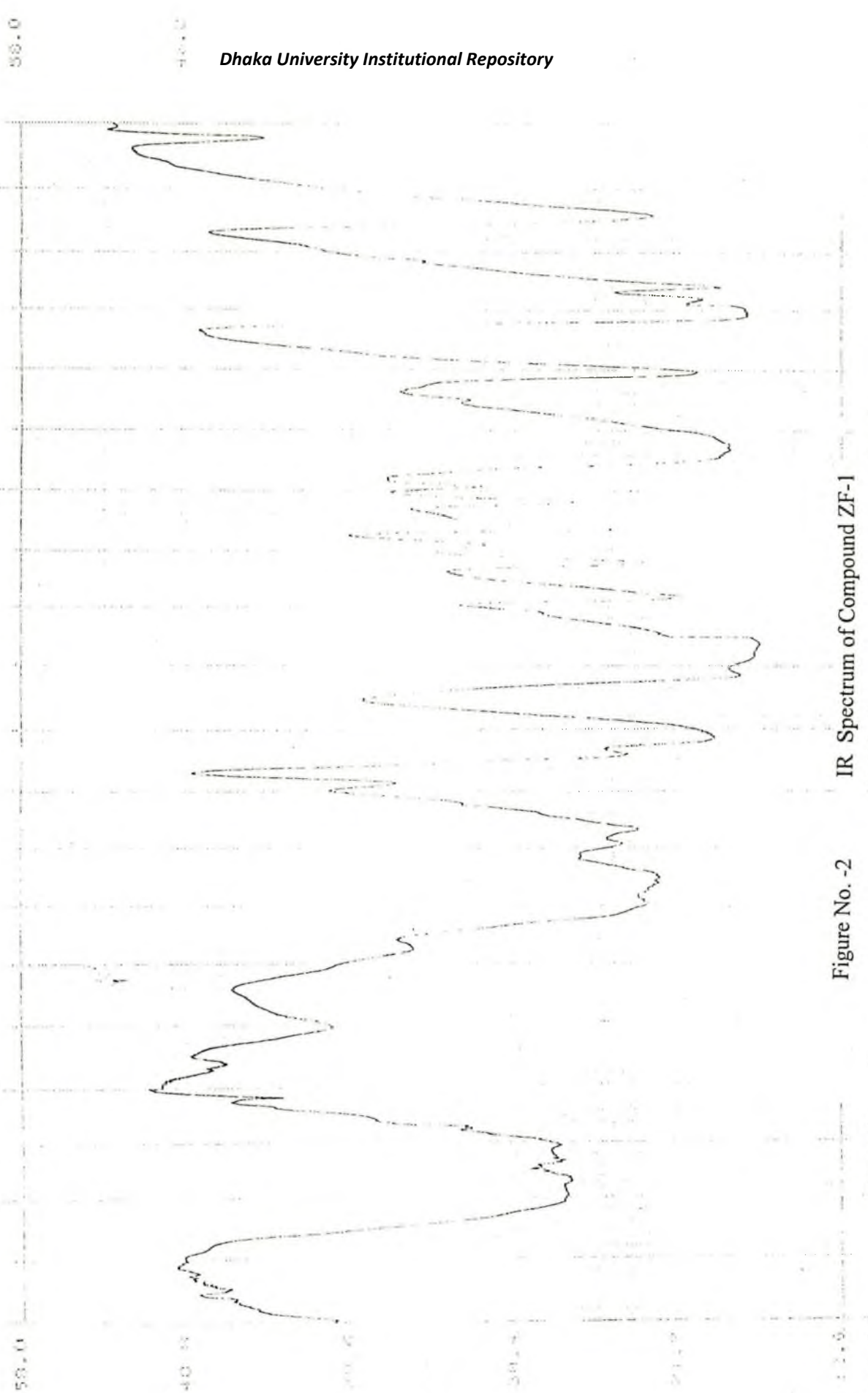


Figure No. -2 IR Spectrum of Compound ZF-1

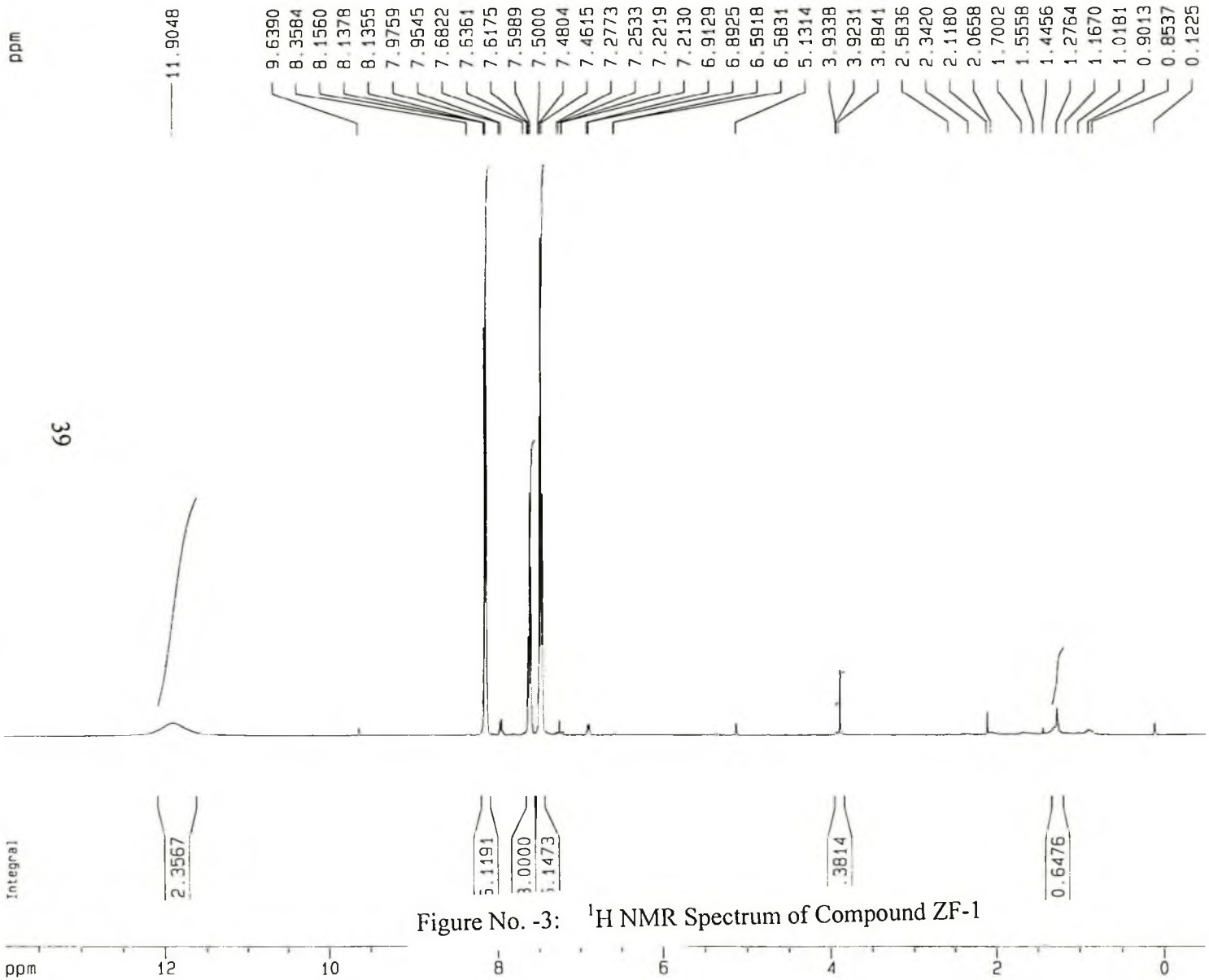


Figure No. -3: ^{1}H NMR Spectrum of Compound ZF-1

```
Current Data Parameters
NAME                  A3280
EXPNO                    1
PROCNO                   1

F2 - Acquisition Parameters
Date_              20070212
Time                 11.29
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                      64
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            400.1400111 MHz
WDW                    EM
SSB                     0
LB                   0.30 Hz
GB                      0
PC                   1.40

1D NMR plot parameters
CX                  20.00 cm
F1P                13.901 ppm
F1                 5562.27 Hz
F2P                -0.497 ppm
F2                 -198.85 Hz
PPMCM           0.71989 ppm/cm
HZCM           288.05582 Hz/cm
```

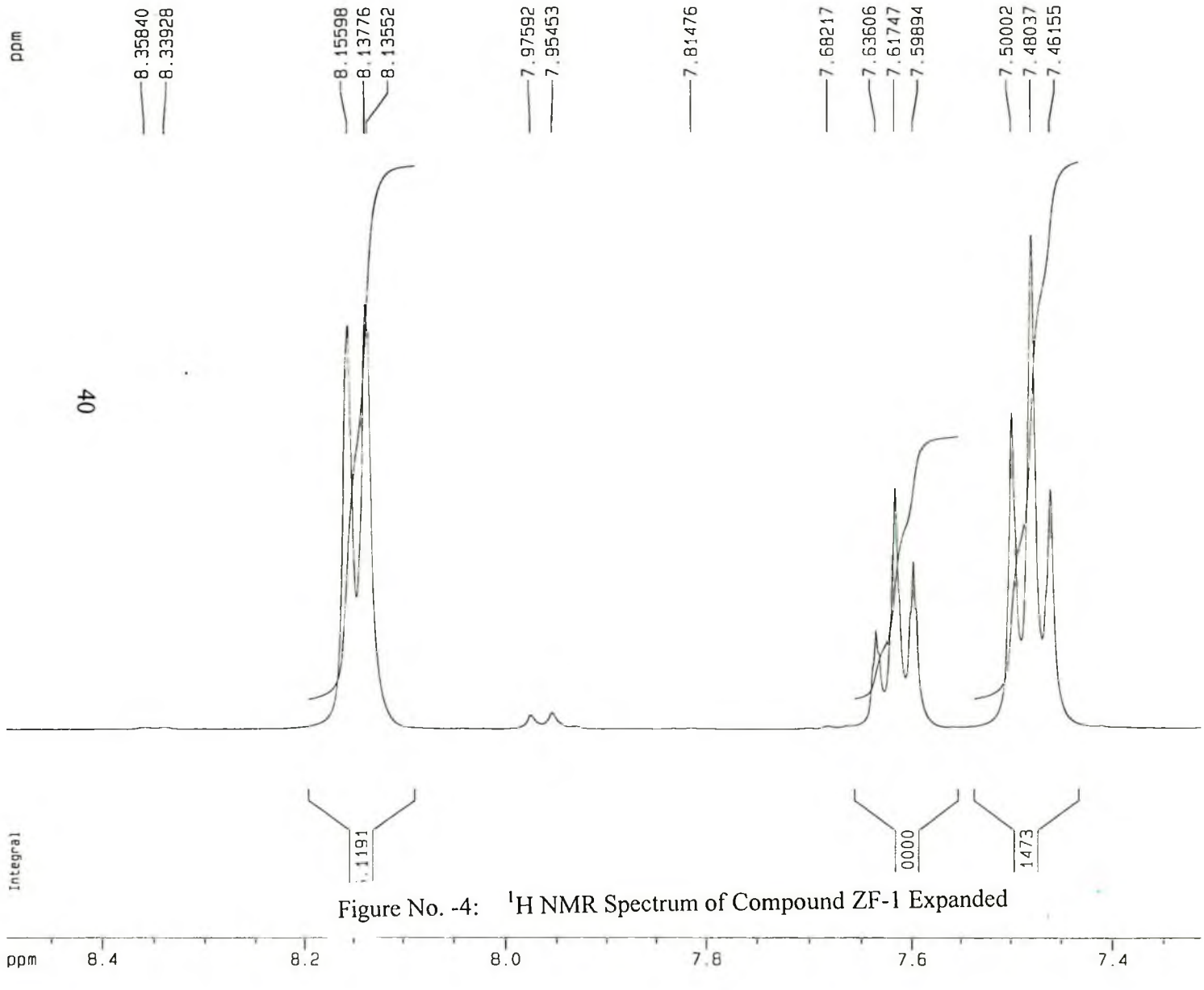
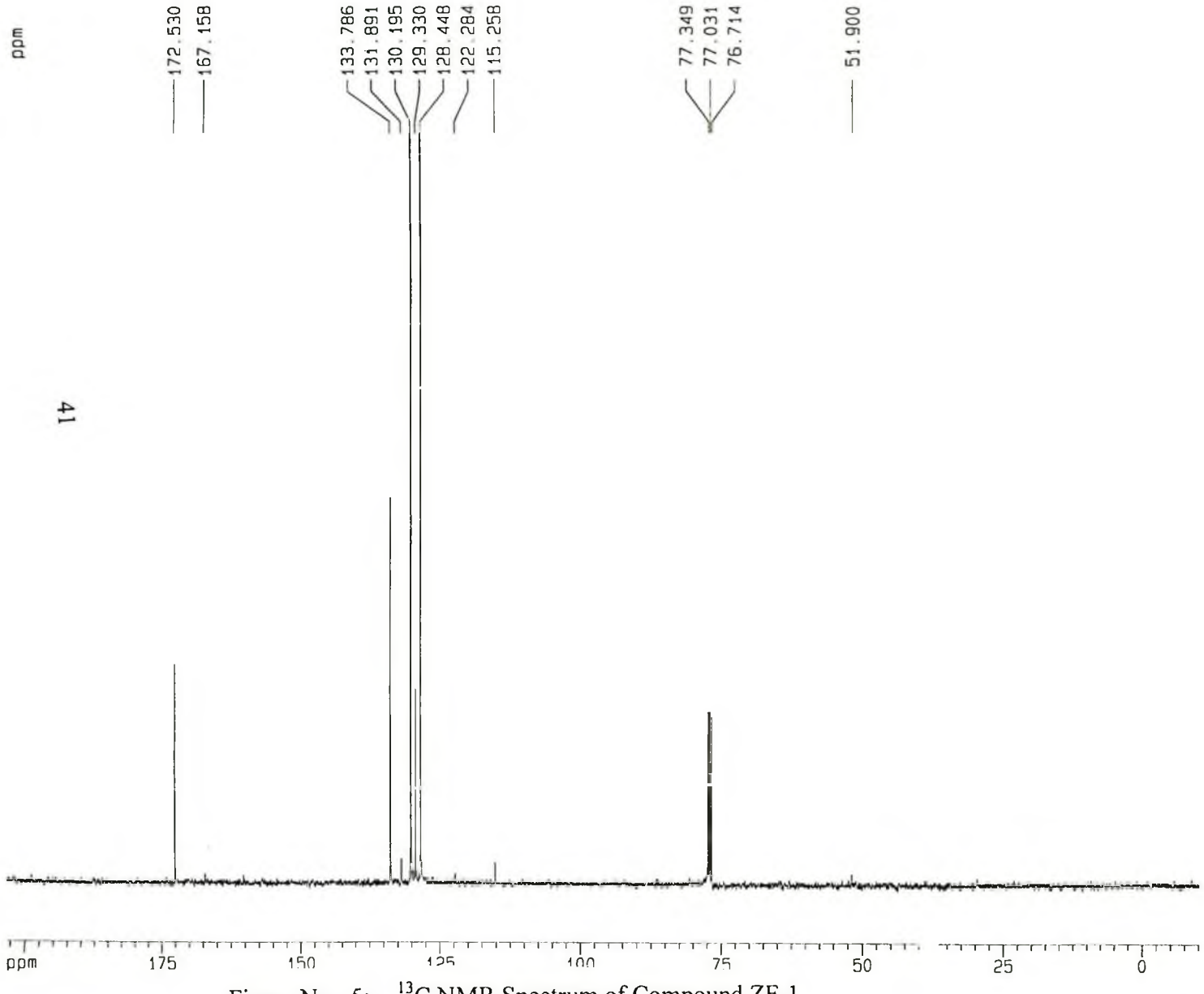


Figure No. -4: ^{1}H NMR Spectrum of Compound ZF-1 Expanded

```
Current Data Parameters
NAME                  A3280
EXPNO                     1
PROCNO                    1

F2 - Acquisition Parameters
Date_              20070212
Time                  11.29
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                       64
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             400.1400111 MHz
WDW                      EM
SSB                       0
LB                    0.30 Hz
GB                        0
PC                    1.40

1D NMR plot parameters
CX                   20.00 cm
F1P                   8.494 ppm
F1                 3398.98 Hz
F2P                   7.312 ppm
F2                 2926.01 Hz
PPMCM             0.05910 ppm/cm
HZCM             23.64823 Hz/cm
```

Analytical, BCSIR Lab. Dhaka 13C Spectrum Z-4 in CDCL3, Ziaul, DU.



Current Data Parameters

NAME	A3487
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20070515
Time	11.00
INSTRUM	dpx400
PROBHD	5 mm Multinuc
PULPROG	zgpg30
TD	32768
SOLVENT	CDCl3
NS	359
DS	2
SWH	24154.590 Hz
FIDRES	0.737140 Hz
AQ	0.6783476 sec
RG	16384
DW	20.700 usec
DE	6.00 usec
TE	300.0 K
D1	1.50000000 sec
d11	0.03000000 sec
d12	0.00002000 sec

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

NUC1	13C
P1	8.30 usec
PL1	-6.00 dB
SFO1	100.6253045 MHz

========== CHANNEL f2 ==========

CPDPRG2	waltz16
NUC2	1H
PCPD2	80.00 usec
PL2	-6.00 dB
PL12	16.00 dB
PL13	120.00 dB
SFO2	400.1400000 MHz

F2 - Processing parameters

SI	32768
SF	100.6152896 MHz
WDW	EM
SSB	0
LB	2.50 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	203.110 ppm
F1	20436.01 Hz
F2P	-10.115 ppm
F2	-1017.75 Hz
PPMCM	10.66128 ppm/cm
HZCM	1072.68799 Hz/cm

Figure No. -5: ^{13}C NMR Spectrum of Compound ZF-1

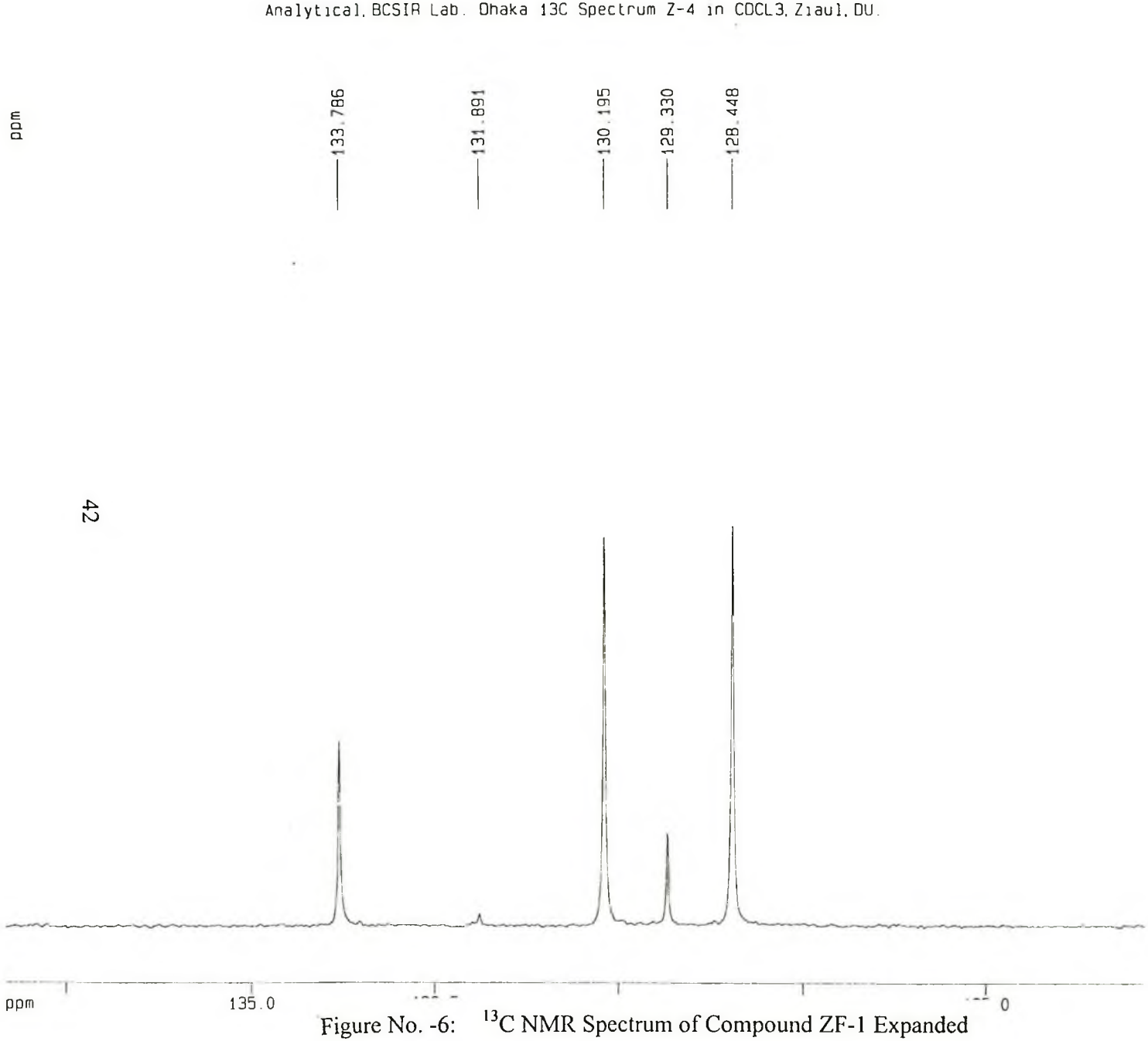
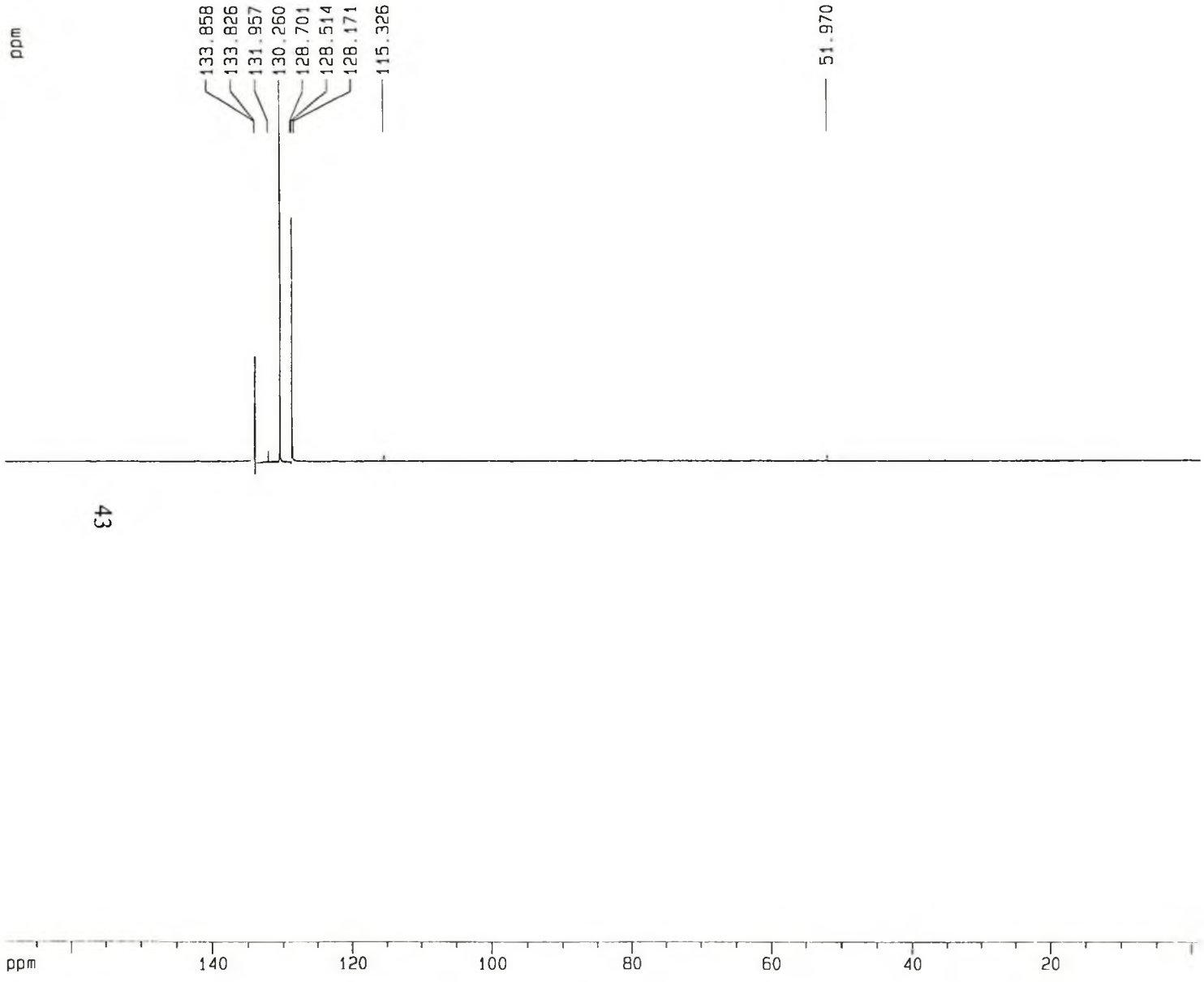


Figure No. -6: ^{13}C NMR Spectrum of Compound ZF-1 Expanded




```
Current Data Parameters
NAME                  A3487
EXPNO                     2
PROCNO                    1

F2 - Acquisition Parameters
Date_              20070515
Time                  11.37
INSTRUM              dpx400
PROBHD     5 mm Multinuc
PULPROG            dept135
TD                    32768
SOLVENT               CDCl3
NS                      425
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                   300.0 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
SFO1             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
SFO2             400.1420007 MHz

F2 - Processing parameters
SI                    32768
SF               100.6152832 MHz
WDW                      EM
SSB                       0
LB                     1.00 Hz
GB                        0
PC                     1.40

1D NMR plot parameters
CX                    20.00 cm
F1P                 169.494 ppm
F1                17053.66 Hz
F2P                  -1.188 ppm
F2                 -119.54 Hz
PPMCM                8.53409 ppm/cm
HZCM               858.65997 Hz/cm
```

Figure No. -7: DEPT- 135 NMR Spectrum of Compound ZF-1

e) 2D- NMR spectroscopy:

2D NMR Spectral data H-H COSY, HSQC and HMBC of the Compound ZF-1 are described in Table 7,8,9 and the spectra are shown in Fig-8-10.

f) Table-7 H-H COSY NMR spectral data of compound ZF-1

^{1}H		^{1}H
8.13 (H-2/H-6)	⟷	7.48 (H-3/H-5)
8.13 (H-2/H-6)	⟷	7.62 (H-4)

d) Table-8 HSQC NMR spectral data of compound ZF-1

δ_H in ppm		δ_C in ppm
8.13(H-2/H-6)	⟷	133.2 (C-2/C-6)
7.62(H-4)	⟷	130.2 (C-4)
7.48(H-3/H-5)	⟷	128.4 (C-3/C-5)

Table-9 HMBC spectral data of compound ZF-1

δ_H in ppm		δ_C in ppm
8. 13(H-2/H-6)	↔	130.2 (C-4), 128.4(C-3), 129.3 (C-2), 172.5(–COO-)
7.62(H-4)	↔	133.2 (C-2), 128.4 (C-3) 129.3 (C-1), 130.2 (C-4),172.5 (–COO-)
7.48(H-3/H-5)	↔	130.2(C-4), 128.4(C-3), 129.3 (C-1), 172.5(–COO-),133.2(C-2)

2.6.5.5. Spectroscopic Analysis of ZF-2, ZF-3 and ZF-4

^{1}H NMR spectrum of ZF-2, ZF-3 and ZF-4 were recorded and found to be very similar to ZF-1,

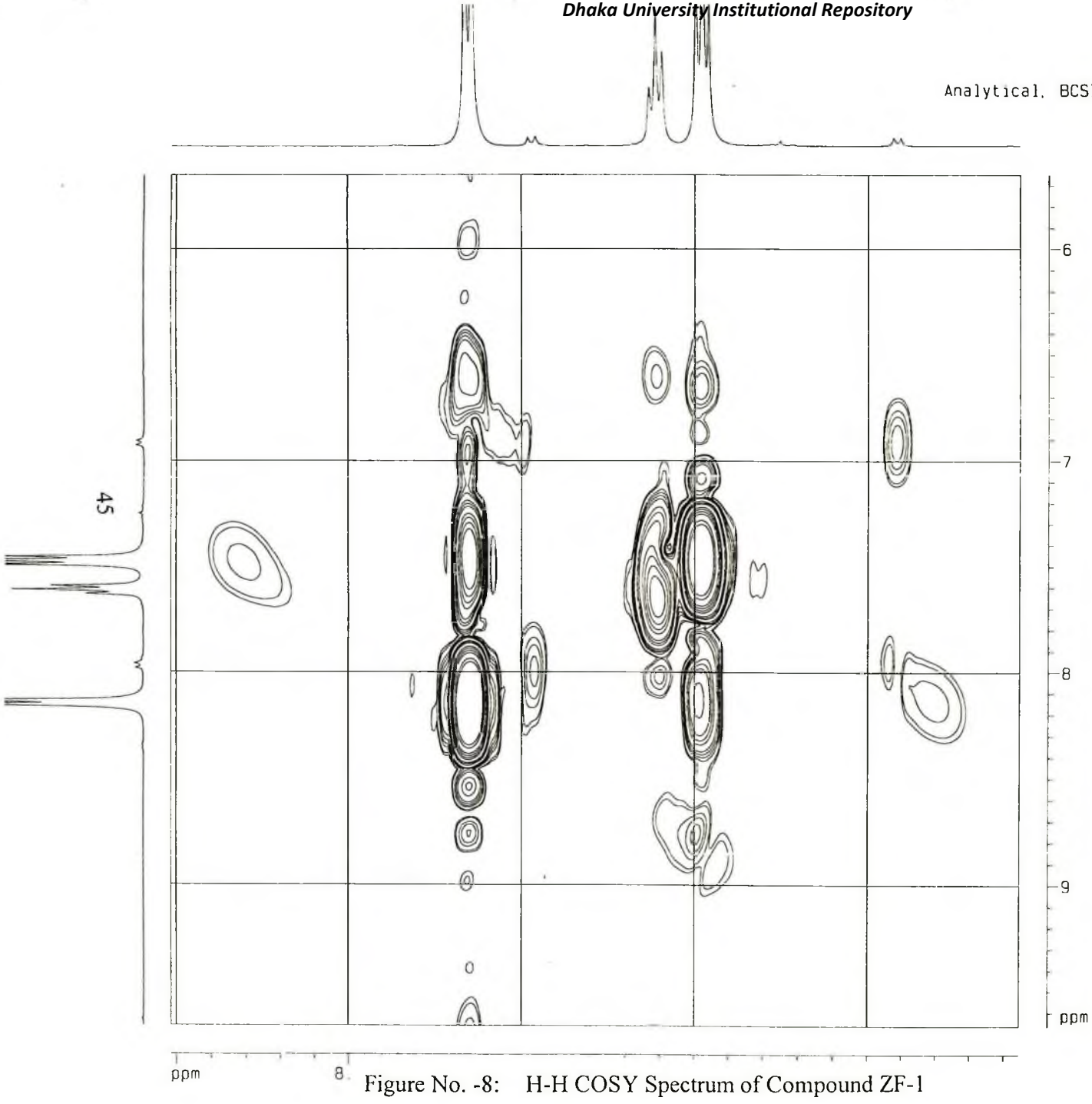


Figure No. -8: H-H COSY Spectrum of Compound ZF-1

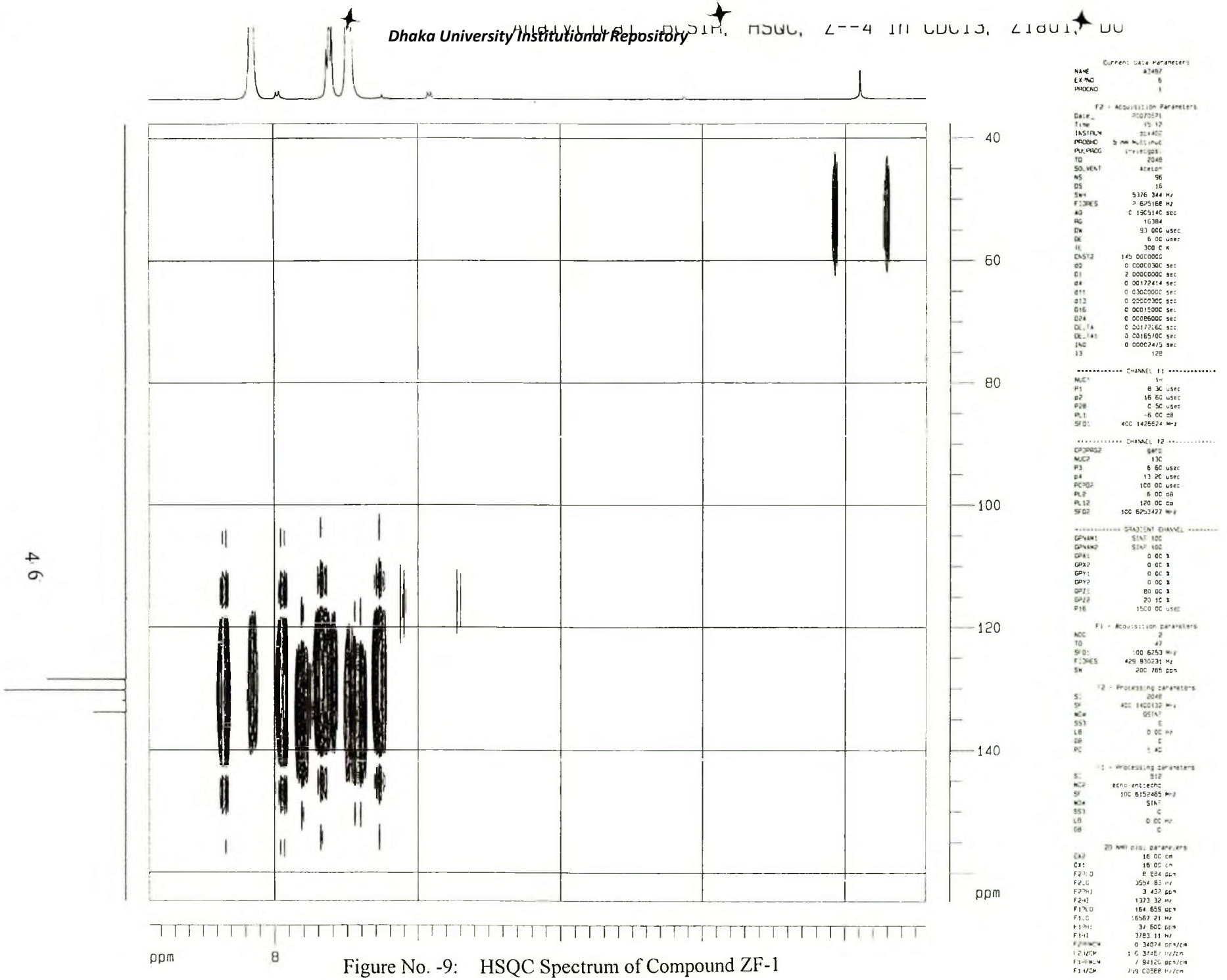


Figure No. -9: HSQC Spectrum of Compound ZF-1

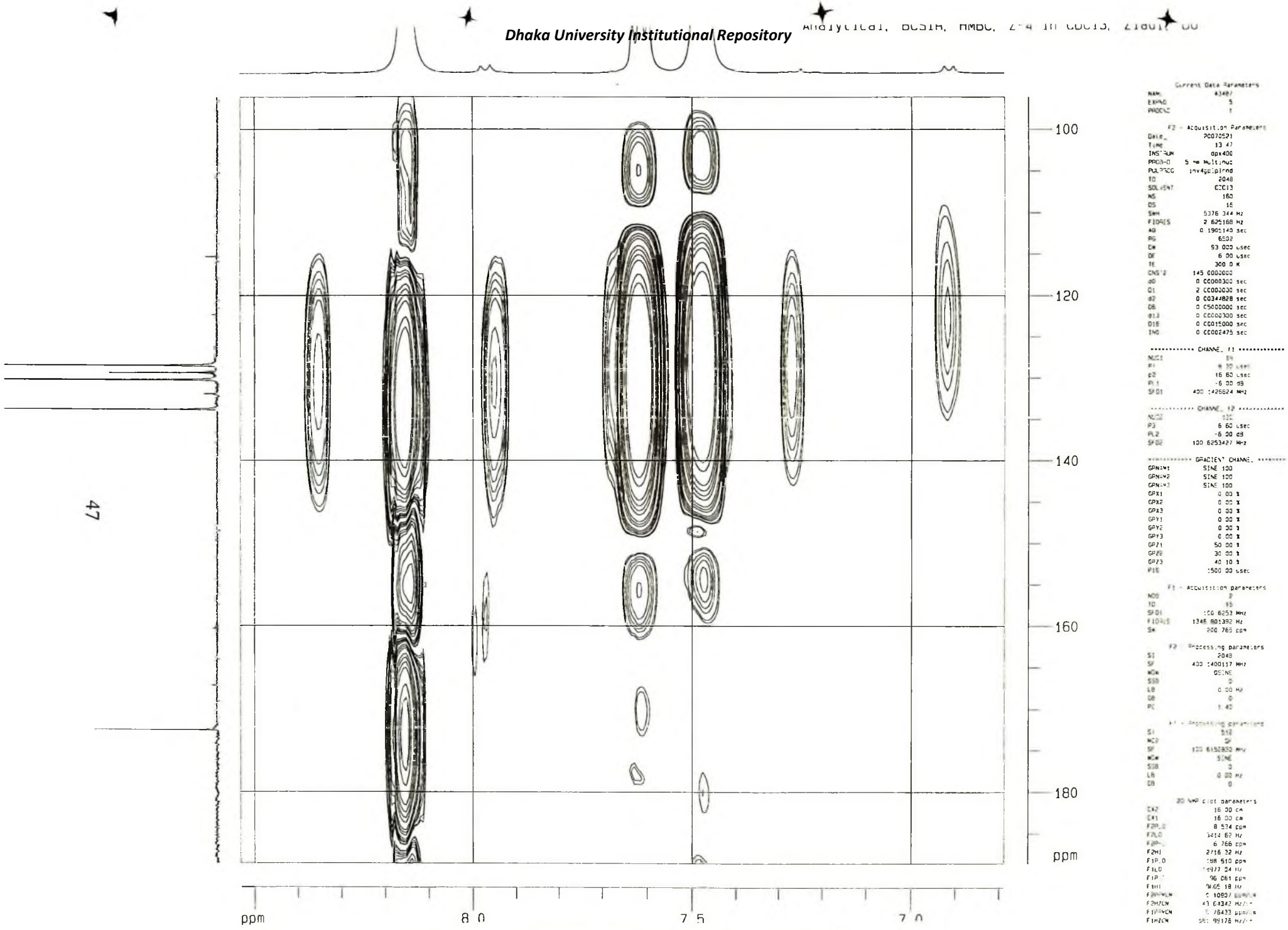
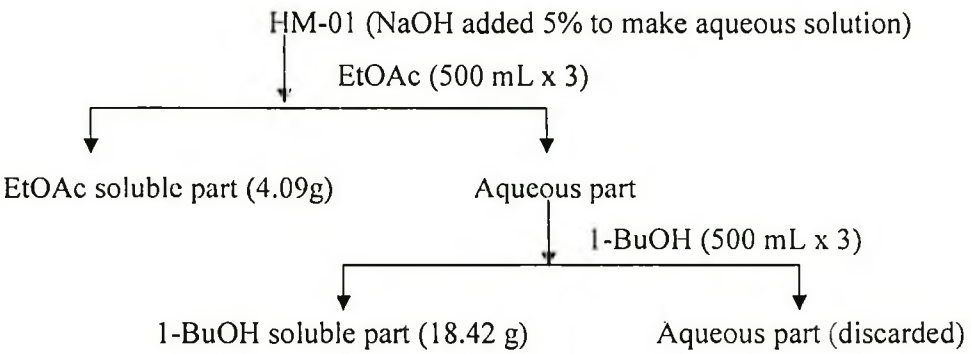


Figure No. -10: HMBC Spectrum of Compound ZF-1

2.6.5.6. Extraction of HM-01 with ethyl acetate

The HM-01 (500 mL) liquid sample was taken in a 1000 mL conical flask and 25g NaOH was added to it to make 5% NaOH solution. The solution was kept for 20 minites and the partitioned with EtOAc (500 mLx3). Two different layers were separated. The whole process was repeated by taking 2300 mL HM-01 liquid sample. The combined EtOAc soluble parts were evaporated to dryness, freeze-dried and 4.09 g solid materials was obtained. The aqueous part was partitioned with 1-butanol. The 1-butanol soluble parts were evaporated separately to dryness followed by freeze-drying to get 18.42g solid material.



Extraction Scheme-II

2.6.5.6.1. Fractionation of ethyl acetate soluble part by column chromatography

The ethyl acetate soluble part was fractioned by silica gel column chromatography. A glass column (250 mL x 3 cm) was packed with 80 g of column grade silica gel in DCM as column equilibrating solvent (column volume 250 mL). The EtOAc soluble part (4.09 g) was dissolved in DCM and column grade silica gel (10 g) was added to it and evaporated to dryness. The dried sample was applied carefully on the top of the column bed. After application of the sample solvent, polarity was increased from DCM to EtOAc. The eluted samples were collected in 190 test tubes and monitored by TLC, 16 Fractions were obtained. The amount of each fraction, the corresponding solvents polarity and TLC pattern are given in table 10 and 11.

Table 10 Fraction of EtOAc extract by column chromatography

DCM %	EtOAc %	MeOH %	Volume of solvent (ml)	Number of test tube
100	0	0	500	1-30
95	5	0	200	31-43
90	10	0	200	44-55
80	20	0	200	56-65
70	30	0	200	66-77
50	50	0	200	78-90
30	70	0	200	91-97
0	100	0	200	98-109
0	95	5	200	110-119
0	90	10	200	120-131
0	80	20	200	132-144
0	70	30	200	145-154
0	50	50	200	155-171
0	0	100	300	172-190

Table 11 Different fractions of EtOAc soluble part

Fraction	Test tube NO	Amount(g)	TLC pattern
ZSF_1	1-10	0.017	Single Spot
ZSF_2	11-18	0.020	Single Spot
ZSF_3	19-30	0.053	Two spots tailing
ZSF_4	31-40	0.290	Two spots tailing
ZSF_5	41-52	0.115	Two spots tailing
ZSF_6	53-63	0.100	Two spots
ZSF_7	64-76	0.057	Single spots
ZSF_8	76-85	0.160	Two spots tailing
ZSF_9	86-98	0.078	Mixture
ZSF_{10}	99-105	0.115	Two spots
ZSF_{11}	106-118	0.054	Mixture
ZSF_{12}	119-125	0.125	Two spots tailing
ZSF_{13}	126-132	0.823	Long tailing
ZSF_{14}	133-155	0.858	Mixture
ZSF_{15}	156-175	0.153	Mixture
ZSF_{16}	176-190	0.205	Mixture

2.6.5.6.2. Isolation of compound ZSF-1

The fraction ZSF-1 (0.017 g) was a white color material. It was soluble in EtOAc. This fraction was purified by washing with hexane and white solid materials obtained. It was re-crystallized from hexane to give nice colorless crystals (0.012 g).

2.6.6. Characterizations of the compound

2.6.6.1. Characterization of the compound (ZS F-1)

2.6.6.2. Physical and chemical characteristics of the compound ZSF-1

The compound ZSF-1 was nice colorless crystalls. It was readily soluble in DCM and EtOAc.

2.6.6.3. Characterization of the compound ZSF-1 by spectroscopic method

a) Ultra violet spectroscopy

In UV spectrum of the compound ZSF-1 (Fig. No-11) had the absorption maxima, λmax at 276, 260, 245 nm.

b) Infrared (IR) spectroscopy

In IR spectrum of the compound ZSF-1 (Fig. No-12) the absorption frequencies at 3050, 1550, 1820, 1360, 1220, 1160, 840, 800 cm^{-1}.

c) ^{1}H NMR (400MHz) spectroscopy

^{1}H NMR (400 MHz) spectral data of the compound ZSF-1 (Fig-13-14) and their assignment are given the Table -12.

Table 12 ^{1}H NMR spectra ZSF-1

δ values in pmm	Integration	Splitting pattern	Coupling constant	Assignment
3.88	3H	Singlet	-	$-CH_3$ proton
6.72	1H	Brood Singlet	-	-OH proton
6.88	2H	Doublet	8.74	*ortho* proton
7.93	2H	Doublet	8.74	*meta* proton

d) ^{13}C NMR Spectroscopy

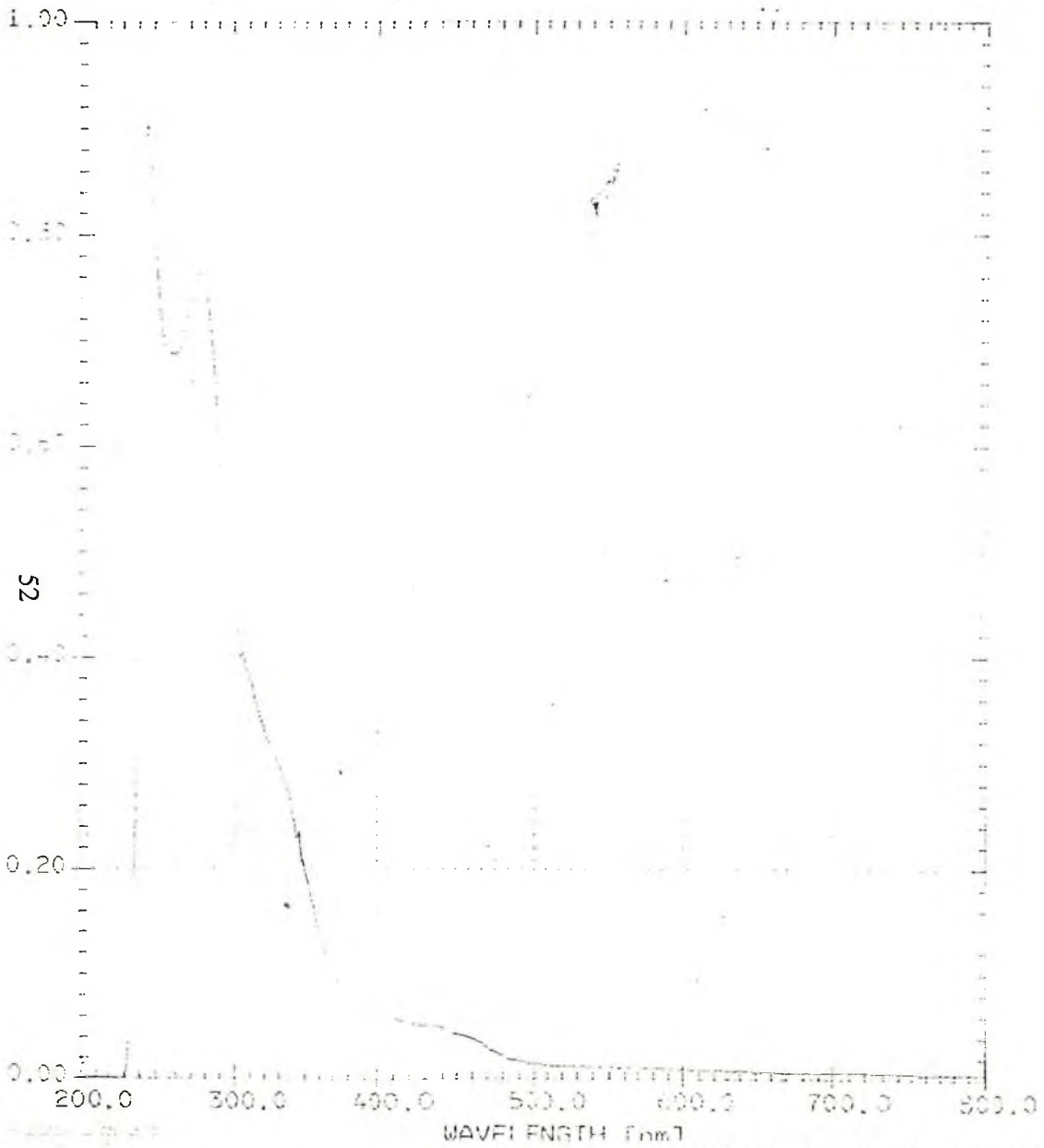
^{13}C NMR (100 MHz, $CDCl_3$) (Fig-15) of compound ZSF-1 was recorded the spectral data and their assignment are presented in the Table-13.

Table 13 ^{13}C NMR spectral data of compound ZSF-1

Carbon No	Chemical Shift in ppm	Types of carbon
C-1	122.2	Aromatic carbon
C-2	115.3	Aromatic carbon
C-3	131.9	Aromatic carbon
C-4	160.3	Aromatic carbon
C-5	131.9	Aromatic carbon
C-6	115.3	Aromatic carbon
CH_3	52.1	$-CH_3$ carbon
-COO-	167.5	Ester carbonyl carbon

Table 14 DEPT Spectral data of Compound ZSF-1

Carbon No	Chemical Shift in ppm	Types of carbon
$-CH_3$	52.1	$-CH_3$
C-2	115.3	=CH
C-3	131.9	=CH



xxx PEAK-PICK xxx

No.	-- PEAK -- λ	ABS	-- VALLEY -- λ	ABS
1	276.0	0.784	260.0	0.688
2	245.0	0.907		

Figure No. -11 Ultraviolet Spectrum of Compound ZSF-1

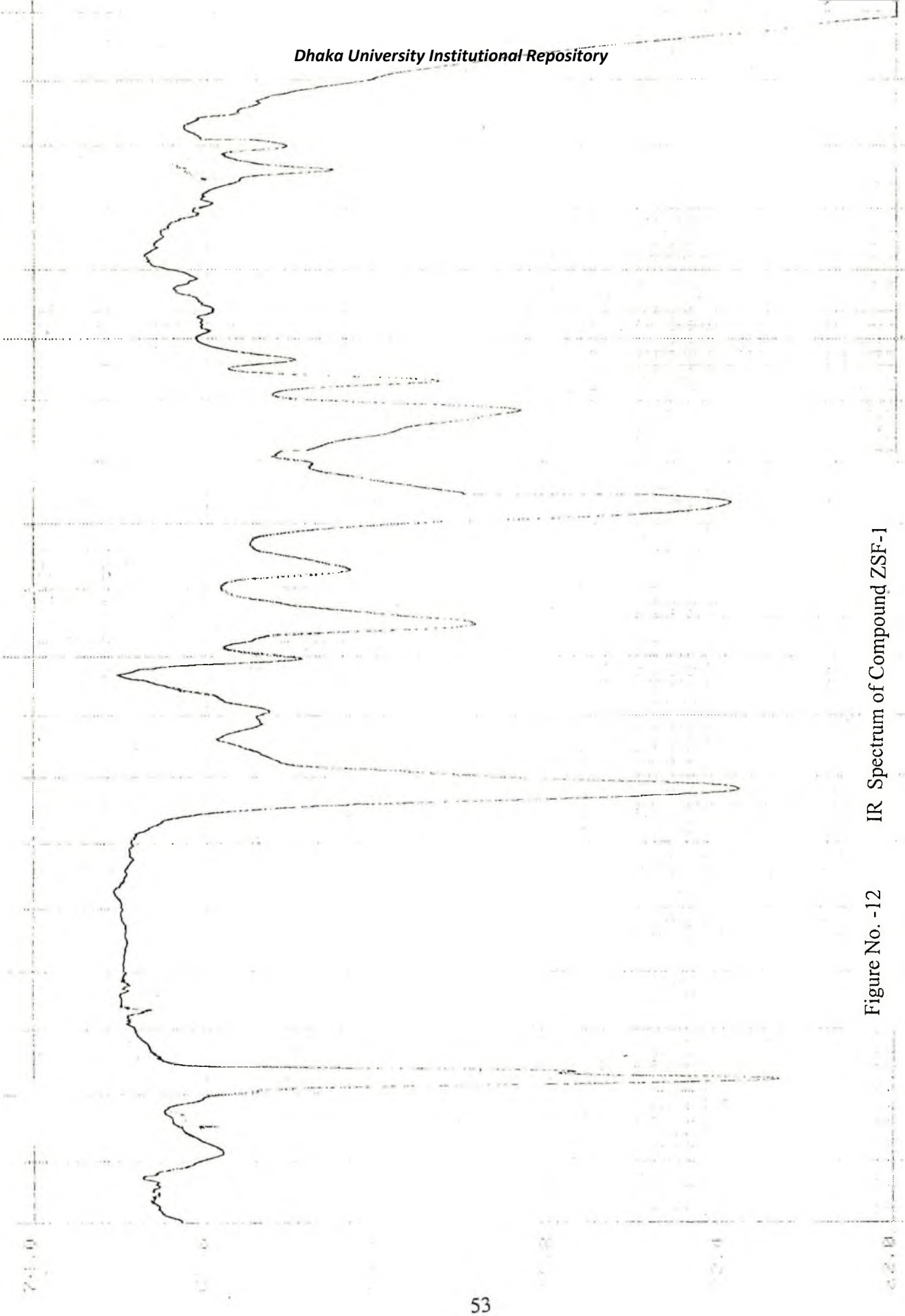


Figure No. -12 IR Spectrum of Compound ZSF-1

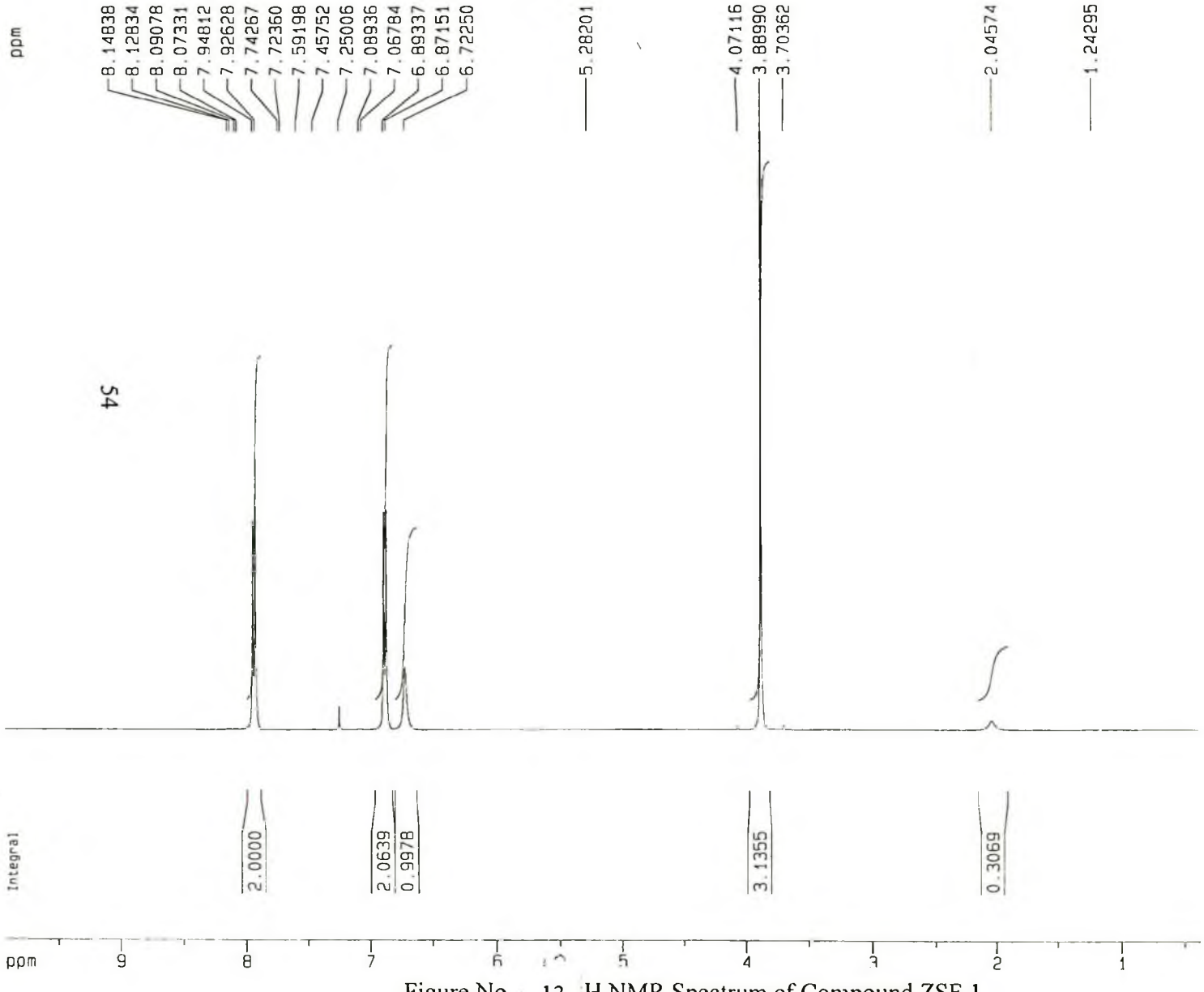


Figure No. 13 H NMR Spectrum of Compound ZSF-1

Current Data Parameters

NAME	A3279
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20070212
Time	9.50
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	256
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	9.926 ppm
F1	3971.86 Hz
F2P	0.378 ppm
F2	151.40 Hz
PPMCM	0.47739 ppm/cm
HZCM	191.02293 Hz/cm

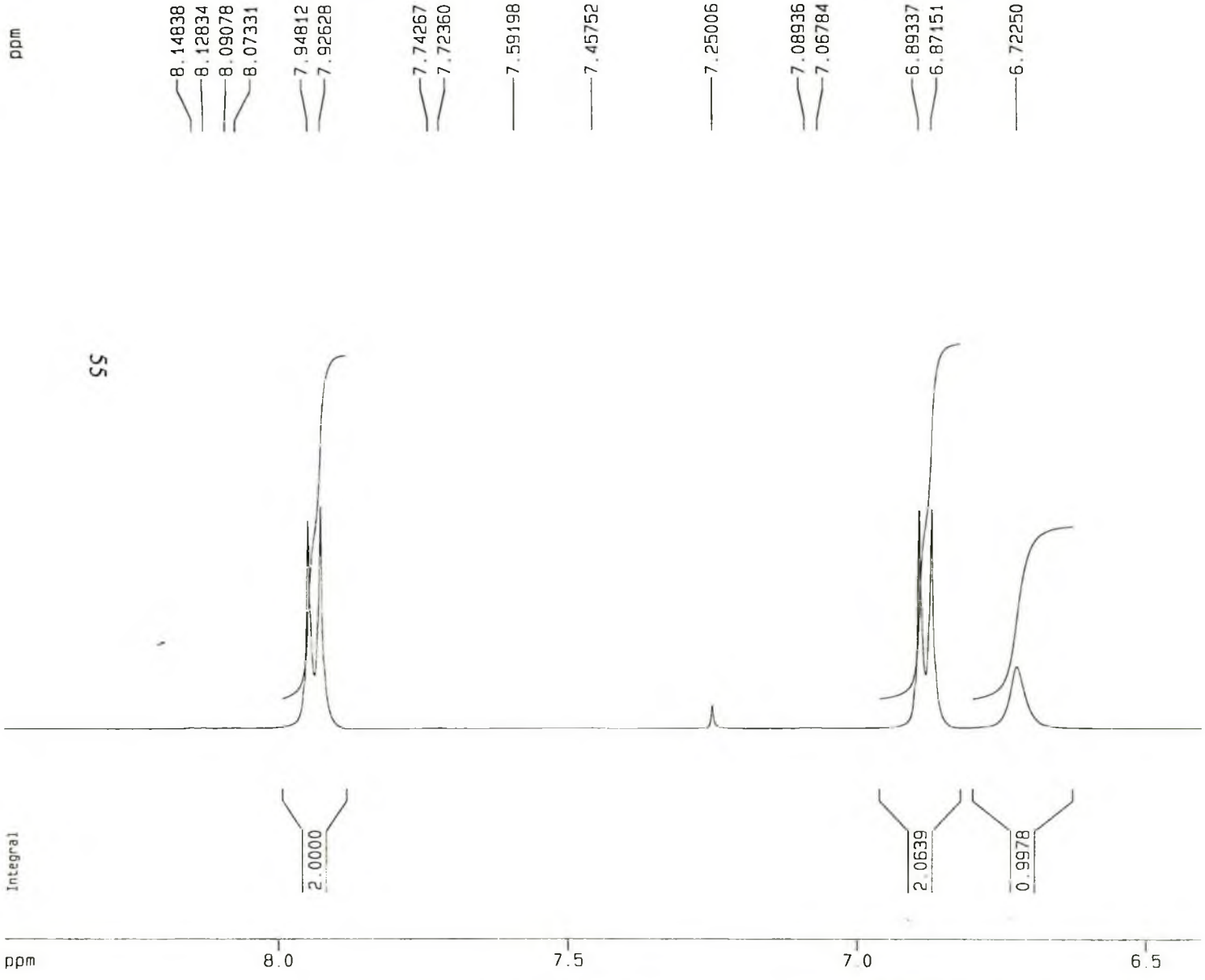


Figure No. 14 : ^{1}H NMR Spectrum of Compound ZSF-1 (Expanded)

```
Current Data Parameters
NAME                  A3279
EXPNO                     1
PROCNO                    1

F2 - Acquisition Parameters
Date_              20070212
Time                   9.50
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                     256
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                8.471 ppm
F1              3389.74 Hz
F2P                6.403 ppm
F2              2561.92 Hz
PPMCM           0.10344 ppm/cm
HZCM           41.39081 Hz/cm
```

Analytical, BCSIR Lab. Dhaka 13C Spectrum Z-3 in CDCL3, Ziaul, DU.

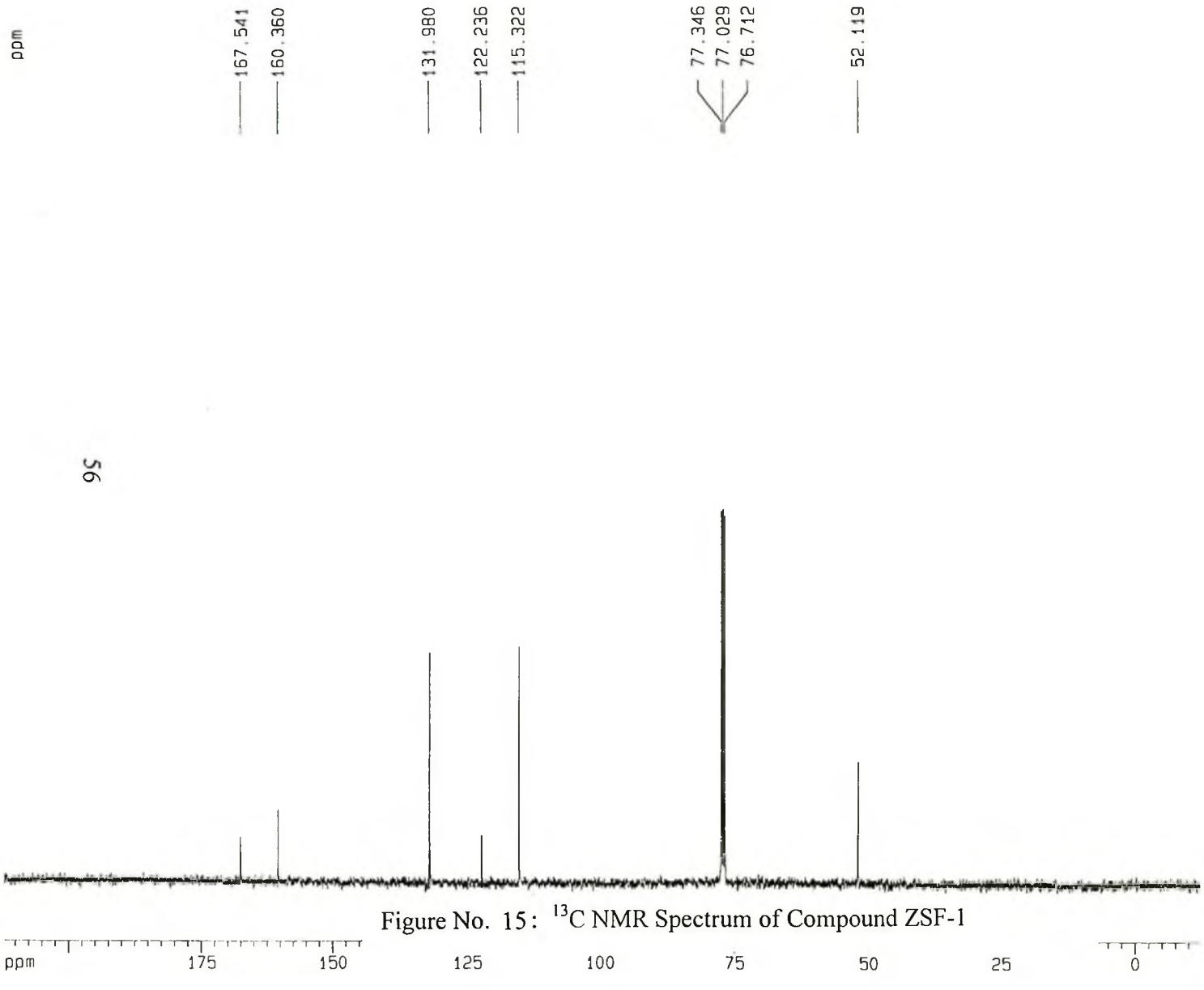


Figure No. 15: ^{13}C NMR Spectrum of Compound ZSF-1

```
Current Data Parameters
NAME                  A3488
EXPNO                    1
PROCNO                   1

F2 - Acquisition Parameters
Date_              20070515
Time                 12.07
INSTRUM              cpx400
PROBHD     5 mm Multinuc
PULPROG              zgpg30
TD                   32768
SOLVENT              CDCl3
NS                     719
DS                       2
SWH              24154.590 Hz
FIDRES           0.737140 Hz
AQ               0.6783476 sec
RG                   16384
DW                  20.700 usec
DE                   6.00 usec
TE                  300.0 K
D1               1.50000000 sec
d11              0.03000000 sec
d12              0.00002000 sec

============ CHANNEL f1 ============
NUC1                   13C
P1                    8.30 usec
PL1                  -6.00 dB
SFO1           100.6253045 MHz

============ CHANNEL f2 ============
CPDPRG2            waltz16
NUC2                   1H
PCPD2                80.00 usec
PL2                  -6.00 dB
PL12                 16.00 dB
PL13                120.00 dB
SFO2           400.1400000 MHz

F2 - Processing parameters
SI                   32768
SF             100.6152830 MHz
WDW                    EM
SSB                     0
LB                   2.50 Hz
GB                      0
PC                   1.40

1D NMR plot parameters
CX                  20.00 cm
F1P                212.040 ppm
F1                 21334.43 Hz
F2P                -12.075 ppm
F2                 -1214.95 Hz
PPMCM              11.20574 ppm/cm
HZCM            1127.46899 Hz/cm
```

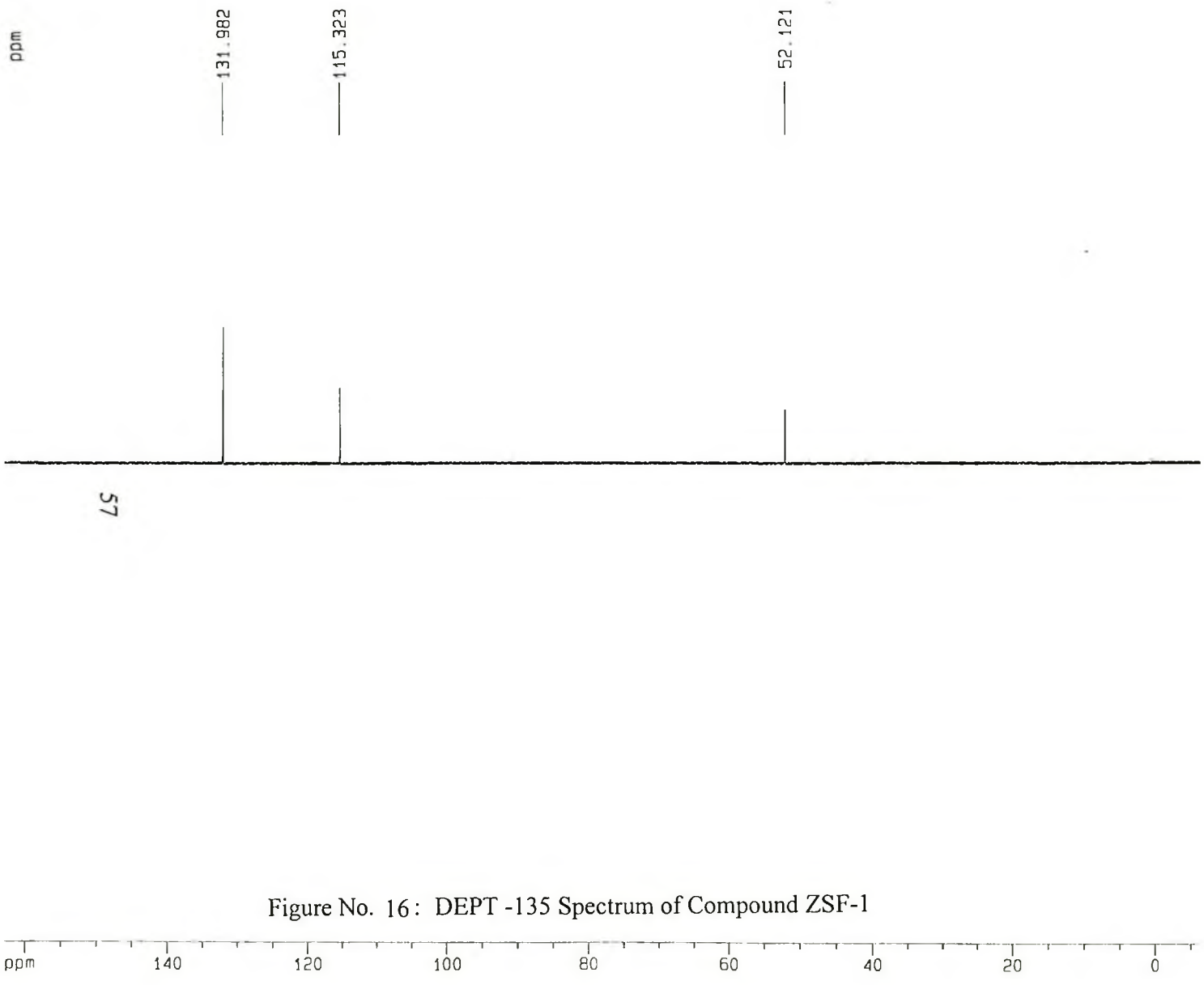


Figure No. 16 : DEPT -135 Spectrum of Compound ZSF-1

```
Current Data Parameters
NAME                  A3488
EXPNO                     2
PROCNO                    1

F2 - Acquisition Parameters
Date_              20070515
Time                  12.22
INSTRUM               dpx400
PROBHD     5 mm Multinuc
PULPROG             dept135
TD                    32768
SOLVENT               CDCl3
NS                      248
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                   300.0 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
SFO1             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
SFO2             400.1420007 MHz

F2 - Processing parameters
SI                    32768
SF               100.6152832 MHz
WDW                      EM
SSB                       0
LB                    1.00 Hz
GB                        0
PC                    1.40

1D NMR plot parameters
CX                   20.00 cm
F1P                 162.910 ppm
F1                16391.19 Hz
F2P                  -6.253 ppm
F2                 -629.13 Hz
PPMCM                8.45812 ppm/cm
HZCM               851.01611 Hz/cm
```

f) 2D NMR spectroscopy

2D NMR Spectra (Fig 17-19) H-H COSY, HSQC and HMBC of compound ZSF-1 were recorded. The spectral data are given in the table 15-17.

Table 15 H-H COSY NMR spectra data of compound ZSF-1

^1H		^1H
6.89 (H-2/H-6)	⟷	7.94(H-3/H-5)

Table16 HSQC NMR spectral data of compound ZSF-1

δ_H in ppm		δc in ppm
3.88 (-CH_3 proton)	⟷	52.1 (-CH_3 carbon)
6.89 (H-2/H-6)	⟷	115.3 (C-2/C-6)
7.94 (H-3/H-5)	⟷	131.9 (C-3/C-5)

Table 17 HMBC spectral data of compound ZSF-1

δ_H in ppm		δc in ppm
7.94(H-3/H-5)	↔	160.3 (C-4), 132.9 (C-3), 122.2 (C-1),
6.89(H-2/H-6)	↔	167.5 (-COO-), 122.2 (C-1), 115.3(C-2/H-6),
3.88(-CH_3)	↔	167.5 (-COO-).

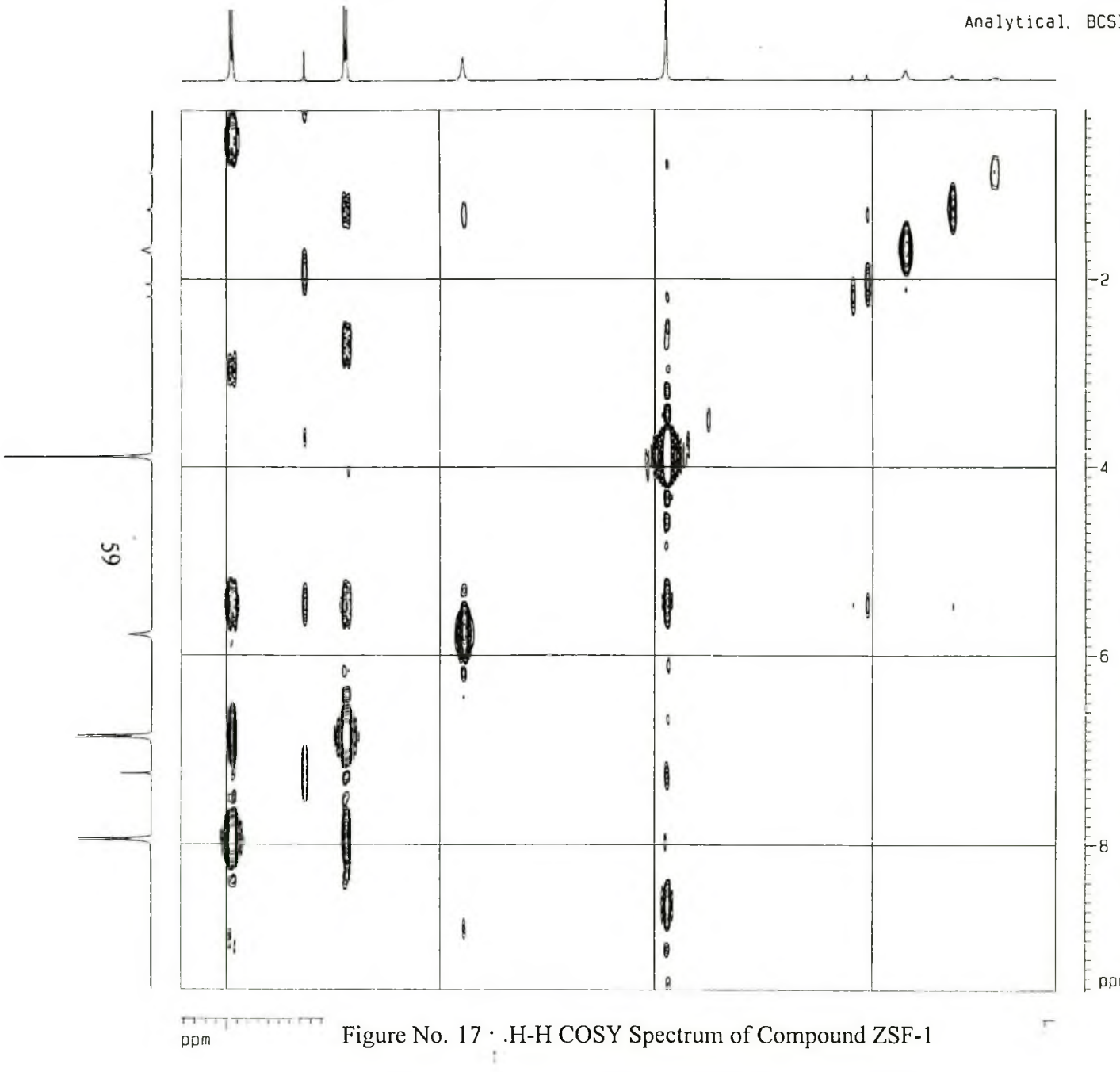


Figure No. 17 · .H-H COSY Spectrum of Compound ZSF-1

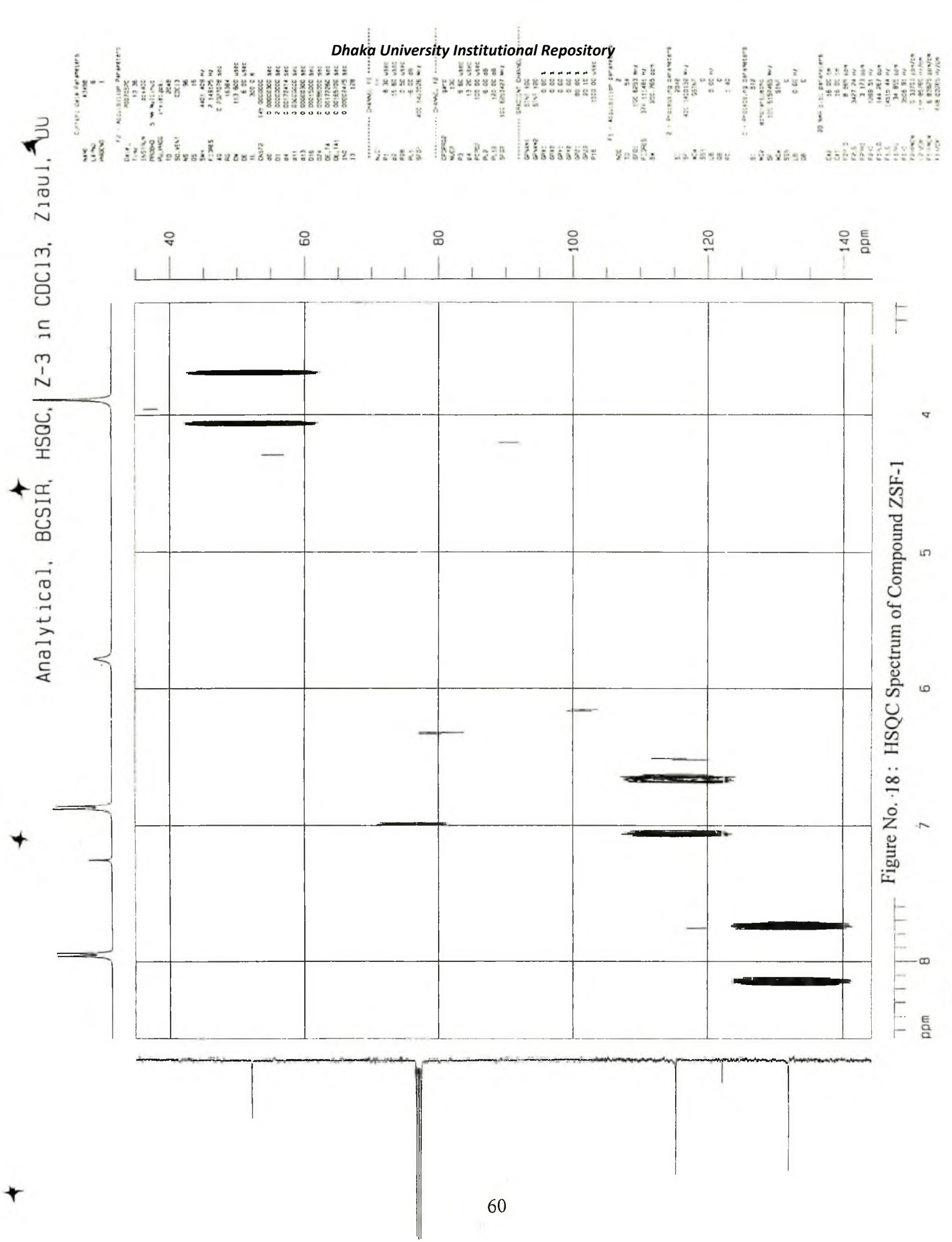


Figure No. 18 : HSQC Spectrum of Compound ZSF-1

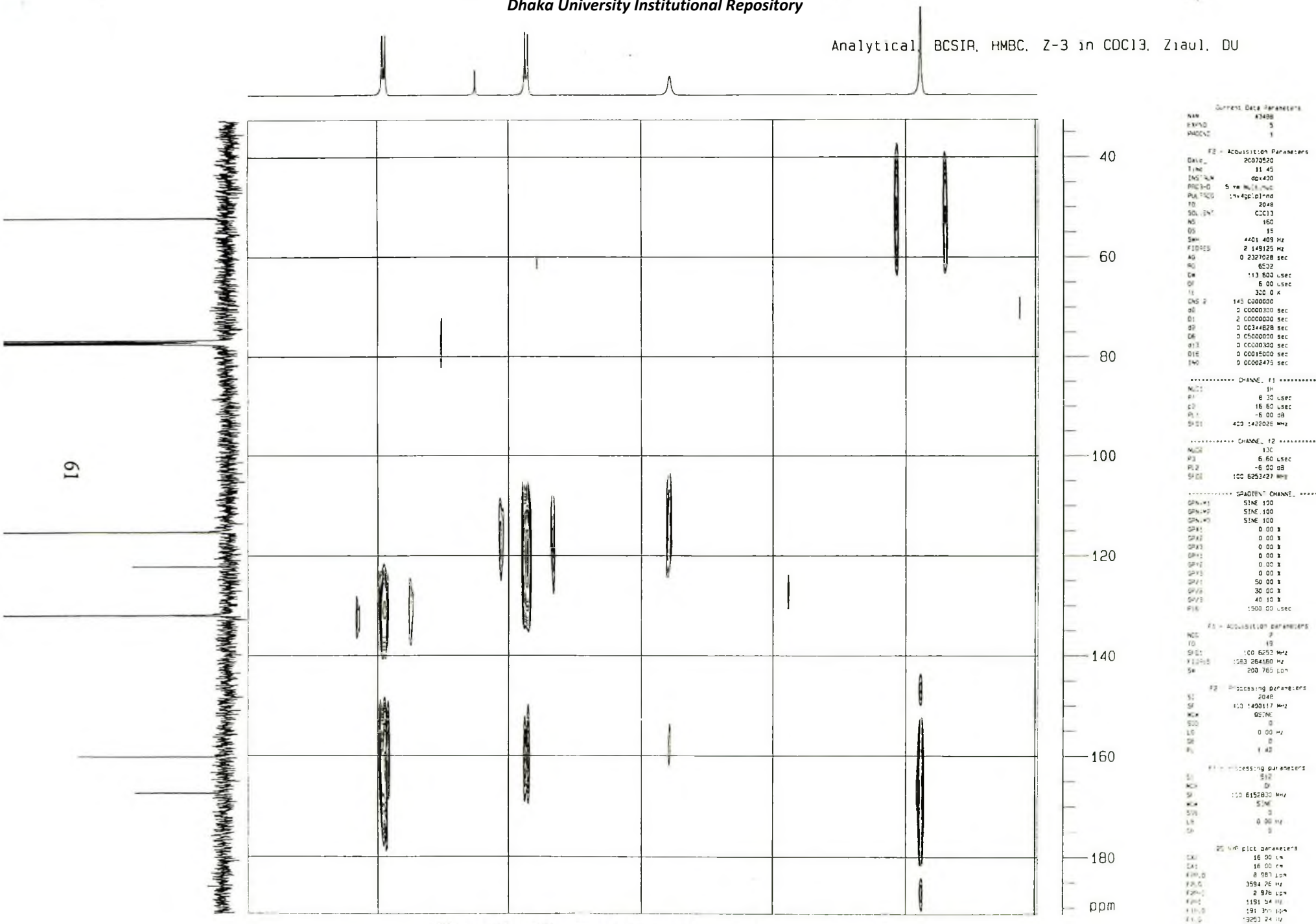


Figure No. 19: HMBC Spectrum of Compound ZSF-1

2.6.6.4. Estimation of Benzoic acid in HM-01

a) Gravimetric method

HM-01 (10 mL) was evaporated by a rotary vacuum evaporator followed by freeze-dryer to get 0.925 g of solid materials. The solid was dissolved in 5% aqueous NaOH (10 mL) and then partitioned with ethyl acetate (10 mL x 3). The aqueous part was acidified with dilute HCl and then again partitioned with ethyl acetate (10 mL x 3). The ethyl acetate soluble part was treated with anhydrous Na_2SO_4 and evaporated to dry-mass (0.012 g; 1.2%) which supposed to be benzoic acid.

b) HPLC Analysis

i) The HM-01 medicine was diluted in to 50.0, 0.50, 0.25 µL/ mL by HPLC grade methanol. Each of the diluted sample was filtered by HPLC sample filter and analyzed by HPLC using Acetonitrile:H_2O (1:1) as mobile phase in a reversed phase C-18 column with a flow rate of 0.7mL/min. HPLC profile of one of the diluted sample is presented in (Fig 20).

ii) Standard stock solution Benzoic acid (1mg/mL) was prepared by dissolving 10mg of benzoic acid in 10 mL of methanol. The stock solution (1mg/mL) was successively diluted with methanol to get 2.5, 1.25 and 0.625µg/ mL sample solution. Each of the diluted sample was filtered through HPLC sample. The filtered benzoic acid solution was analyzed by HPLC in same condition in which HM-01 was analyzed. The presence of benzoic acid in HM-01 was detected considering the same time of standard benzoic acid. A calibration curve of the benzoic acid was made using sigma plot and amount of benzoic acid present in the HM-01 sample quantified using the standard calibration curve. The calibration curve is presented in Fig 21 and formula for quantification is given below.

Natural Product Research Laboratory

Department of Chemistry
University of Dhaka

HPLC Analysis Report

Sample ID: HM-01.2
Filename: C:\CLASS-VP\Data\PDA Data
Method: C:\CLASS-VP\Methods\HM-02 &HM-01.met
User: System
Acquired: 6/14/2008 3:47:45 PM
Printed: 6/16/2008 12:05:01 PM

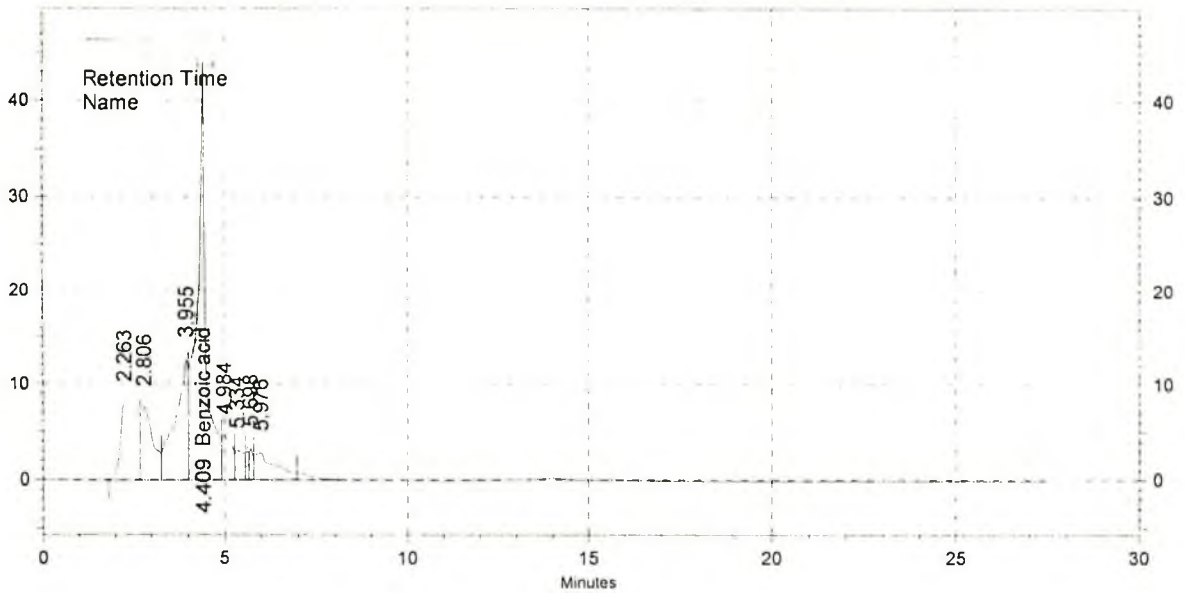


Figure No. 20.: Estimation of Benzoic acid in HM-01 .2 by HPLC Analysis

Pk #	Name	Retention Time	Height
1		2.263	7989
2		2.806	7529
3		3.955	12699
4	Benzoic acid	4.409	44189
5		4.984	4532
6		5.334	3035
	5.698	21908	

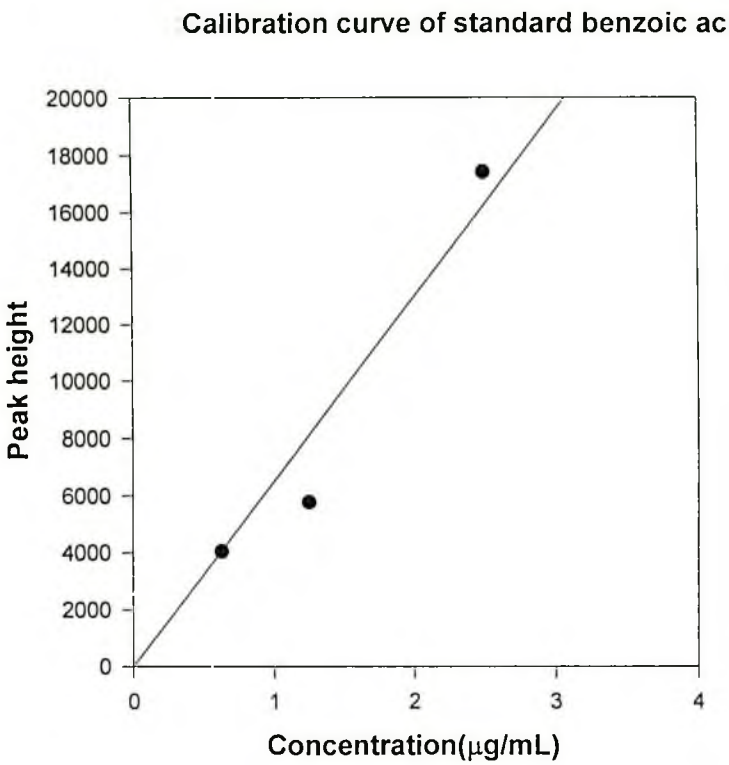


Fig 21. Calibration curve of Standard Benzoic Acid

$$\textbf{Concentration of unknown sample} = \frac{\text{Peak Height}_{\text{Sample}} \times \text{Conc.}_{\text{Std}}}{\text{Peak Height}_{\text{Std}} \times \text{Conc.}_{\text{sample}}}$$

2.7. Analysis of Herbal medicine of HM-02.

Physical characteristics of the sample

The liquid medicine is greenish in color. It is sour and bitter in taste.

i) Flavor: No smell of fermentation
No smell of Alcohol
Gives flavor of plan material.

The composition of the medicine as described by the company is given bellow:

ii) Plants with amount are used to prepare the medicine

Name of the Plants.		**Amount of plants in each 5ml (as aqueous extract)**
Cassia angustifolia	–	17.00 mg
Rheum emodi	–	13.00 mg
Cassia Occidentalis	–	12.50 mg
Ocimum sanctum	–	2.50 mg
Ipomoca turpethum	–	2.00 mg
Rosa damasceua	–	2.00 mg
Sphaeranthus indicus	–	2.00 mg
Gentiana Kurroo	-	2.00 mg
Oldenlandia corymbosa	–	2.00 mg
Clitoria termatea	–	2.00 mg
Artemisia absinthium	–	2.00 mg
Nymphae alba	–	1.25 mg
Dalbergia sissoo	–	1.25 mg
Pterocarpus santalinus	-	1.25 mg
Tinospora cordifolia	–	1.25 mg
Terminalia chebula	–	1.25 mg
Zingiber zerumbet	–	1.25 mg
Swertia chirata	–	1.25 mg
Andrographis Paniculata	–	1.25 mg
Bautinia Varieguta	–	1.25 mg
Azadirachta indica	–	1.25 mg
Curcuma longa	–	1.25 mg

iii) pH of the medicine was determined and found to be 5.37.

iv) Boiling point of the liquid sample was determined and found to be 110 -111^{0}C.

iv) Determination of Dry mass:

Procedure: Liquid sample was taken into a 100mL round bottom flask. Then the sample was evaporated by a rotatory vacuum evaporator at bath temperature below 400cto small volume (5 mL) the concentrated sample was dried by a freeze-dryer. The total dry mass of HM-02 was found to be (5.025g; 5.196%).

v) Ash content

Ash content of HM-02

The freeze dried sample (HM-02; 1.0937 g) was ignited about 2-3 hours in direct flame until the fume disappeared and then it was burned at 750^{0}C about 4-5 hours. It was repeatedly burnt and cooled until and constant weight was obtained (0.0271 g; 2.71%).

vi) Charcoal Treatment

10 mL of the sample HM-02 was taken in a beaker and diluted into 30 mL with water. The sample solution color was brown. Then small amount of conditioned charcoal was added to it. Then the beaker was shacked for a few minite. After that it was filtered. Filtrate was colorless. Then it was concentrated by vacuum rotavapor. The concentrated sample was 10 mL.

Test for steroid

1. Salkowsti Test

Small amount of charcoal treated colorless liquid was mixed with CH_3OH, then a few drops of conc. H_2SO_4 was added to it followed by a few drops of Ac_2O. No characteristic color of steroid was observed.

2. Test for alkaloid:

About 0.5 mL charcoal treated HM-01 was mixed with 5 mL 1% HCl then it was fillered.To the 1 mL filtrate 3 drops Dragendorff's reagent was added. No characteristic color of alkaloid was observed.

vii) Determination of Heavy metals HM-02

4.655 g of HM-02 sample was supplied to the analytical Research laboratory of BCSIR, Dhaka to determine the Quantitative elementary analysis of heavy metals. The results are given in the Table No .18

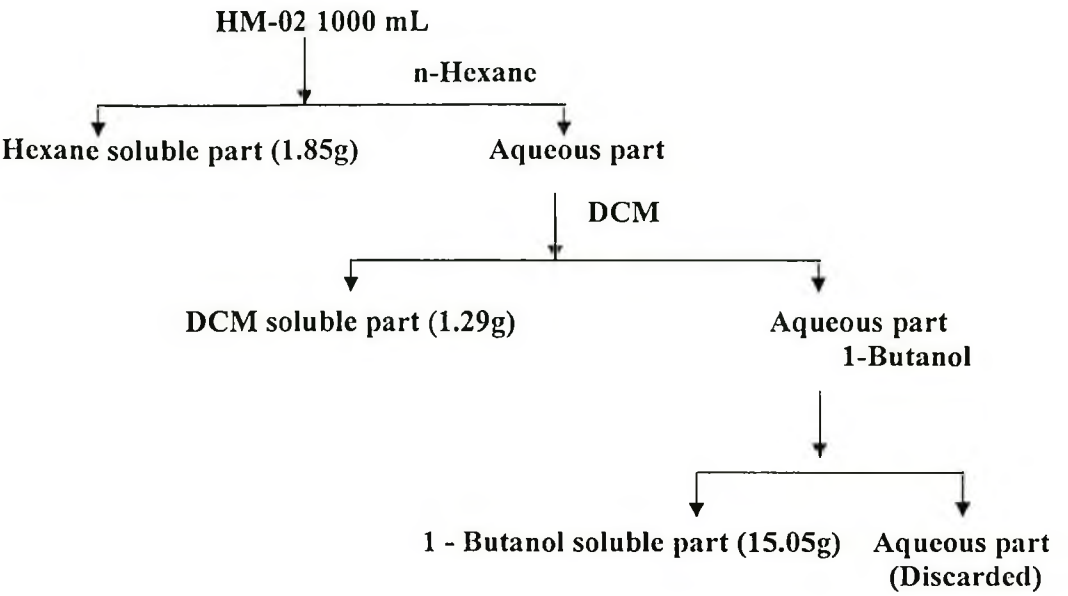
Table 18: presence of heavy metal in HM-02

Sample Name	Elements	Concentration mg/L
HM-02	Pb	4.55
	As	0.09
	Cr	0.88

2.7.1. Extraction of HM-02

2.7.2. Extraction (Scheme III) of HM-02 with organic solvent

HM-02 1000 mL was taken into a separating funnel and it was partitioned with 1000 mL hexane three times, at room temperature. The hexane soluble part and aqueous part was then separated .The hexane soluble part was evaporated to dryness by a rotary vacuum evaporator and finally dried by high vacuum freeze-dryer to give 1.85 g of solid material.The aqueous part was further partitioned with 1000 mL DCM three times. The DCM-soluble part was evaporated to dryness to get 1.29 g of DCM Extract. Then the remaining aqueous part was partitioned with 1000 mL 1-butanol three times, at room temperature. The 1-butanol soluble part and the aqueous part were evaporated separately to dryness finally dried by high vacuum freeze-dryer.The extraction Scheme is shown below.



Extraction Scheme III

2.7.3. Analysis of hexane soluble part:

Hexane soluble part gave white crystalline solid. It was re-crystallised and nice crystals were obtained. Melting point of the crystals was found to be 110-120^{0}C.

2.7.4. Fractionation of DCM extract by column chromatography

The DCM extract was fractionated by silica gel column chromatography. A glass column (50 cm × 3 cm) was packed with 40 g of column grade silica gel in as column equilibrating solvent (column volume 250 ml). The DCM extract (1.10g) was dissolve in DCM with added MeOH and column grade silica gel (5.0g) was added to the solution .It was then evaporated to dryness. The dried sample was applied carefully on the top of the column bed. After application of sample solvent, it was eluted hexane, DCM and finally MeOH with increasing polarity. The eluted samples were monitored by TLC and were combined on the basis of their R_f values in TLC. Finally 4 fractions were obtained .The amount of each fraction, the corresponding solvent polarity and TLC pattern are given in Table 19 and 20.

Table 19 Fractions of DCM extract by column chromatography

Hexane	DCM	MeOH	Volume of solvent	Number of test tube
100	0	0	300	1-17
60	40	0	200	18-28
50	50	0	200	29-40
40	60	0	200	41-51
20	80	0	200	52-60
0	95	5	300	61-77
0	90	10	400	78-103
0	70	30	400	104-131
0	60	40	500	132-164
0	50	50	500	165-193
0	0	100	200	194-221

Table 20 Different fraction of DCM soluble part obtained by column chromatography

Fraction Number	Test tube Number	Amount (g)	TLC pattern of the sample
SFZ-1	18-60	0.305	Single spots
SFZ-2	61-131	0.135	Two spots with tailing
SFZ-3	132-193	0.225	Mixture of spots
SFZ-4	194-221	0.130	Mixture of spots

2.7.5. Isolation of compound SFZ-1

The compound SFZ-1(0.325 g) was a white solid material. It was soluble in DCM. This fraction was purified by washing with hexane and a white solid materials obtained. It was crystallised from hexane and it was labeled SFZ-1 (0.260g).

2.7.5.1. Charac6terization of the compound

2.7.5.2. Characterization of the compound SFZ-1

2.7.5.3. Physical and chemical characteristics of the compound SFZ-1

The compound SFZ-1 was a colorless solid .It was readily soluble in DCM.

2.7.5.4. Characterization of the compound SFZ-1 by spectroscopic method

a) Ultra violet spectroscopy

In UV spectrum of the compound SFZ-1 (Fig. 22) the absorption maxima λmax at 644, 782, 221, 633 nm.

b) Infrared (IR) spectroscopy

In IR spectrum of the compound SFZ-1 (Fig. 23) had absorption frequencies at 3010, 2000, 1750,1670, 1510, 1360, 1280, 1220, 1160, 1120, 1030, 905, 810, 760, 640 cm^{-1}.

c) ^{1}H NMR (400MHz) spectroscopy

^{1}H NMR spectrum of the compound SFZ-1 (Fig:24-25) was recorded and their assignment is given in Table -21.

Table 21 ^{1}H NMR spectral data of compound SFZ-1

δ values in ppm	Integration	Splitting pattern	Coupling constant (*J* in Hz)
8.13	2H	Doublet	*ortho* proton,7.69
7.62	1H	Triplet	*para* proton, 1.05
7.48	2H	Triplet	*meta* proton, 2.82

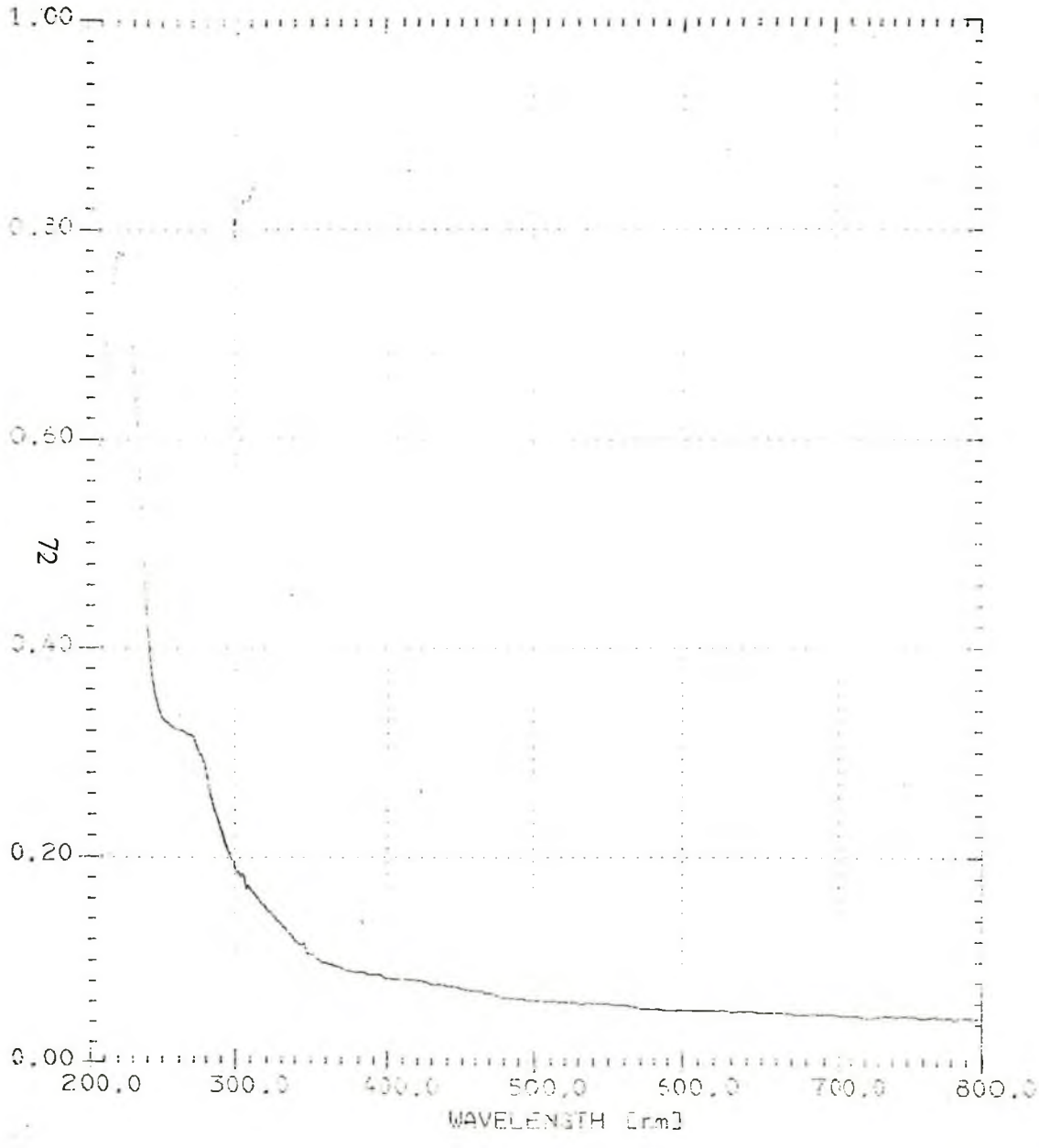
d) ^{13}C NMR (100 MHz , $CDCl_3$) (Fig-26-27) of the compound including detp SFZ-1 was recorded. The spectral data and their assignment are presented in the Table-22-23.

Table 22 ^{13}C NMR spectra data of compound SFZ-1

Carbon No	Chemical Shift in ppm	Types of carbon
C-1	133.2	Aromatic carbon
C-2	130.2	Aromatic carbon
C-3	128.5	Aromatic carbon
C-4	129.3	Aromatic carbon
-COO-	172.4	Carboxyl carbon

Table 23 DEPT Spectral data of the compound (SFZ-1)

Carbon No	Chemical Shift in ppm	Types of corbon
C-2	133.2	=CH
C-3	128.4	=CH
C-4	130.2	=CH
C-5	128.4	=CH
C-6	133.2	=CH



*** PEAK-PICK ***

No.	PEAK λ	PEAK ABS	VALLEY λ	VALLEY ABS
1	644.0	0.052	782.0	0.044
2	221.0	0.779	633.0	0.051

Figure No. -22 Ultraviolet Spectrum of Compound SFZ-1

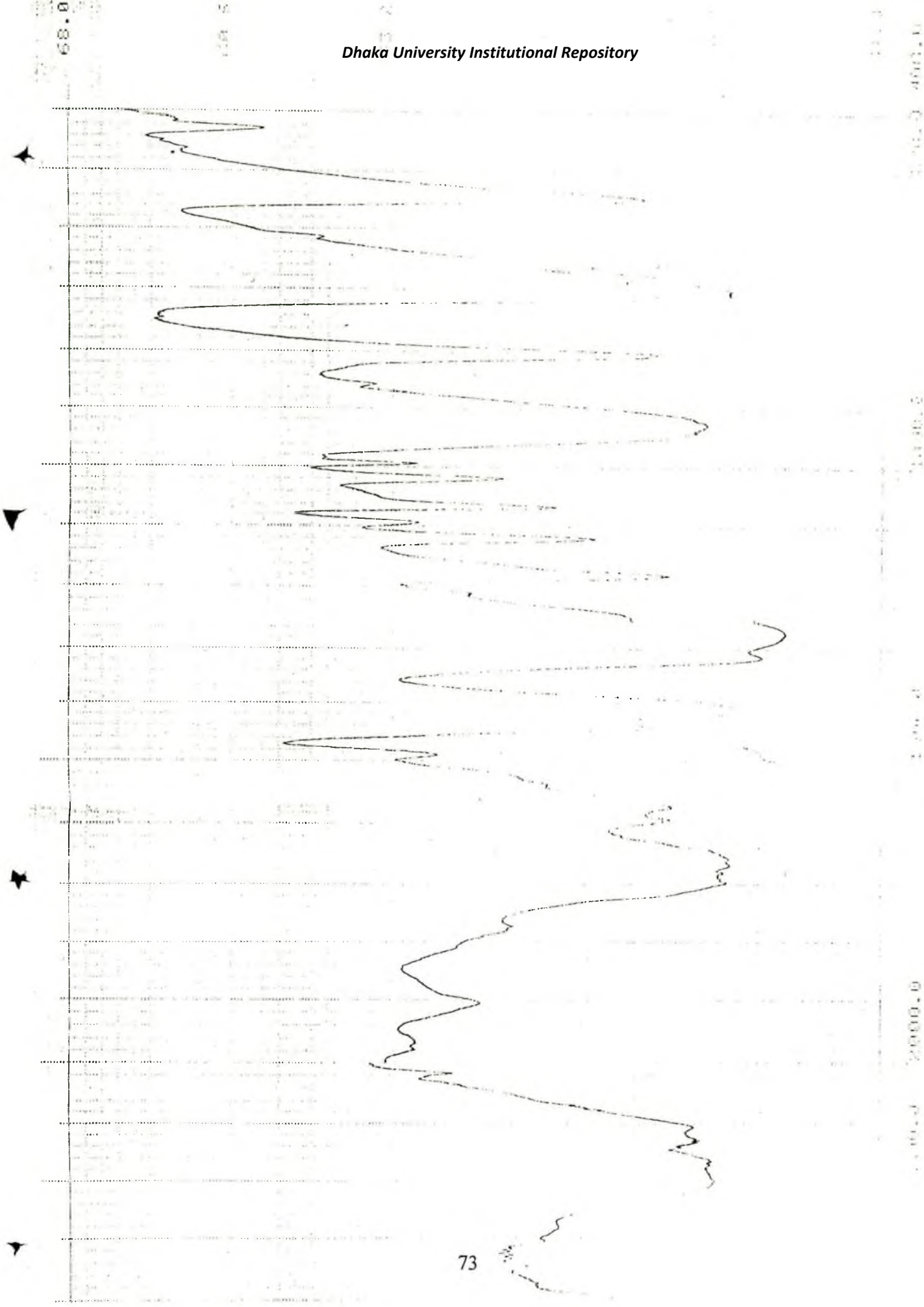


Figure No. -23 IR Spectrum of Compound ZSF-1

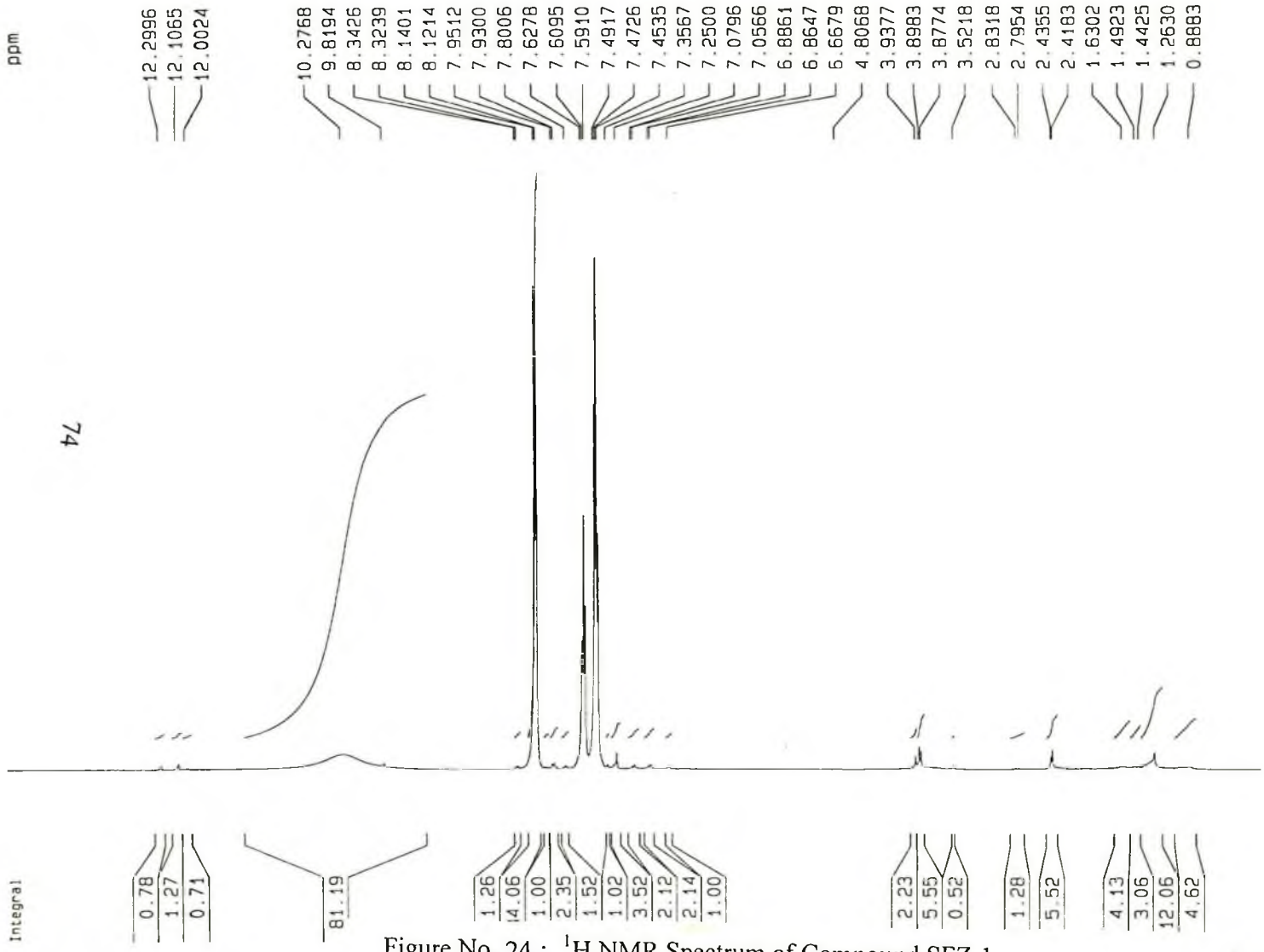


Figure No. 24 : ^{1}H NMR Spectrum of Compound SFZ-1

```
Current Data Parameters
NAME                  A3482
EXPNO                     1
PROCNO                    1

F2 - Acquisition Parameters
Date_              20070509
Time                  15.40
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                    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              400.1400119 MHz
WDW                     EM
SSB                      0
LB                    0.30 Hz
GB                       0
PC                    1.40

1D NMR plot parameters
CX                   20.00 cm
F1P                  14.051 ppm
F1                 5622.34 Hz
F2P                   0.109 ppm
F2                   43.80 Hz
PPMCM              0.69707 ppm/cm
HZCM             278.92728 Hz/cm
```

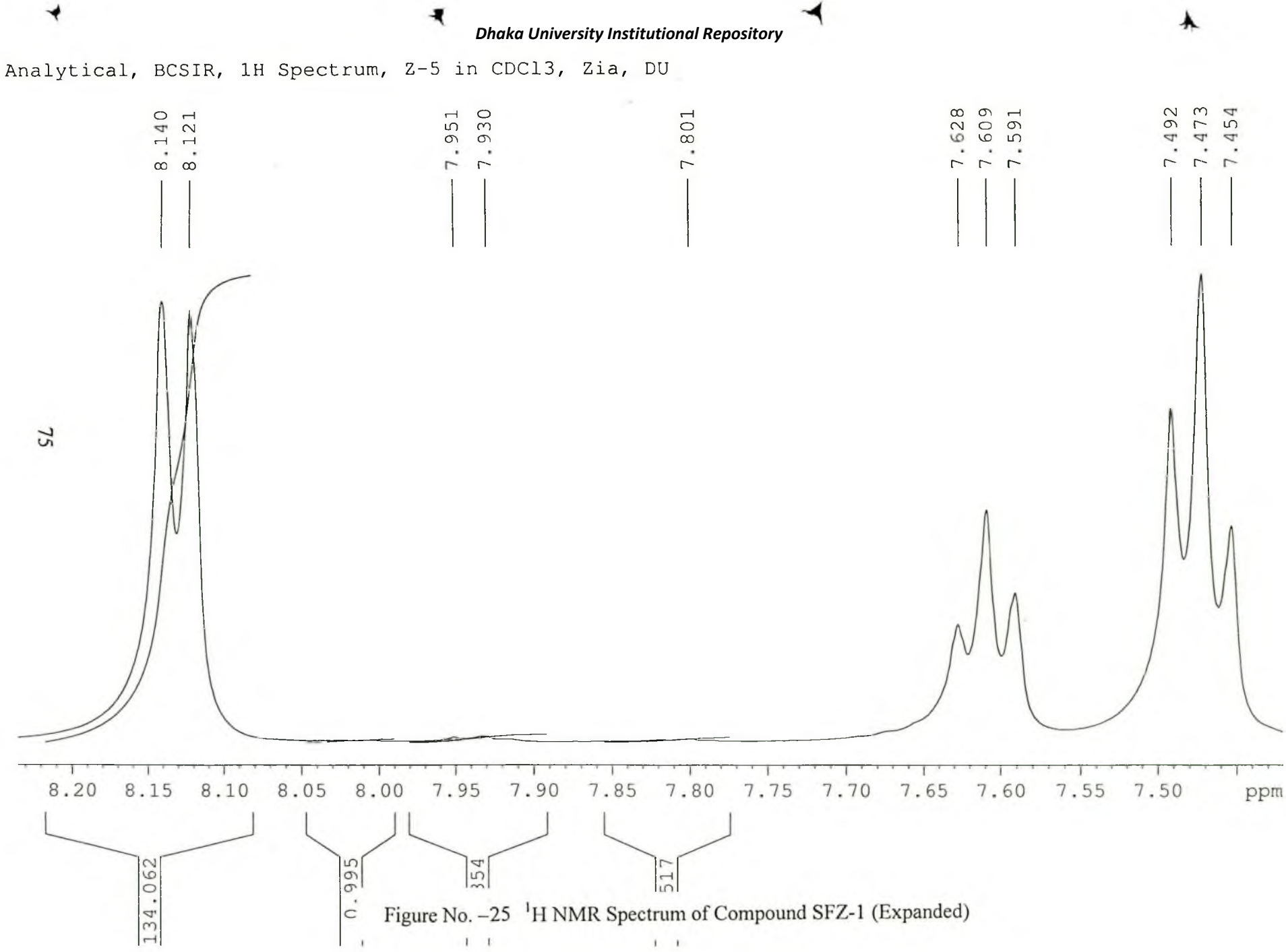


Figure No. –25 ^{1}H NMR Spectrum of Compound SFZ-1 (Expanded)

Analytical, BCSIR Lab. Dhaka 13C Spectrum Z-5 in CDCL3, Ziaul, DU.

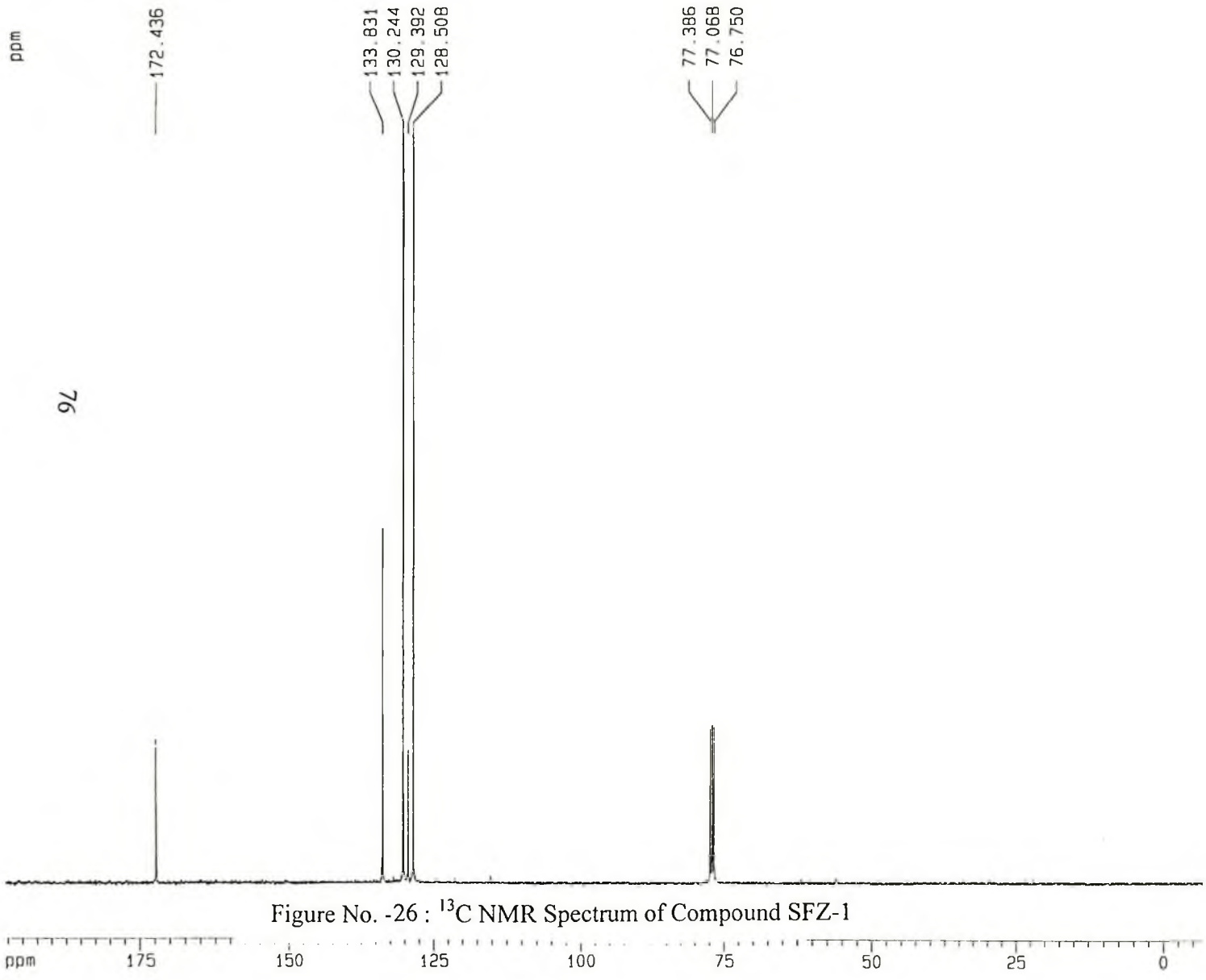


Figure No. -26 : ^{13}C NMR Spectrum of Compound SFZ-1

Current Data Parameters

NAME	A3482
EXPNO	2
PROCNO	1

F2 - Acquisition Parameters

Date_	20070514
Time	10.34
INSTRUM	dpx400
PROBHD	5 mm Multinuc
PULPROG	zgpg30
TD	32768
SOLVENT	CDCl3
NS	824
DS	2
SWH	24154.590 Hz
FIDRES	0.737140 Hz
AQ	0.6783476 sec
RG	16384
DW	20.700 usec
DE	6.00 usec
TE	300.0 K
D1	1.50000000 sec
d11	0.03000000 sec
d12	0.00002000 sec

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

NUC1	13C
P1	8.30 usec
PL1	-6.00 dB
SFO1	100.6253045 MHz

========== CHANNEL f2 ==========

CPDPRG2	waltz16
NUC2	1H
PCPD2	80.00 usec
PL2	-6.00 dB
PL12	16.00 dB
PL13	120.00 dB
SFO2	400.1400000 MHz

F2 - Processing parameters

SI	32768
SF	100.6152827 MHz
WDW	EM
SSB	0
LB	2.50 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	198.115 ppm
F1	19933.40 Hz
F2P	-6.501 ppm
F2	-654.06 Hz
PPMCM	10.23078 ppm/cm
HZCM	1029.37268 Hz/cm

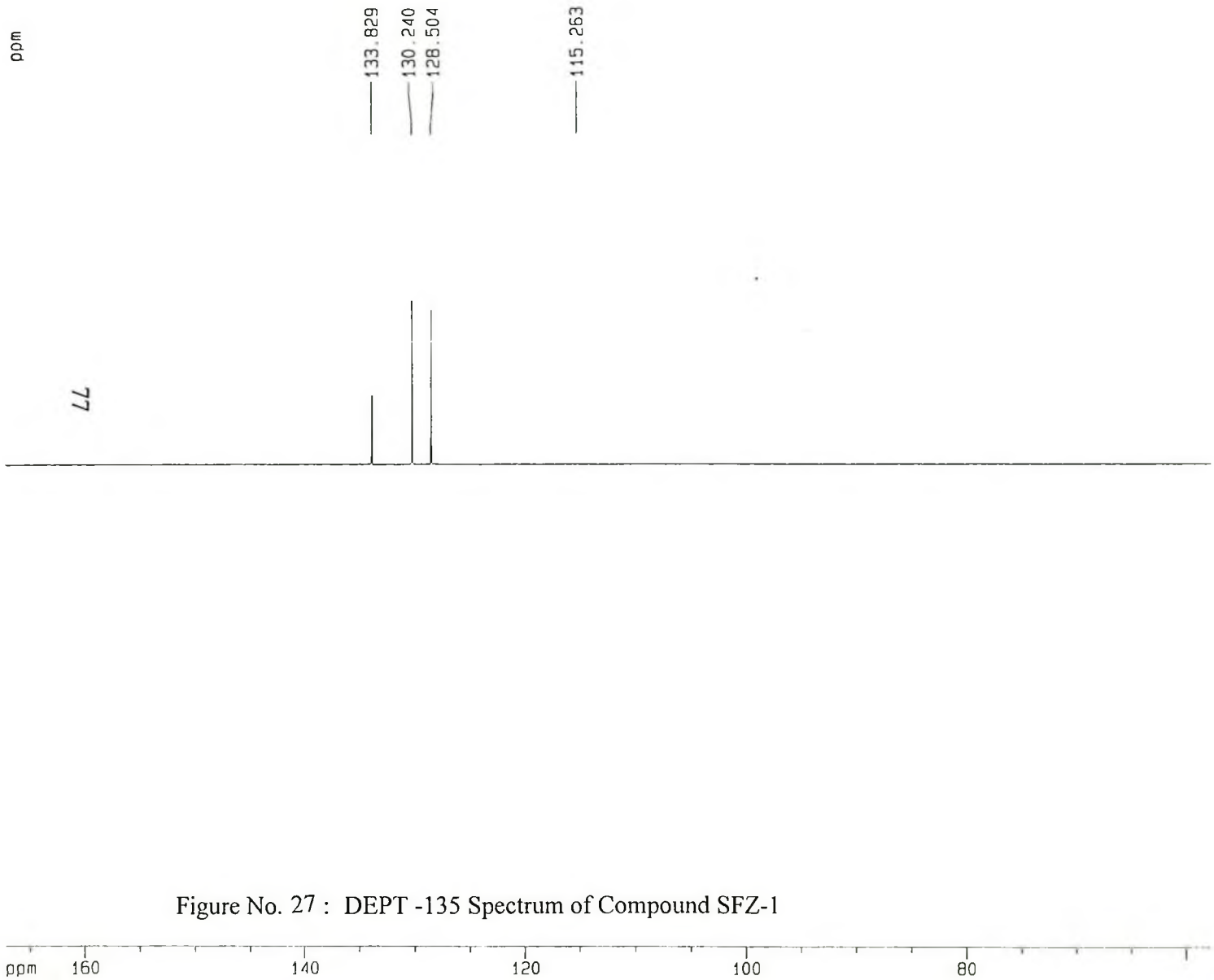


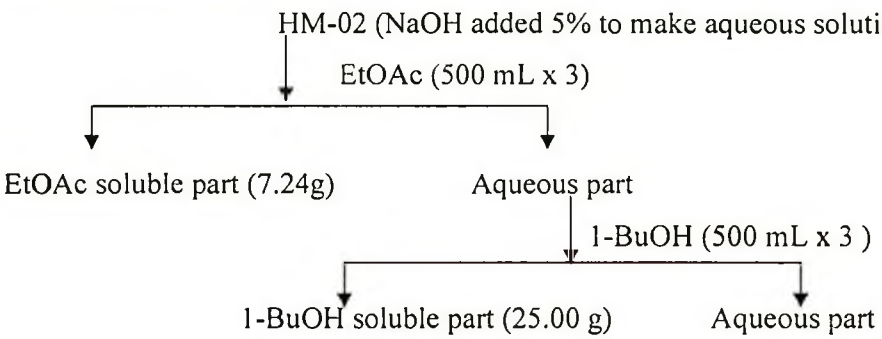
Figure No. 27 : DEPT -135 Spectrum of Compound SFZ-1

```
Current Data Parameters
NAME                  A3482
EXPNO                     3
PROCNO                    1

F2 - Acquisition Parameters
Date_              20070514
Time                 10.57
INSTRUM              dpx400
PROBHD     5 mm Multinuc
PULPROG           dept135
TD                   32768
SOLVENT              CDCl3
NS                     266
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                   300.0 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
SFO1             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
SFO2             400.1420007 MHz

F2 - Processing parameters
SI                   32768
SF               100.6152832 MHz
WDW                     EM
SSB                      0
LB                    1.00 Hz
GB                       0
PC                    1.40

1D NMR plot parameters
CX                   20.00 cm
F1P                 167.215 ppm
F1                16824.35 Hz
F2P                  57.816 ppm
F2                 5817.19 Hz
PPMCM                5.46992 ppm/cm
HZCM               550.35767 Hz/cm
```

2.7.5.5. Spectroscopic analysis of SFZ-2, SFZ-3 and SFZ-4 were very similar to SFZ-1.

2.7.5.6. Extraction of HM-02 with ethyl acetate

The HM -02 (500 mL) liquid sample was taken in a 1000 mL conical flask and 25g NaOH was added to it to make 5% NaOH solution. The solution was treated for 20 minutes and then partitioned with EtOAc (500 mLx3). Two different layers were separated. The whole process was repeated by taking 2400 mL HM-02 liquid sample. The combined EtOAc soluble parts were evaporated to dryness and freeze-dried, 7.24g solid materials was obtained. The aqueous part was partitioned with 1-butanol. The 1-butanol soluble part was evaporated separately to dryness followed by freeze-drying to get 25.00g solid materials.



Extraction Scheme-IV

2.7.5.7. Fractionation of ethyl acetate soluble part by column chromatography

The ethyl acetate soluble part was fractioned by silica gel column chromatography. A glass column (250 mL x 3 cm) was packed with 80 g of column grade silica gel in DCM as column equilibrating solvent (column volume 250 mL). The EtOAc soluble part (7.4. g) was dissolved in DCM and column grade silica gel (10g) was added to it and evaporated to dryness. The dried sample was applied carefully on the top of the column bed. After application of the sample solvent, polarity was increased from DCM to EtOAc. The eluted samples were collected in 190 test tubes and monitored by TLC, 14 Fractions were obtained. The amount of each fraction, the corresponding solvents polarity and TLC pattern are given in Table 24 and 25.

Table 24 Different Fractions of EtOAc extract obtained by column chromatography

DCM %	EtoAc %	MeOH %	Volume of solvent (ml)	Number of test tube
100	0	0	500	1-30
95	5	0	200	31-43
90	10	0	200	44-55
80	20	0	200	56-65
70	30	0	200	66-77
50	50	0	200	78-90
30	70	0	200	91-97
0	100	0	200	98-109
0	95	5	200	110-119
0	90	10	200	120-131
0	80	20	200	132-144
0	70	30	200	145-154
0	50	50	200	155-171
0	0	100	300	172-190

Table 25 Different fraction of EtOAc soluble part obtained by column chromatography

Fraction	Testable NO	Amount (g)	TLC pattern
DFZ-1	1-15	0.035	Single Spot
DFZ-2	16-24	0.038	Single Spot
DFZ-3	25-36	0.060	Two spots tailing
DFZ-4	37-48	0.290	Two spots tailing
DFZ-5	49-60	0.130	Two spots tailing
DFZ-6	61-71	0.105	Two spots
DFZ-7	72-85	0.085	Two spots
DFZ-8	86-96	0.170	Two spots tailing
DFZ-9	97-108	0.095	Mixture
DFZ-10	109-123	0.130	Two spots
DFZ-11	124-136	0.065	Mixture
DFZ-12	137-150	0.135	Two spots tailing
DFZ-13	151-171	0.890	Long tailing
DFZ-14	172-190	0.845	Mixture

2.7.5.8. Isolation of compound DFZ-1

The fraction DFZ-1(0.035 g) was a white solid material. It was soluble in EtOAc. TLC of the fraction showed a single spot. It was re-dissolved in MeOH and was attempted to crystallize but amorphous solid material was obtained.

2.7.6. Characterizations of the compound

2.7.6.1 Characterization of the compound (DFZ-1)

2.7.6.2. Physical and chemical characteristics of the compound DFZ-1

The compound DFZ-1 was a nice colorless crystal. It was readily soluble in MeOH.

2.7.6.3. Characterization of the compound DSF-1 by spectroscopic method

a) Ultra violet spectroscopy

UV spectrum of the compound DFZ-1 (Fig. 28) the absorption maxima, λmax at 644, 222, 782, 633 nm.

b) Infrared (IR) spectroscopy

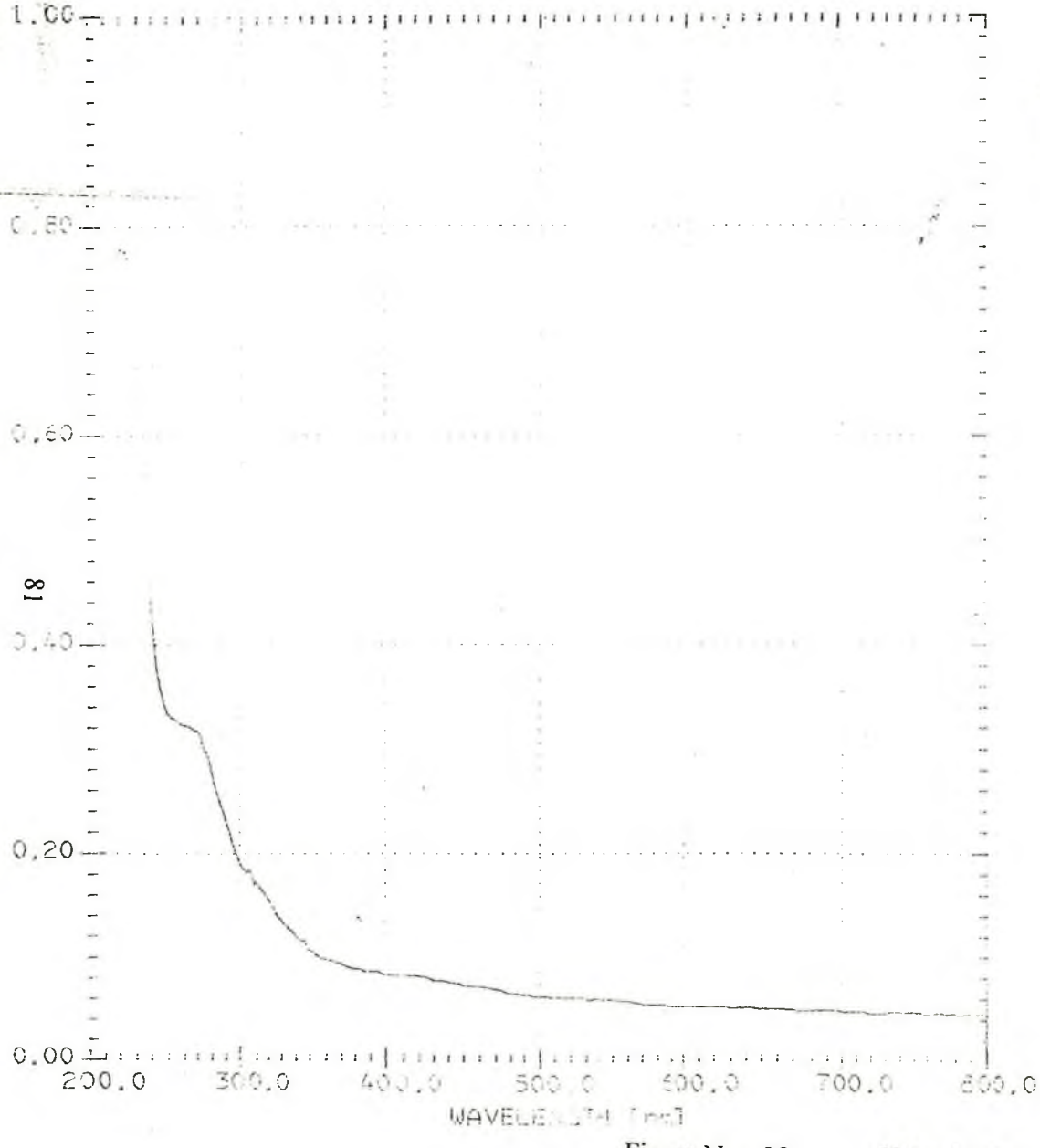
In IR spectrum of the compound DFZ-1 (Fig. 29) the absorption frequencies at 3010, 2000, 1750, 1510, 1360, 1220, 1160, 1120, 905, 810, 760, 640 cm^{-1}.

c) ^{1}H NMR (400MHz) spectroscopy

^{1}H NMR (400 MHz) spectrum of DF-2, DFZ-1 was recorded (Fig 30-31), spectral data of the compound DFZ-1 and their assignment is given in the Table 26.

Table 26 ^{1}H NMR spectra DFZ-1

δ values in pmm	Integration	Splitting pattern	Coupling constant (*J*, in Hz)
8.13	2H	Doublet	*ortho* proton,7.69
7.62	1H	Triplet	*para* proton, 1.05
7.48	2H	Triplet	*meta* proton, 2.82



*** PEAK-PICK ***

	-- PEAK --		-- VALLEY --	
No.	λ	ABS	λ	ABS
1	644.0	0.052	762.0	0.044
2	221.0	0.779	635.0	0.051

Figure No. -28 Ultraviolet Spectrum of Compound DFZ-1

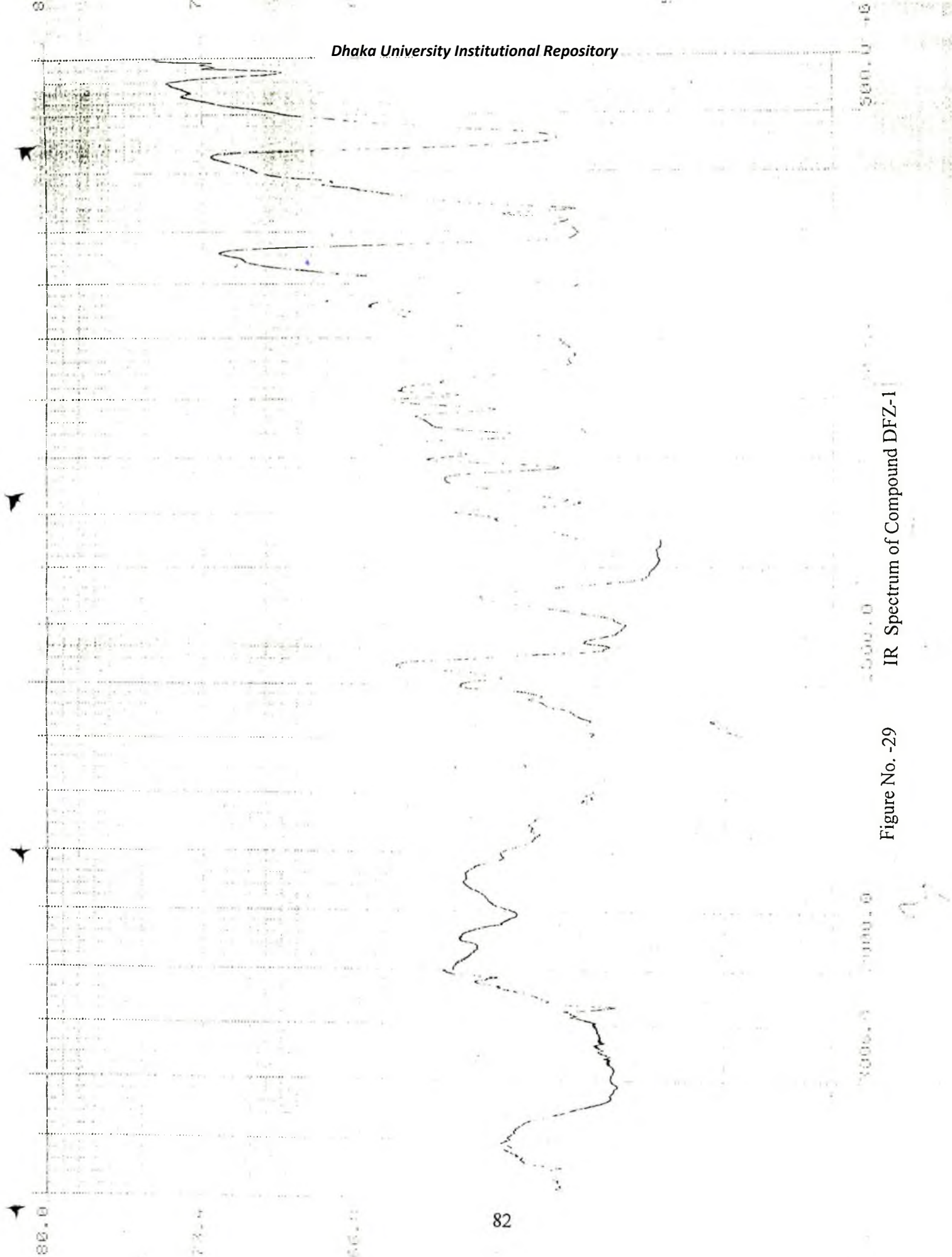


Figure No. -29 IR Spectrum of Compound DFZ-1

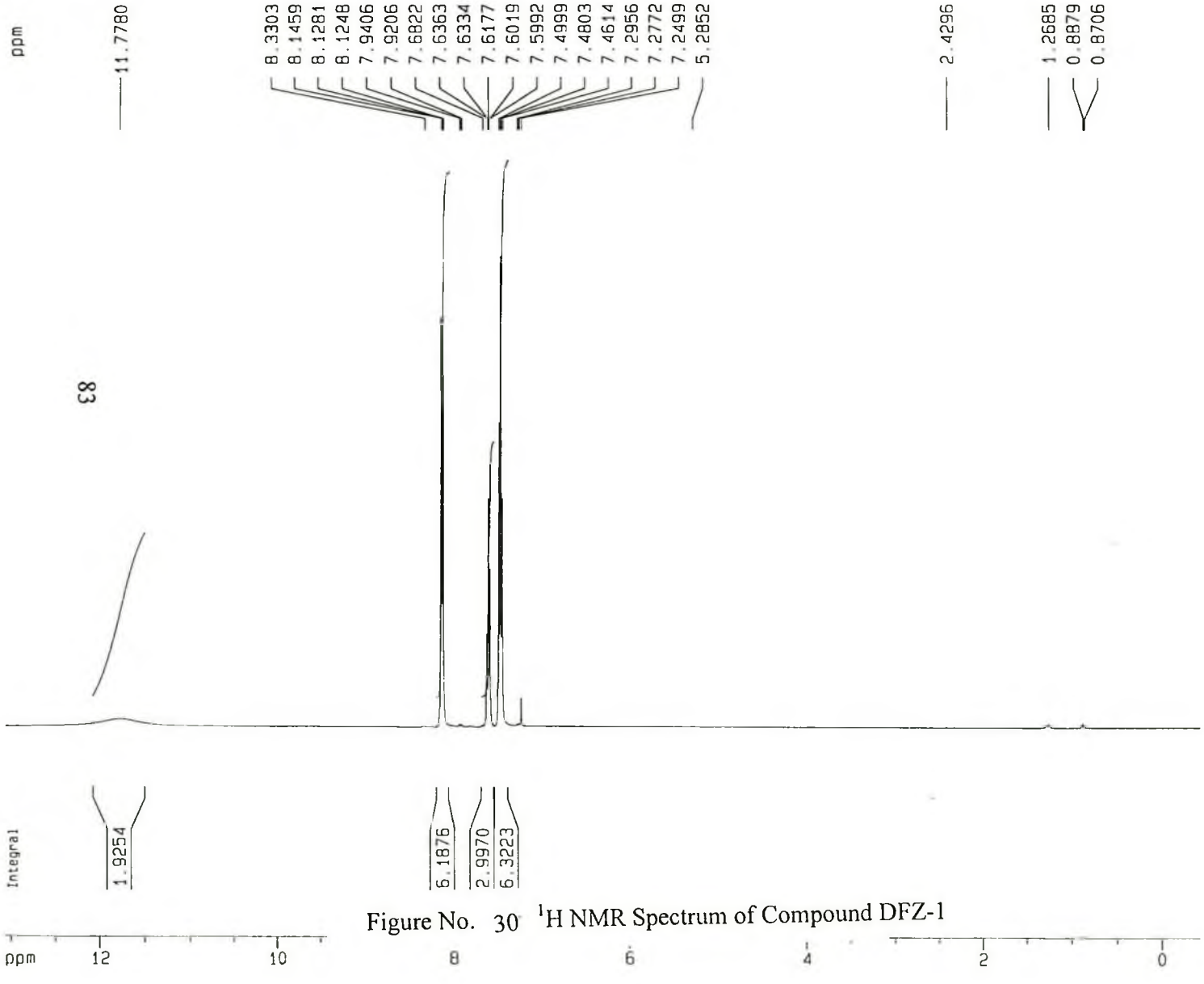


Figure No. 30 ^{1}H NMR Spectrum of Compound DFZ-1

```
Current Data Parameters
NAME                  A3281
EXPNO                     1
PROCNO                    1

F2 - Acquisition Parameters
Date_              20070212
Time                  11.45
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                      128
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                  13.069 ppm
F1                 5229.57 Hz
F2P                  -0.433 ppm
F2                 -173.17 Hz
PPMCM              0.67511 ppm/cm
HZCM             270.13687 Hz/cm
```

Analytical, BCSIR Lab. Dhaka 1H Spectrum Z-6 in CDCL3, Z.Haque, DU.

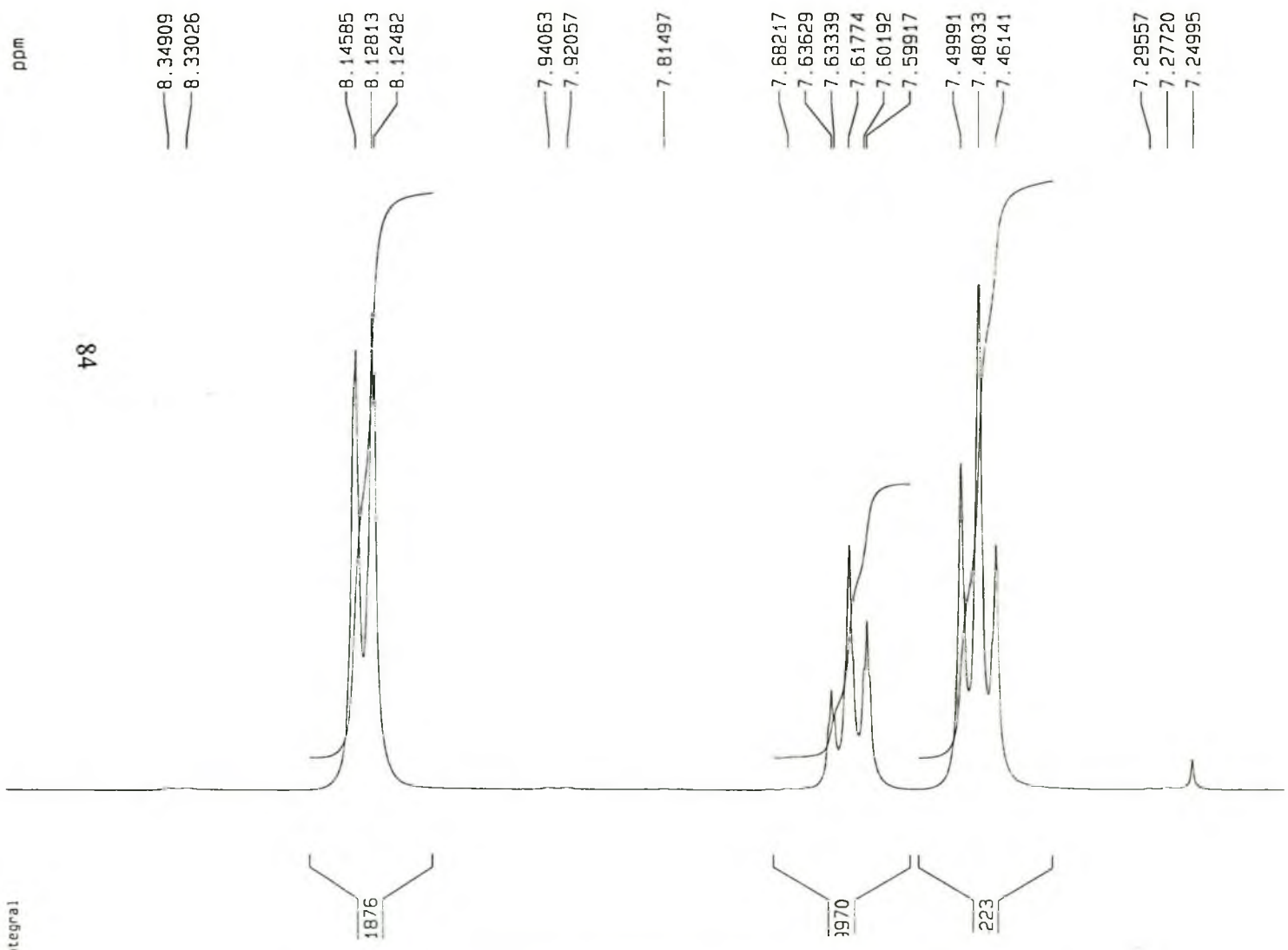


Figure No. 31: ^{1}H NMR Spectrum of Compound DFZ-1 (Expanded)

Current Data Parameters

NAME	A3281
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20070212
Time	11.45
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	128
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	8.523 ppm
F1	3410.33 Hz
F2P	7.149 ppm
F2	2860.77 Hz
PPMCM	0.06867 ppm/cm
HZCM	27.47827 Hz/cm

2.7.7. Estimation of Benzoic acid in HM-02

a) Gravimetric method

HM-01 (10 mL) was evaporated by a rotary vacuum evaporator followed by freeze-dryer to get 0.925 g of solid material. The solid was dissolved in 5% aqueous NaOH (10 mL) and then partition with ethyl acetate (10 mL x 3). The aqueous part was acidified with dilute HCl and then again partition with ethyl acetate (10 mL x 3). The ethyl acetate soluble part was treated with anhydrous Na_2SO_4 and evaporated to dry-mass (0.012 g; 1.2%) which supposed to be benzoic acid.

b) HPLC Analysis

i) The HM-02 medicine was diluted in to 50.0, 0.5, 0.25 µg/ mL by HPLC grade methanol. Each of the diluted sample was filtered by HPLC sample filter and analyzed by HPLC using Acetonitrile:H_2O (1:1) as mobile phase in a reversed phase C-18 column with a flow rate of 0.7ml/min. HPLC profile of the sample is presented in (Fig 32).

ii)Benzoic acid standard stock solution(1mg/mL)was prepared by dissolving 10mg of benzoic acid in 10 mL of methanol. The stock solution(1mg/mL) was successi vely diluted with methanol to get 2.5, 1.25 and 0.625 µg/ mL sample solution. Each of the diluted samples was filtered through HPLC sample filter. The filtered benzoic acid solution was analyzed by HPLC in same condition in which HM-02 was analyzed. The presence of benzoic acid in HM-02 was detected considering the same time of standard benzoic acid. A calibration curve of the benzoic acid was made using sigma plot (Fig-33) and amount of benzoic acid present in the HM-02 sample was quantified using the standard calibration curve and found to be 6.26 mg/mL The calibration curve is presented in Fig. No-34 and formula for quantification are given in next page.

Natural Product Research Laboratory

Department of Chemistry
University of Dhaka

HPLC Analysis Report

Sample ID: HM-02.2
Filename: C:\CLASS-VP\Data\PDA Data
Method: C:\CLASS-VP\Methods\HM-02 &HM-01.met
User: System
Acquired: 6/14/2008 5:51:32 PM
Printed: 6/15/2008 3:28:45 PM

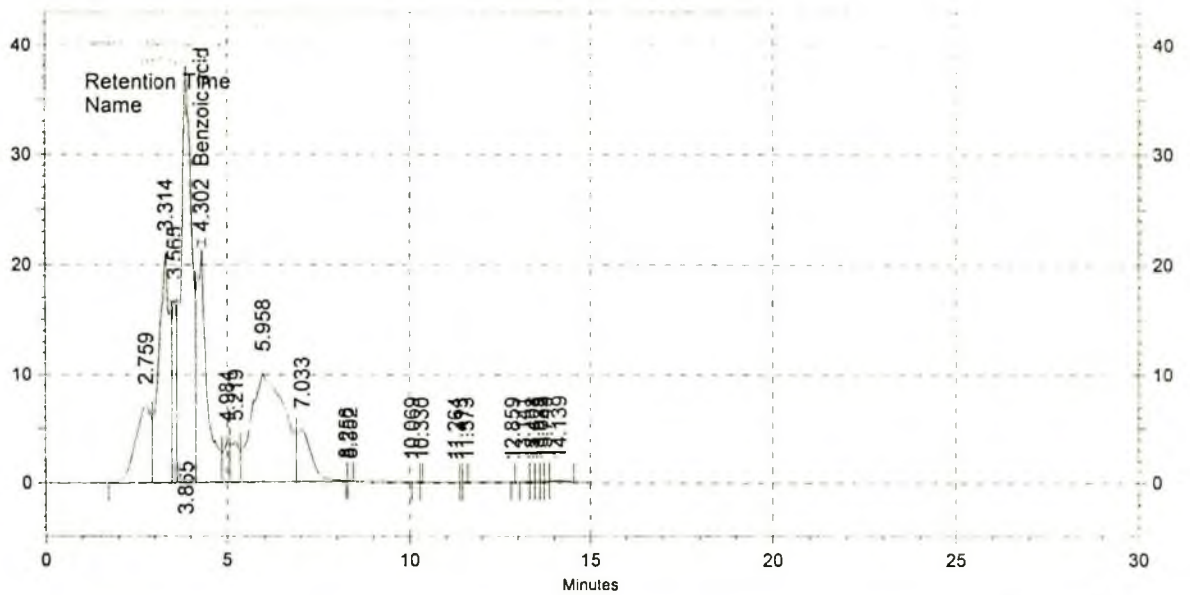


Figure No. -32 Estimation of Benzoic acid in HM-02 .2 by HPLC Analysis

Pk #	Name	Retention Time	Height
1		2.759	*7095*
2		3.314	*21016*
3		3.565	*16732*
4		3.865	*38189*
5	Benzoic acid	4.302	*21308*
6		4.984	*3565*
7		5.219	*3649*
8		5.958	*10021*
9		7.033	*4736*

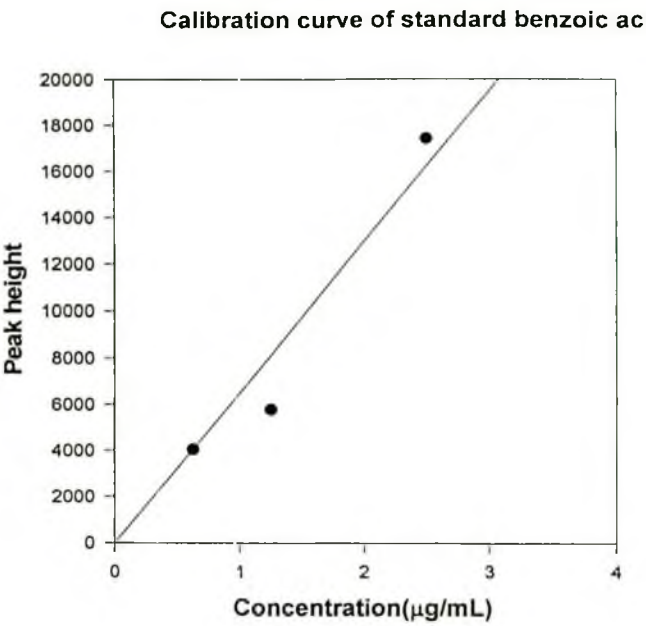


Figure No. -33 Calibration curve of standard benzoic acid

$$\textbf{Concentration of unknown sample} = \frac{\text{Peak Height}_{\text{Sample}} \times \text{Conc.}_{\text{Std}}}{\text{Peak Height}_{\text{Std}} . \times \text{Conc.}_{\text{sample}}}$$

PART A

RESULTS AND DISCUSSION

3 Results and discussion

General

Herbal medicines as old as the civilization of human being. The medicine is going to be popular day by day around the world. World Health Organization (WHO) also gave importance on herbal medicine as no new modern medicines are coming in to the market for many incurable diseases, like asthma, diabetes, arthritis, hepatitis, cancer, HIV etc. The herbal medicine is also popular in the developed countries (Germany, France, U.K. etc). People in the Indian Sub-continent and China are mostly dependent on herbal medicine. In Bangladesh, more than 600 herbal companies[20] are producing herbal medicine for local consumption as well as to export abroad. Their total sale is near about 40 billion taka. But there is a big question about the quality of medicines (except few companies). Most of the company do not have own plant cultivation, which indicated random collection of raw materials (plant parts/herbs), do not have proper quality control (no proper standardization). Efficacy and safety of the medicines are also doubtful. Citation of the chemical composition of the medicines is not available. In the presence study two herbal medicines from two different companies were chosen to determine their chemical composition. For secrecy trade name of the medicines were not mentioned in any where of the thesis. Both the medicines are in liquid form available in nice bottle with reasonable price. The cited efficacy and name of the plants used in the preparation of the medicine were given in the experimental part and our findings are described gradually.

i) HM-01

The HM-01 medicine is useful for skin diseases such as acne vulgaris, boils, skin rasher, blemisher, urticarea, check nose bleeding, cures constipation, corrects indigestion prevents burning sensation during urination. It also relives general depression, improves complexion and helps to become slim and smart.

Twenty different plants are used to make the medicine HM-01 in different amount 1.25 mg to 17 mg. But it has not been mentioned which part of the plant has been taken for the medicine and how medicine has been prepared likes decoction, infusion etc. It has only mentioned that the medicine aqueous extracts of the parts.

Our findings showed that the HM-01 did not give any smell of fermentation and did not give any smell of alcohol. It's smell like plant materials. It has neutral pH (6.90) and boiling point 119- 121^{0}C. The medicine contained nearly 9.25% solid material where the solid material contained 6.63% ash. High amount of ash content indicated the presence substantial amount of inorganic materials.

To find out any adulteration of the medicine with steroid or with other modern medicine, the medicine was decolorized with charcoal, and the colorless medicine did not show the presence of steroid and alkaloid, which indicated that the natural medicine (plant origin) has not been adulterated with modern medicine, likes steroid and alkaloid. Presence of heavy metals in the herbal medicine is a big concern for the consumers. Heavy metals Pb, As, and Cr were determined at BCSIR Lab Dhaka (on payment). The concentration of the three metals (Tables-1) were more than tolerance level. The HM-01 was successively partitioned with hexane, DCM and 1-butanol to isolate secondary metabolites from the medicine. Hexane soluble part gave only benzoic acid which was characterized from its melting points 119-120°C and super impossible IR spectrum with standard benzoic acid. Fractionation of DCM soluble part by silica gel column chromatography, four fractions were obtained (ZF-1-ZF-4). The first fraction ZF-1 on purification by recrystallisation gave pure crystal which had melting point 120^{0}C.

The compound was characterized by UV, IR, ^{1}H and ^{13}C NMR spectroscopy including DEPT, H-H COSY, HSQC and HMBC as benzoic acid. Other three fractions were mixture of compounds. ^{1}H NMR spectra of the compound had major signals in the aromatic regions which were characteristics of mono-substituted benzene ring. Other signals were insignificant in comparison with aromatic signals. Therefore, no further attempt was taken to purity these fractions. For isolation of

secondary metabolites from HM-01, it was treated with NaOH to make it 5% aqueous solution with large amount of medicine so that the benzoic acid can form soluble salt. Then the solution was partitioned with EtOAc followed by 1 butanol. Benzoic acid free EtOAc soluble part on silica gel column chromatography gave 16 fractions (ZSF-1–ZSF-16). One fraction ZSF-1 gave single spot which was purified by recrystalization. It was characterized by UV, IR, ^{1}H and ^{13}C NMR spectroscopy including DEPT, H-H COSY, HSQC and HMBC as *para* hydroxyl methyl benzoate. IR, ^{1}H NMR spectra ZSF-2 was same as ZSF-1. So it was not studied again. Rest of the fractions from ZSF-3 to ZSF-16 didn't show any characteristics peaks of secondary metabolites. From the analysis the entire fractions major constituent was benzoic acid and small amount of *para* hydroxy methyl benzoate was obtained. There were also presence of other signals besides that signals which indicated of some compounds but there were too small in amount which was impossible to isolate under classical condition of isolation of compound. Large amount of benzoic acid identified in HM-01 should not be constituent of the plant/herbs. This must be added in the preparation of the medicine as sodium benzoate to avoid fermentation or any microbial growth in the medicine.

Amount of sodium benzoate was quantified by gravimetric method. A HPLC Profile of the medicine was carried out at different concentration of the sample 50, 0.50, 0.25 µL/mL. A calibration curve of standard benzoic acid was made and amount of sodium benzoate presence in the quantified using calibration curve. It revealed that amount of sodium benzoate added in the medicine much more than standard protocol (12.99 mg/mL). Three heavy metals, arsenic (As), chromium (Cr) and lead (Pb) were quantified in HM-01 by Atomic Absorption Spectroscopy (AAS), and found 0.16, 1.18 and 10.5, mg/L, respectively which were also very high in amount and more than tolerance level[21-23].

ii) HM-02

The HM-02 medicine is useful for skin diseases such as acne vulgaris, boils, skin rasher, blemisher, urticarea, dermatitis, check nose bleeding, cures constipation, rheumatic pain and inflammation.

Twenty different plants are used to make the medicine HM-02 in different amount (1.25 mg to 17 mg). But it has not been mentioned which part of the plant has been taken for the medicine and how medicine has been prepared likes decoction, infusion etc. It has only mentioned that the medicine aqueous extracts of the parts.

It has not also not been mentioned whether any preservative, additives were used in it.

Our findings showed that HM-02 did not give any smell fermentation and did not give any smell of alcohol. It's smell like plant materials. It is slightly acidic pH (5.37) and boiling point 110-111^{0}C. The medicine contained nearly 5.96% solid material where the solid material contained 2.71% ash. The amount of ash content indicated the presence substantial amount of inorganic materials in the medicine.

To find out any adulteration of the medicine with steroid or with other modern medicine was decolorized. The colorless medicine did not show the presence of steroid and alkaloid, which indicated that the natural medicine (plant origin) has not been adulterated with modern medicine, likes steroid and alkaloid. Presence of heavy metals in the herbal medicine is a big concern for the consumers. Heavy metals, Pb, As, and Cr in HM-02 were determined by Atomic Absorption Spectroscopy (AAS), (0.09, 0.88 and 4.55 mg/L, respectively) which were also very high in amount and more than tolerance level[21-23].

The HM-02 was successively partitioned with hexane, DCM and 1- butanol to isolate secondary metabolites from the medicine. Hexane soluble part gave only benzoic acid which was characterized from its melting points (119-120^{o}C) and super-impossible IR spectrum with standard benzoic acid. Fractionation of DCM soluble part by silica gel column chromatography four fractions are obtained (SFZ-1-SFZ-4). The first fraction SFZ-1 on purification by recrystallisation gave pure crystal which had melting point 120^{0}C.

The compound was characterized UV, IR, ^{1}H and ^{13}C NMR spectroscopy including DEPT as benzoic acid. Other three fractions were mixture of compounds. ^{1}H NMR spectra of the compound had major signals in aromatic regions which were characteristics of mono-substituted benzene ring. Other signals were insignificant in comparison with aromatic signals. Therefore, no further attempt was taken to purity these fractions. For isolation of secondary metabolites, HM-02 was treated with NaOH to make it 5% aqueous solution with large amount of medicine so that the benzoic acid can form soluble salt. Then the solution was partitioned with EtOAc followed by 1-butanol Benzoic acid free EtOAc soluble part on silica gel column chromatography gave 16 fractions (DFZ-1– DFZ -14). Two fractions ZSF-1 gave single spot which was purified by recrystalization. It was characterized by UV, IR and ^{1}H. IR and ^{1}H NMR spectra DFZ-2 was same as DFZ-1. So it has not been studied again. Rest of the fractions from DFZ-3 to DFZ-14 didn't show any characteristics peak of secondary metabolites. From the analysis all of the fractions major constituent was only benzoic acid found. There were also presence of other small signals besides the aromatic signal which indicated presence of some compounds but these were too small in comparison with benzoic acids which was impossible to isolate under classical condition of isolation of compounds. Large amount of benzoic acid identified in HM-02 should not be the constituent of the plant/herbs. This must be added in the preparation of the medicine as sodium benzoate to avoid fermentation or any microbial growth in the medicines.

Amount of sodium benzoate was quantified by gravimetric method. A HPLC Profile of the medicine was carried out at different concentration of the sample 50, 0.50, 0.25µL/mL. A calibration curve of standard benzoic acid was made and amount of sodium benzoate presence in the quantified using calibration curve. It revealed that amount of sodium benzoate added in the medicine much more than standard protocol (6.26 mg/mL). Three heavy metals, arsenic (As), chromium (Cr) and lead (Pb) were quantified in HM-01 and HM-02 (0.16, 1.18, 10.5 & 0.09, 0.88, 4.55, respectively) by Atomic Absorption Spectroscopy (AAS), which were also very high in amount and more than tolerance level[21-23].

PART B

INTRODUCTION

PART-B

1 INTRODUCTION

1.1. General description of *Myristica fragrans*[24]

Scientific classification

Kingdom : Plantae

Division : Magnoliophypa

Class : Magnoliopside

Order : Magnolial

Family : Myristicaceae

Genus : *Myristica*

Species : *fragrans*

Myristica fragrans is a spreading aromatic evergreen tree usually growing to around 5 to 13 metres high, occasionally 20 metres. It is now cultivated in tropical regions, Specially Indonesia, Grenada in the West Indies and Sri Lanka(purseglove, 1968, Bown, 1995) and other tropical areas.The bark contains watery pink or red sap. The pointed dark green leaves (5 to 15 cm × 2 to 7 cm) are arranged alternately along the branches and are borne on leaf stems about 1 cm long. Upper leaf surfaces are shiny. Flowers are usually single sexed; occasionally male and female flowers are found on the same tree. Female flowers arise in groups of 1 to 3; males in groups of 1 to 10. Flowers are pale yellow, waxy, fleshy and bell-shaped. Male flowers are 5 to 7 mm long; female flowers are up to1 cm long. The fruits are fleshy, drooping, yellow, smooth, 6 to 9 cm long with a longitudinal ridge. When ripe, the succulent yellow fruit coat splits into 2 valves revealing a purplish-brown, shiny seed (nutmeg) surrounded by a red aril (mace). Seeds (nutmegs) are broadly ovoid (2 to 3 cm long), firm, fleshy, whitish and transverse by red-brown veins. When fresh, the aril (mace) is bright scarlet becoming more horny, brittle and a yellowish-brown cooler when dried. The tree does not flower until around 9 years old, when it fruits; it can continue to do so for a further 75 years. The tree bears 2 to 3 crops a year.

1.2. Pharmacologically active parts of the plant[25]

The most important part of the plant in terms of its pharmacological activity and also in commerce is of course the dried kernel (seed), the nutmeg. Intoxication from the use of the aril of the fruit (seed case), generally known as mace, has also been reported, but only rarely. The oil of nutmeg has also been used for medicinal purposes and it is this fraction of the nutmeg which is strongly suspected of harbouring the pharmacologically active components of nutmeg.

1.3. General description of Myristicaceae family [24-27]

The family Myristicavceae contains only 18 genera and about 300 species. Myristica is the largest genus for which has been listed 72 species, spreading from India and Sri Lanka eastwards through Malaysia to North–Estern Australia, Taiwan and the pacific, including the Solomon Islands ,Fiji and Samoa.Since 40 known species of Myristica of which endemics are found in New Guinea (Indonisia) representing an area of concentration , this location has been designated the center of origin and distribution of this genus. Some of the species of Myristica genus are *Myristica fragragns, Myristica otoba, Myristica malabarica, Myristica argentea , Myristica sebifera, Myristica officinalis, Myristica fatua, Myristica madagascariensis, Myristica acuminate* etc.

According to Groome another species found growing locally of the Myristicavceae family is Virola surinamensis and with common names wild nutmeg or wild ceder.

1.4. Medicinal uses of other *Myristica species*[28]

The major components in *Myristica fragrans* are terpenes, terpene alcohols and phenolic ethers. The major phenolic ether is myristicin (4-methoxy-6-(2- propenyl)-1, 3-benzadioxole) accompanied by safrole 5-(2-propenyl) 3-benzodioxole and eugenol methyl ether (3, 4,-dimethoxy-(2-propenyl) benzene). Myristicin accounts for about 2.12 to 2.88% of the total weight of the nutmeg where as safrole accounts for 0.27 to 0.39%. The volatile oil content of nutmeg depends on the geographical origin and length of storage. Chemical analysis has shown that even though there is a real variability between the quality (differences in composition) and quantity of nutmeg oil from various samples of nutmegs oil accounts for 84

to 95% of the total aromatic fraction of the volatile oil from all the samples tested. In thesamples, myristicin, safrole and elemicin accounted for 3.86 to 12.7%, 0.53 to 3.42% and 0.02 to 2.36%, respectively of the nutmeg oil samples. Early work on myristicin used myristicin distilled from nutmeg oil. It has been subsequently proven that myristicin extracted from nutmeg via this method is not elemicin free and therefore the effects reported may be due to either substance found in the extract.

1.5. Medicinal use of *Myristica fragrans*[28,29]

Used as an anti-diarrhoea agent for patients with medullary carcinoma of the thyroid. The effectiveness of the treatment may be due to the inhibition of prostaglandin synthesis in the mucosa and submucosa of the colon. The dosage given was 9 tablespoons orally per day but it may vary between patients to avoid toxic symptoms.

Domestic

Used as a spice in various dishes, as components of teas and soft drinks or mixed in milk and alcohol.

Traditional and folk medicine

It is widely used as a traditional medicine in the Middle East and Asia.

In Western medicine nutmeg is sometimes used as a stomachic, stimulant, carminative as well as for intestinal catarrh and colic, to stimulate appetites, to control flatulence, and it has a reputation as an emmenagogue and abortifacient.

1.6. Products of Myristica: their uses and composition[30]

The finest mace and the finest nutmegs come from Penang, and, in general the East Indian species are preferred West Indian.

Nutmeg Husks

The pericarp of the nutmeg fruits can be preserved in the while unripe, salted and dried as a condiment , or made into jellies.All of these preparation have the flavour of nutmeg.

Nutmeg Ground nutmeg, a granular orange brown powder with characteristic aroma ,is a widely- known kitchen spice. It has a warm aromatic, slightly bitter taste and is often added to custards, puddings, pies, certain vegetables and milk drinks like egg-nog. In the past, nutmeg was much used in medicine. Whole nutmeg, depending on the variety, contains from 5 to15 percent of a volatile oil that accounts entirely for the aroma and flavor of the spice.

Mace

Mace, though not quite so well–known in the kitchen as nutmeg, is none the less a popular spice. It is a brownish–yellow or brownish–orange granular powder with a strong aroma closely resembling but not identical to that of nutmeg. The flavor of mace is softer and some-what less pungent then the flavor of nutmeg.

Mace is used in the manufacture of pickles tomato ketchup, in meat and fish sauce in chocolate dishes, cherry pie and pound cake. Like nutmeg, mace has been used in medicine.

Whole mace contains from 4 to 40 percent of volatile oil very similar to that found in nutmeg 59, along with moisture, fat, starch, etc.

1.7. Chemical investigation on *Myristica fragrans*[31,32]

Myristica fragrans Houtt. has been reported to contain 25-30% fixed oils and 5-15% volatile oils such a camphene elemicin eugenol isoeugenol methoxyeugenol, pinene sabinene, safrol, etc and also chemical substances such as dihydroguaiaretic acid, myristicine and lignan compound. From the ethanolic extract of the leaves of virola calophylla were isolated the compounds, designated hydroxyotobain, otobaene, sitosterol, dihydro-chalcone (2, 4 – dihydroxy-4, 6 dimetoxydihydro chalcone) and vanillin. From the ethanolic extract of the bark of virola calophylla were isolated two compounds safrole and methylparaben isolated substances for the first time in the family myristicaceae.

Fixed oil of nutmeg

The fixed oil of nutmeg is known by many names nutmeg butter,balsam of nutmegs, oil of mace, butter of mace, Banda soap, and *Oleum Myris tica Expressum.*

It is obtained by exposing the nuts to hydraulic pressure and heat. Two odorous fixed oils have been separated a yellow one insoluble in boiling alcohol but soluble in ether, a red one soluble compound either.

Essential oils of nutmeg and mace[33]

The essential or volatile oils of nutmeg and mace are obtained by steam distillation. In commerce, both products go under the name "oil of nutmeg" (officially, myristica oil or oleum Myristicae), and in fact the commercial oil probably distilled only from nutmegs since they are cheaper than mace.Oil of nutmegs is chemically complex ,as shown in the analysis by power and Slaway.

Myricin, $C_{11}H_{12}O_3$, constituting 4% of the oil is interesting as the fraction responsible for many of the pharmacological effects of nutmegs and mace.

PART B

EXPERIMENTAL

PART B
2 Experimental

2.1. Collection of plant material

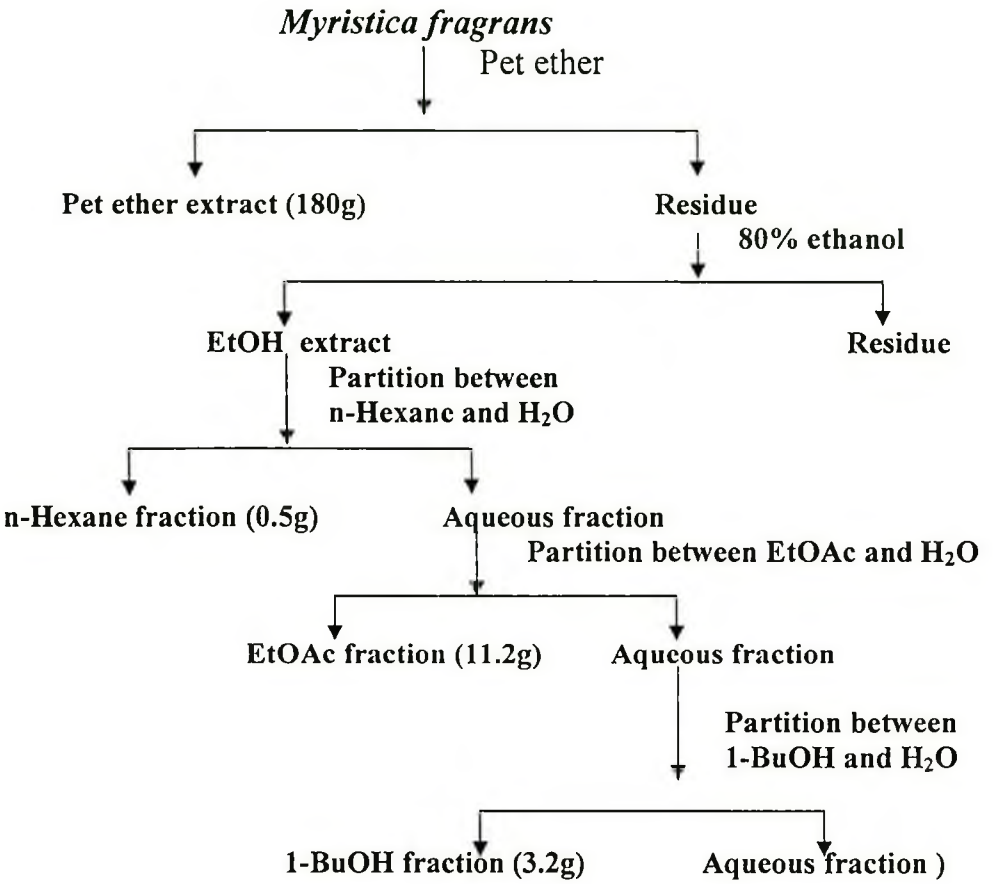
The seeds of *Myristica fragram* (450 g) were brought from the market of Dhaka city. After peeling there were powdered and dried in a oven at 40^0C.

2. 2. Extraction

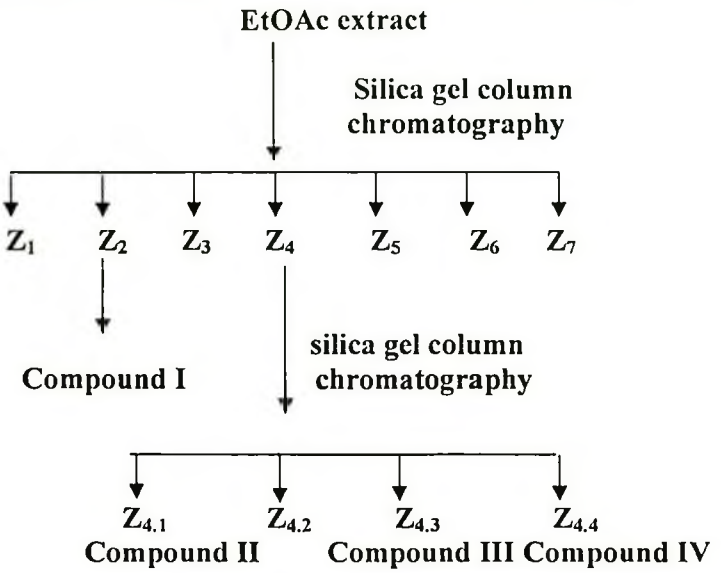
The seed powder of *Myristica fragrans* (450g) was taken in a conical flask and 500 mL of pet ether was added to it and kept it over night. The extract was decanted and these was repeated for three more times. The residue after pet ether extraction was further extracted with aqueous 80% ethanol (300 mL x 3). The extract was evaporated by a rotary vacuum evaporator with added water to remove ethanol completely. The resulting aqueous solution was partitioned with hexane then the aqueous suspension was further partitioned with ethyl acetate followed by 1-butanol. The ethyl acetate and 1-butanol soluble parts were evaporated separately to dryness to get 11.2g of ethyl acetate and 3.2g of 1- butanol extracts. The aqueous part also dried into solid material. The extraction Scheme-I is given in next page.

Extraction of *Myristica fruagrans* seed powder

SCHEME I



Fractionation of ethyl acetate extract (Scheme II)



2.3. Fractionation ethyel acetate extract by column chromatography

Ethyl acetate extract (10g) was fractionated by silica gel column chromatography (Scheme II). The sample was dissolved in DCM-MeOH mixture was added to the solution silica gel (1:2) ratio. The mixture was evaporated to dryness. The dried mixture was made into power by a pestle in a mortar. The dried sample was applied to the column and was eluted with hexane, DCM and MeOH in different ratios with increasing polarity. The fractions were monitored by their TLC and seven (Z_1- Z_7) fraction were obtained. The elution pattern, amount of each fraction and their TLC behavior are given in the Table 1.

Table 1 Elution pattern of ethyl acetate soluble part of 80% ethanol extract of *Myristica fragrans*

Fraction no	Eluting solvent	Amount (g)	TLC pattern
Z1	20% - 30% DCM in Hexane	0.08	Four spots
Z2	35% - 45% DCM in Hexane	0.09	One spots with tailing
Z3	50% DCM in Hexane	2.00	Two spots with tailing
Z4	60% - 90% DCM in Hexane	0.06	Three spots with tailing
Z5	100% DCM	0.19	Two spots with tailing
Z6	1% - 2% MeOH in DCM	2.50	Two spots with tailing
Z7	3% - 20% MeOH in DCM	3.08	Broad tailing

2.4. Isolation of Compound I

Fraction Z_2 gave one spot in TLC with tailing. It was re crystallized from hexane to get a pure compound I.

2.5. Characterization of the Compound I by spectroscopic methods

a) UV spectroscopy

UV spectrum (Fig 1) of Compound I in dichloromethane showed characteristic absorption maxima, λ_{max} at 322, 294 and 260 nm.

b) Infrared spectroscopy

The IR spectrum (Fig 2) of Compound 1 had absorption frequencies at 2910, 1494, 1420, 1200, 1045, 915, 805 cm^{-1}.

c) NMR spectroscopy

i) ^{1}H-NMR spectroscopy

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) (Fig 3-4) of Compound I had signals at 3.28 (2H, d, *J*= 6.4 Hz), 3.87 (3H, s), 5.07 (1H,d, *J*= 9.6), 5.91 (2H, s), 5.91 (1H, m), 6.34 (1H, s), and 6.37 (1H, s) ppm.

ii) ^{13} C-NMR spectroscopy

^{13}C-NMR spectrum (100 MHz, $CDCl_3$) (Fig 5-6) of compound I had 11 signal at 40.2, 56.5, 101.2, 102.6, 107.7, 115.8, 133.5, 134.6, 137.3, 143.5, and 148.8 ppm. The dept spectrum of the compound also recorded (Fig 7)

iii) 2D – NMR spectroscopy

The H-H COSY, HSQC and HMBC spectra ($CDCl_3$ 400 MHz) of compound I are shown in Fig 8-10 and the spectral data are given in Table 2-4.

Table-2. H-H COSY spectral data of the compound I (δ value in ppm).

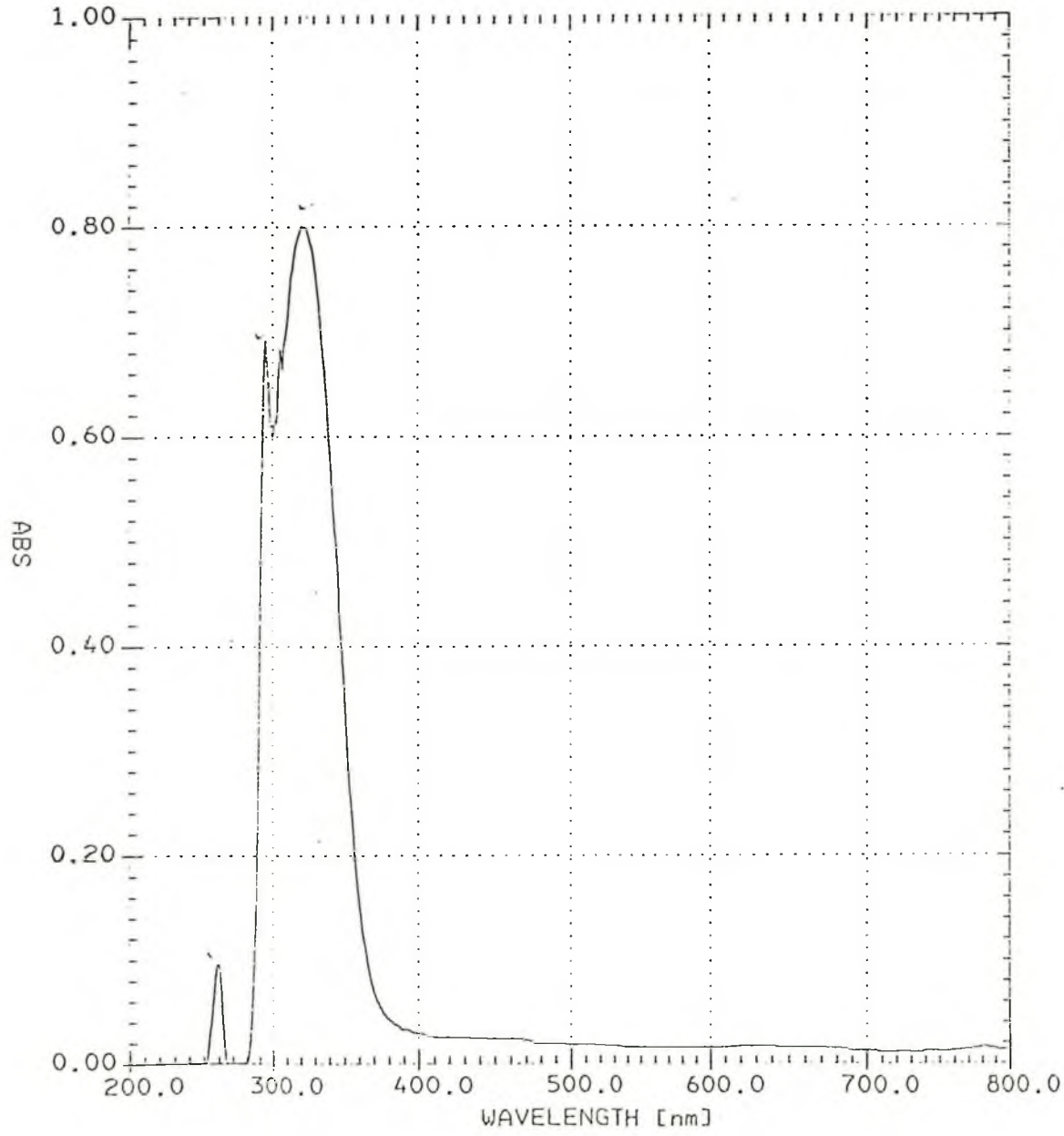
H		H
3.28 (H-1)	⟷	5.91 (H-2)
3.28 (H-2)	⟷	5.07 (H-3)

Table 3. HSQC spectral data of Compound 1 (δ value in ppm)

^{1}H		^{13}C
3.28 (H-1)	⟷	40.2(C-1)
3.87 (4-OCH_3)	⟷	56.5 (4-OCH_3)
5.07 (H-3)	⟷	115.8 (C-3)
5.91 (H-2)	⟷	101.2 (C-2)
5.91 (H-2)	⟷	137.3 (C-2)
6.34 (H-7)	⟷	102.6 (C-7)
6.37(H-5)	⟷	107.7(C-5)

Table 4. HMBC (Heteronuclear multiple Bond Correlation) spectral data of Compound I (δ value in ppm)

^{1}H	^{13}C
3.28 (H-1)	107.7 (C-5), 133.5(C-6), 102.6 (C-7),
	134.6 (C-9), 137.3(C-2), 115.8 (C-3),
3.87 (4-OCH_3)	143.5 (C-4)
5.07 (H-3)	40.2 (C-1), 137.3 (C-2)
5.91 (H-2)	148.8 (C-8),134.6 (C-9),
5.91 (H-2)	133.5 (C-6),40.2 (C-1),
6.34 (H-7)	143.5 (C-4),107.7 (C-5),133.5 (C-6), 148.8 (C-8),134.6 (C-9),40.2 (C-1),
6.37(H-5)	143.3 (C-4),133.5(C-6),148.8 (C-8), 143.6 (C-9),40.2 (C-1),137.3 (C-2),



*** PEAK-PICK ***

No.	-- PEAK -- λ	ABS	-- VALLEY -- λ	ABS
1	322.0	0.800	736.0	0.010
2	294.0	0.692	300.0	0.600
3	260.0	0.096	270.0	-0.056

Figure No. 1 : Ultraviolet Spectrum of Compound –I

Figure-5: Infrared spectrum of Compound I

Figure No. - 2 : IR Spectrum of Compound -I

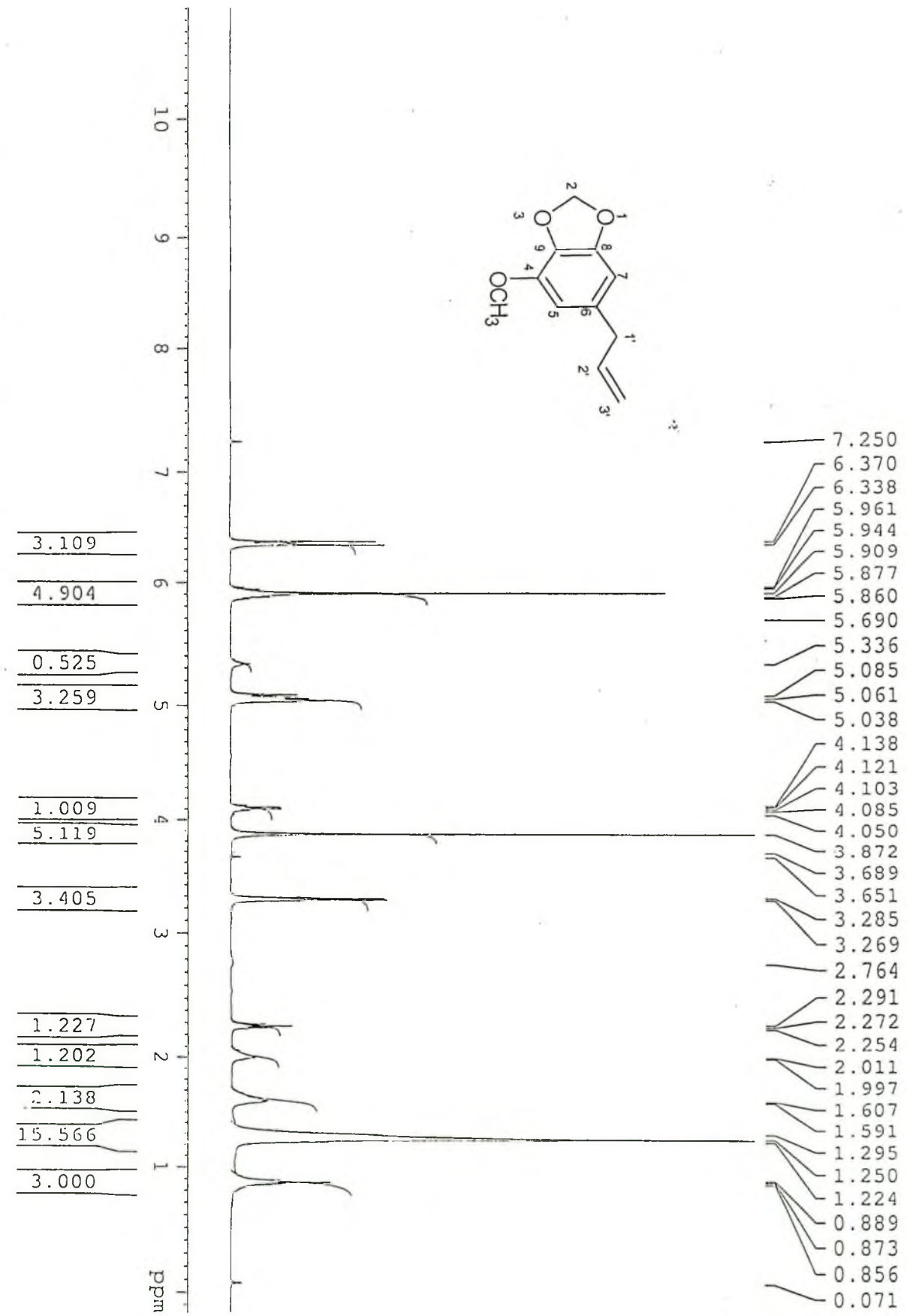


Figure No. 3 : ^{1}H NMR Spectrum of Compound –I

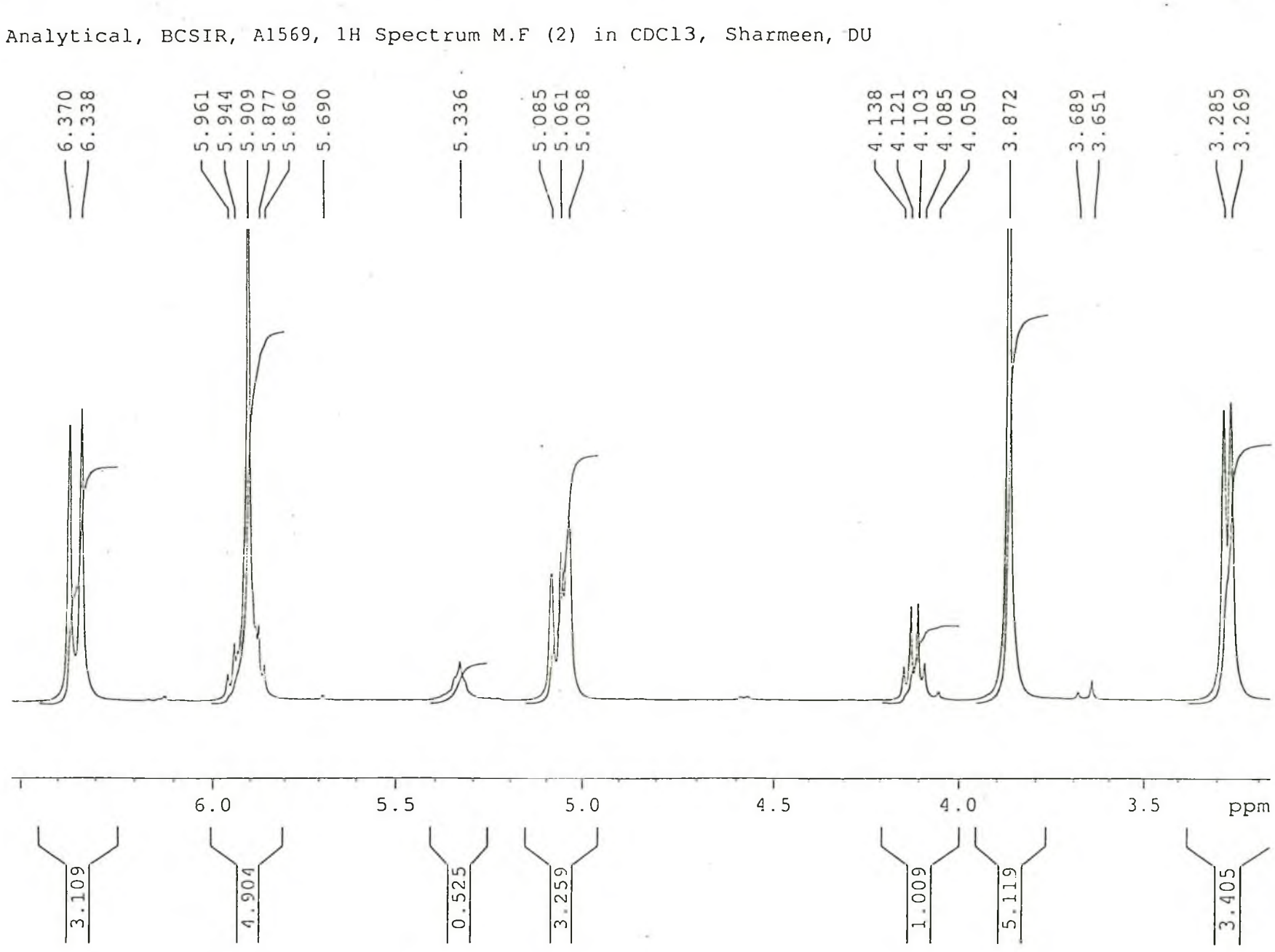


Figure No. 4: ^{1}H NMR Spectrum of Compound –I (Expanded)

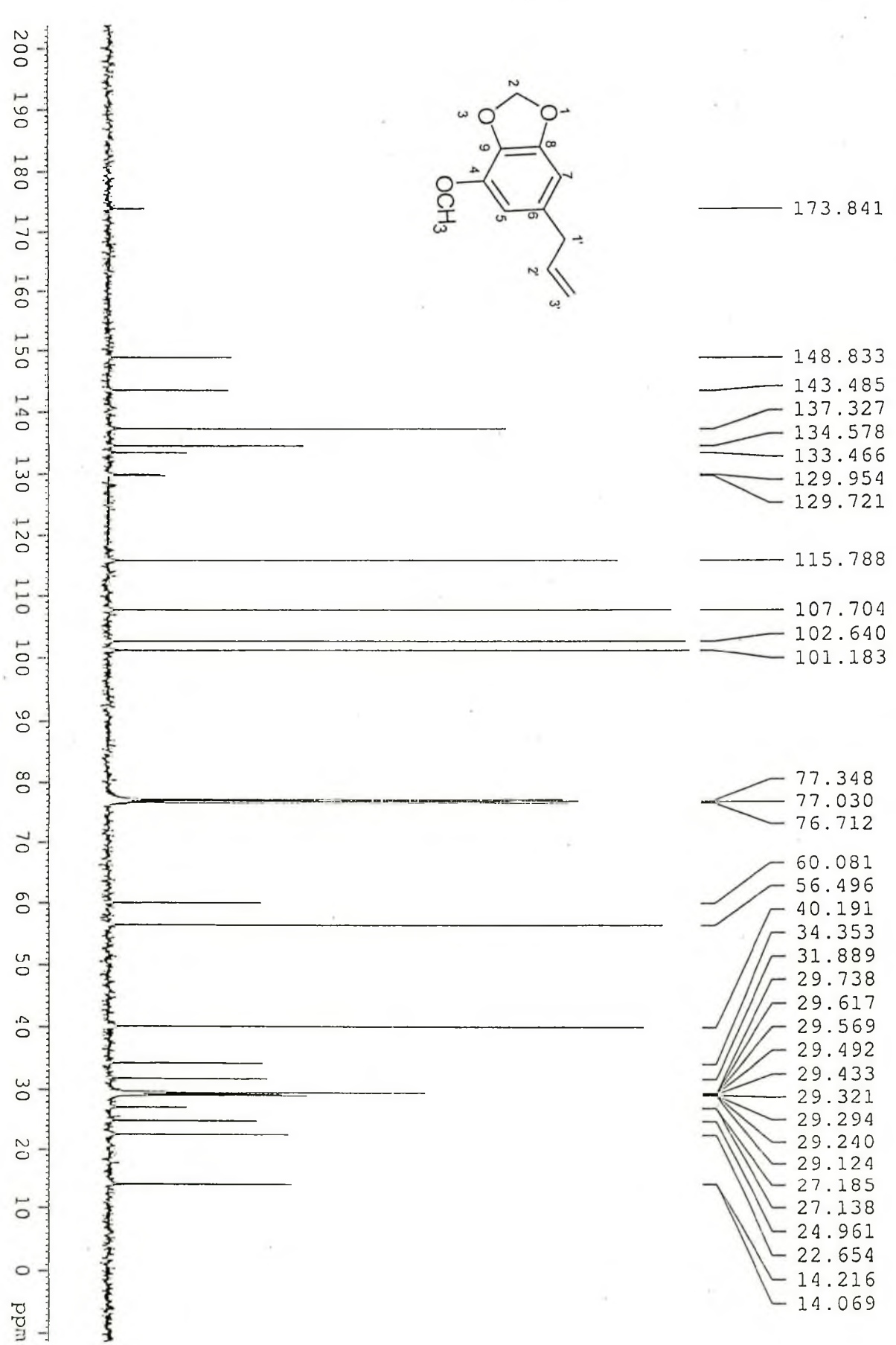


Figure No. 5: ^{13}C NMR Spectrum of Compound -I

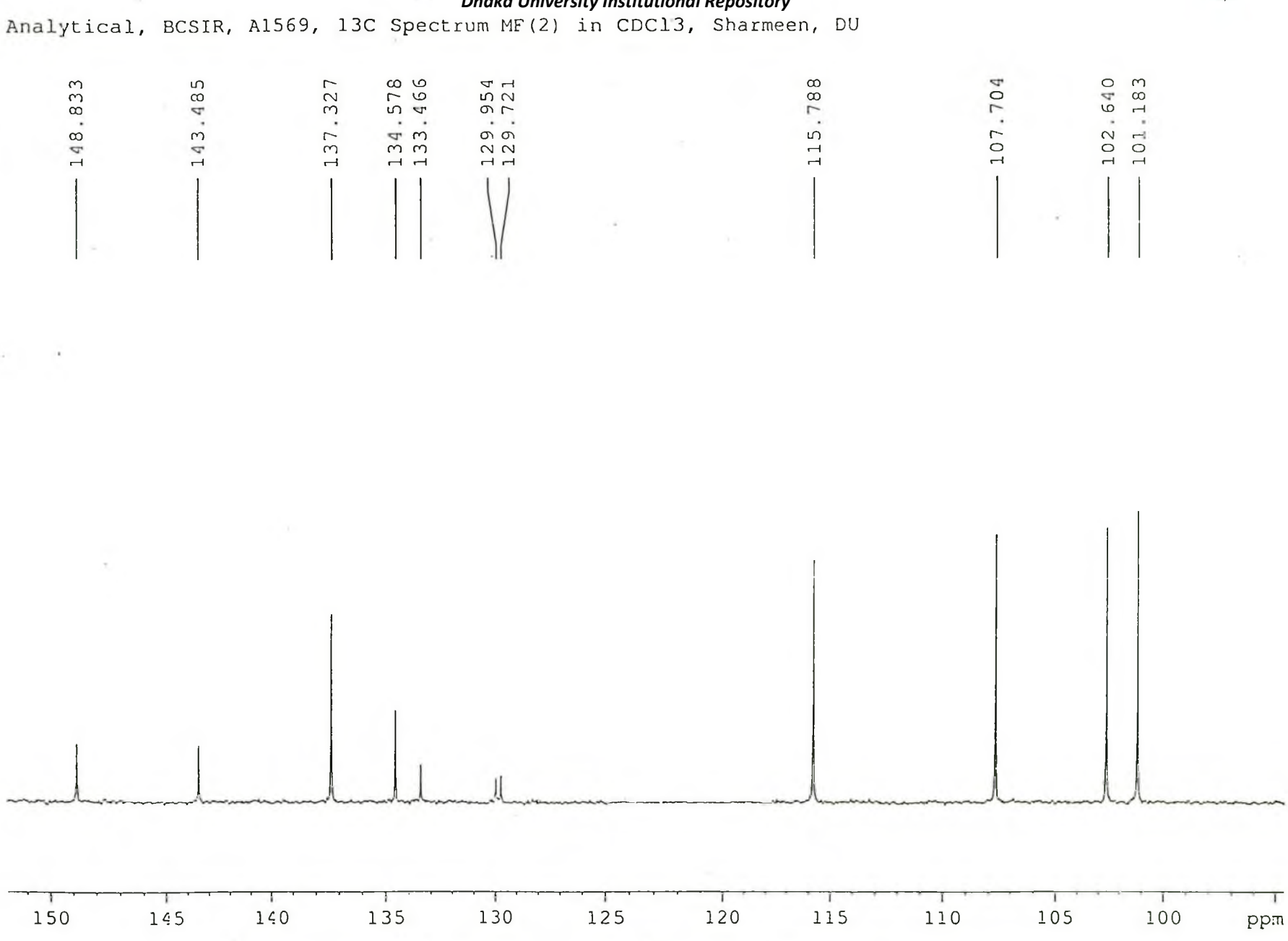


Figure No. 6 ^{13}C NMR Spectrum of Compound -I (Expanded)

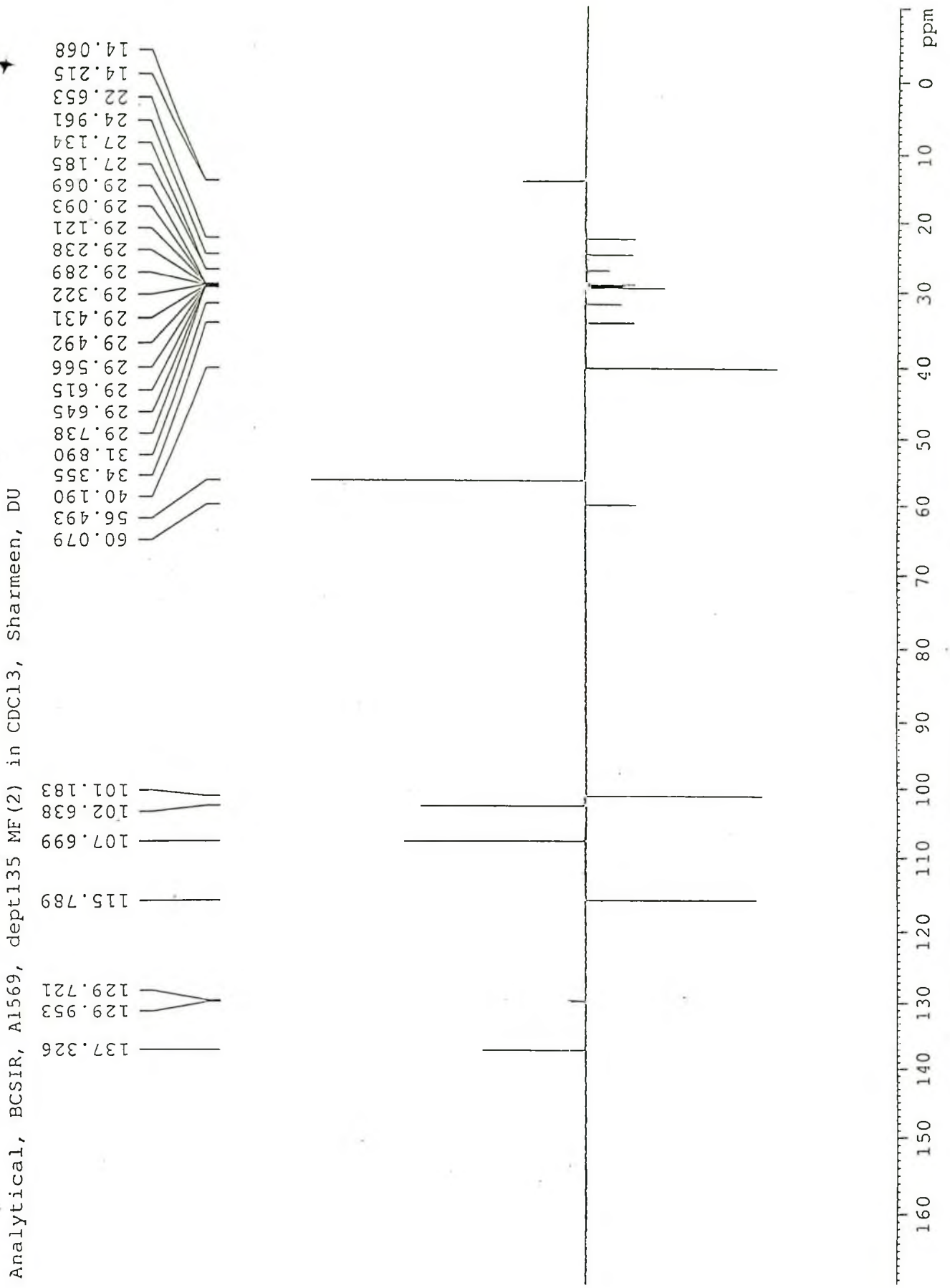


Figure No. 7 DEPT- 135 NMR Spectrum of Compound -I

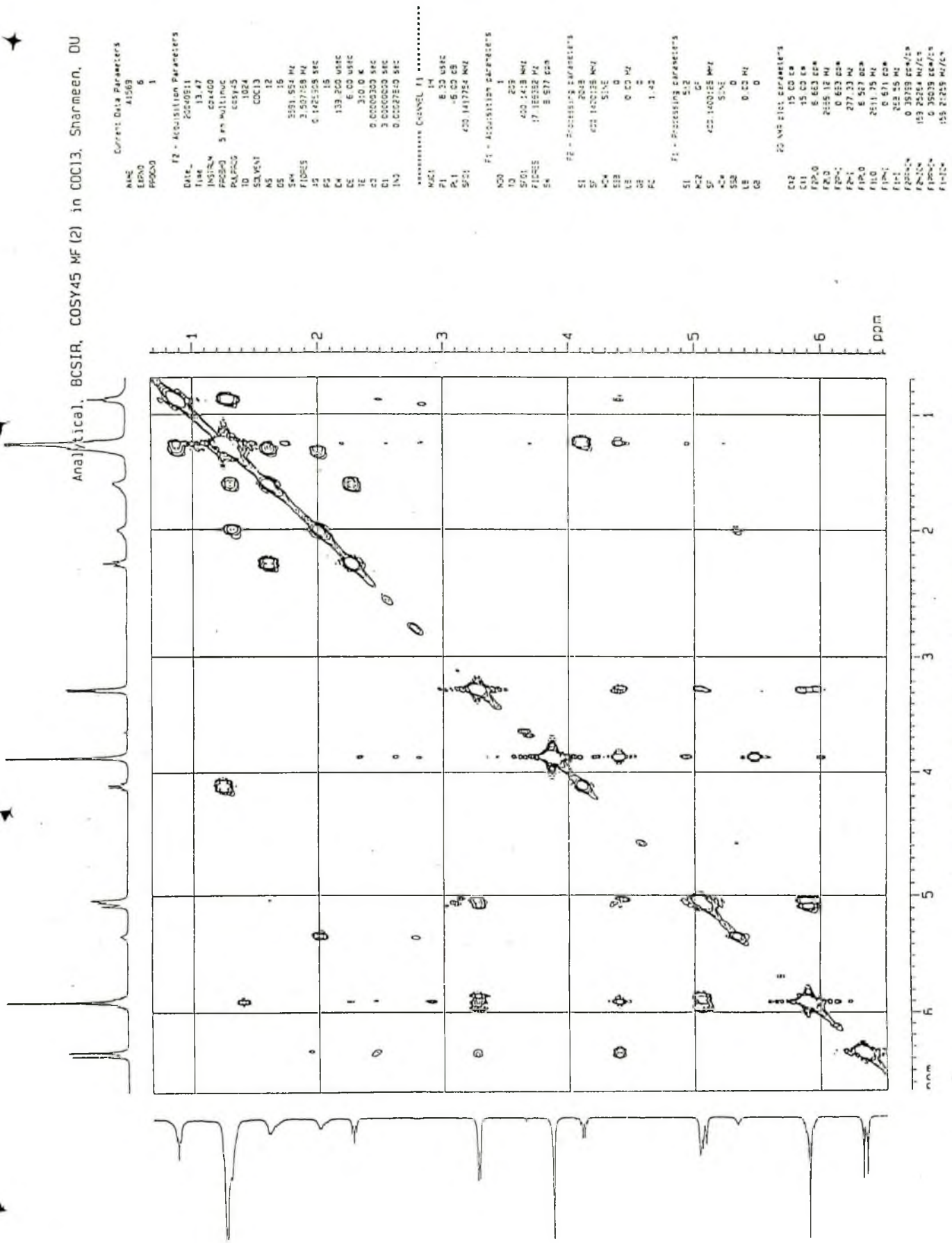


Figure No. 8 : H-H COSY Spectrum of Compound -I

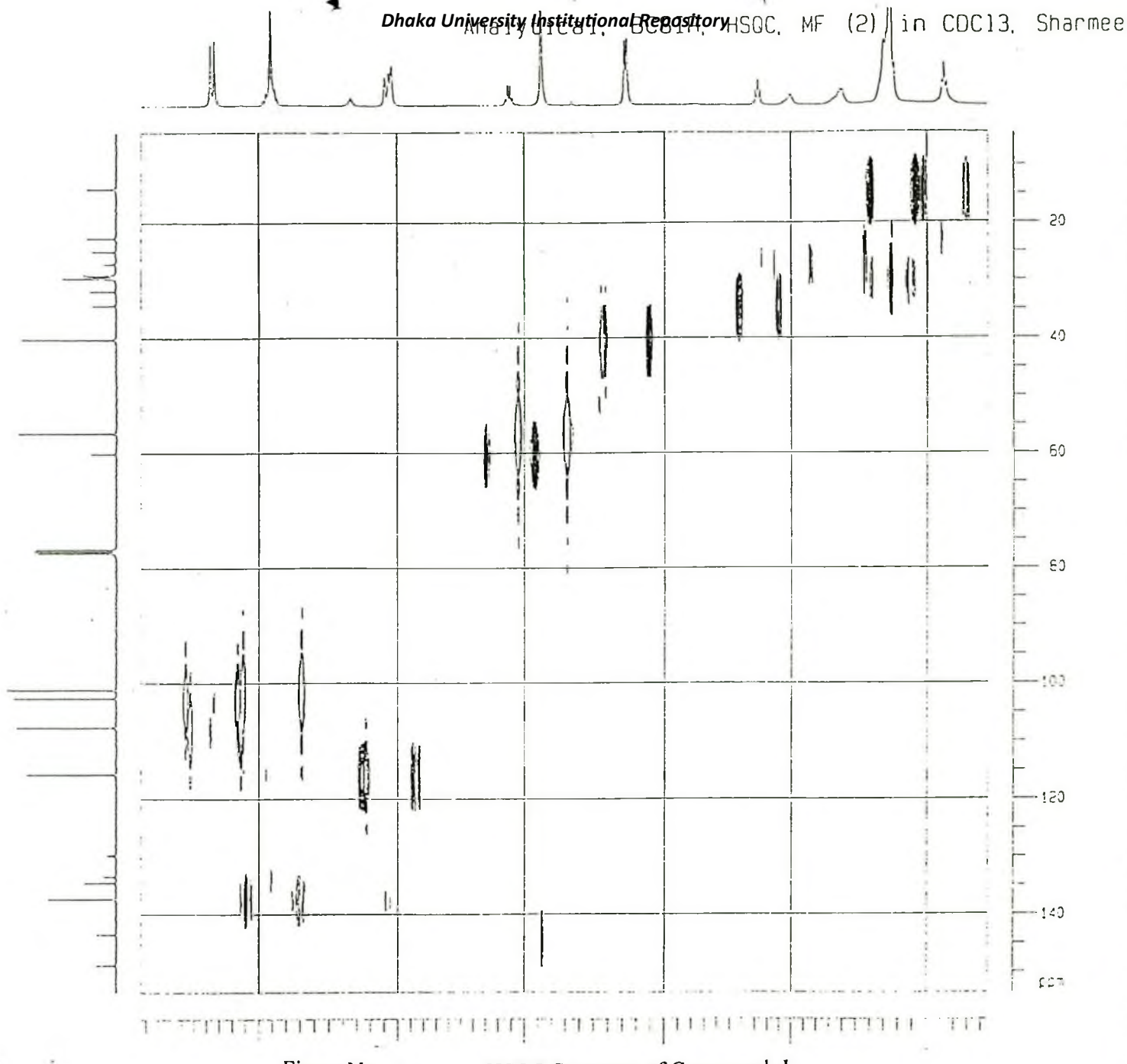


Figure No 9 : .. HSQC Spectrum of Compound -I

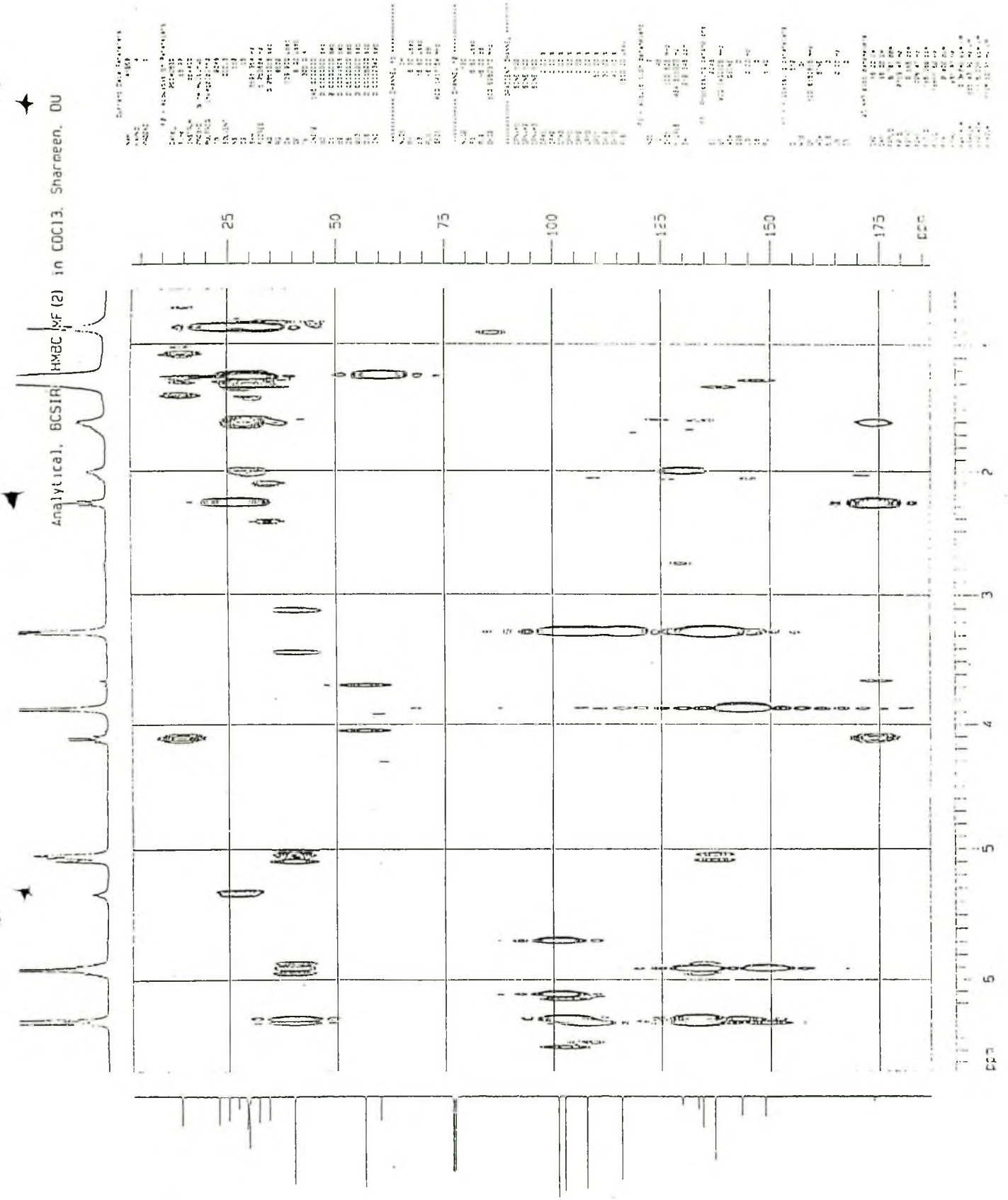


Figure No. 10. HMBC Spectrum of Compound -I

Table 5. Different fractions of Z_4 obtained from silica gel column chromatography.

Faction No.	Eluting solvent	Amount (mg)	TLC pattern
Z4.1	60%-80% DCM in Hexane	22.0	Single spot
Z4.2	90%-100% DCM in Hexane	37.4	Two spot
Z4.3	1% -2% Methanol in DCM	2.3	Single spot
Z4.4	2% -5% Methanol in DCM	2.1	Single spot with tailing

2.6.1. Isolation of Compound II from $Z_{4.1}$

The fraction $Z_{4.1}$ was purified by small silica gel column in a pasteur pipette the small column was eluted first with hexane then hexane with 5% DCM to get pure compound II.

2.6.2. Characterization of Compound II by spectroscopic methods

a) UV spectroscopy

The UV spectrum (Fig 11) of compound II in chloroform showed characteristics absorption maxima, λ_{max} at 244 and 283 nm.

b) Infrared spectroscopy

The IR spectrum of the Compound II (Fig 12) had important absorption frequencies at 3400, 2910, 1600, 1500, 1440, 1420, 1200, 1040, 960, 920, 840, 810 and 720 cm^{-1}.

c) NMR spectroscopy

i) ^1H NMR spectroscopy

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) (Fig 13-14) of compound II had signals at 3.89 (3H, s), 4.29 (2H, d, *J*=5.8Hz), 5.95 (2H, s), 6.20 (1H, dt, *J*=15.6Hz), *J*=5.8Hz), 6.49 (1H, d, J=16Hz), 6.53 (1H,s), 6.61 (1H,s) ppm.

ii) ^{13} C NMR spectroscopy

^{13}C NMR spectrum (100MHz, $CDCl_3$) (Fig 15) of Compound II had 17 signals at 56.6, 63.7, 100.1, 101.5, 106.8, 127.3, 131.0, 131.7, 135.1, 143.6 and 149.7 ppm. Dept spectrum of the compound also recorded (Fig16).

iii) 2D NMR spectroscopy. 2D NMR spectra (H-H COSY, HSQC and HMBC of Compound II were shown in (Fig 17-23) and the spectral data are given in the Table 6-8.

Table 6. H-H COSY spectral data of the Compound II (δ value in ppm).

^{1}H		^{1}H
4.29 (H-1)	⟷	6.20 (H-2)
6.20 (H-2)	⟷	6.49 (H-3)

Table 7. HSQC spectral data of Compound II (δ value in ppm)

^{1}H		^{13}C
3.89 (7′-OCH_3)	⟷	56.6 (7′-OCH_3)
4.29 (H-1)	⟷	63.7 (C-1)
5.95 (H-2′)	⟷	101.5 (C-2′)
6.20 (H-2)	⟷	127.3 (C-2)
6.49 (H-3)	⟷	131.0 (C-3)
6.53 (H-6′)	⟷	106.8 (C-6′)
6.61 (H-4′)	⟷	100.1(C-4′)

Table 8. HMBC (Heteronuclear Multiple Bond Correlation) spectral data of Compound II (δ value in ppm.)

^{1}H		^{13}C
3.89 (7`-OCH_3)	⟷	143.6 (C-7`),
4.29 (H-1)	⟷	127.3 (C-2),131.0 (C-3)
5.95 (H-2′)	⟷	135.1 (C-8′),149.7 (C-9′)
6.20 (H-2)	⟷	63.7 (C-1),131.0 (C-3),131.7(C-5′),
6.49 (H-3)	⟷	63.7 (C-1′), 101. (C-4′),106.8(C-6′),
6.53 (H-6′)	⟷	131.0 (C-3′),100.1 (C-4′),131.7(C-5′), 143.6 (C-7′), 135.1 (H-8′),
6.61 (H-4′)	⟷	131.0 (C-3),131.7 (C-5′),106.8 (C-6′), 135.1 (H-8′), 149.7 (C-9′),

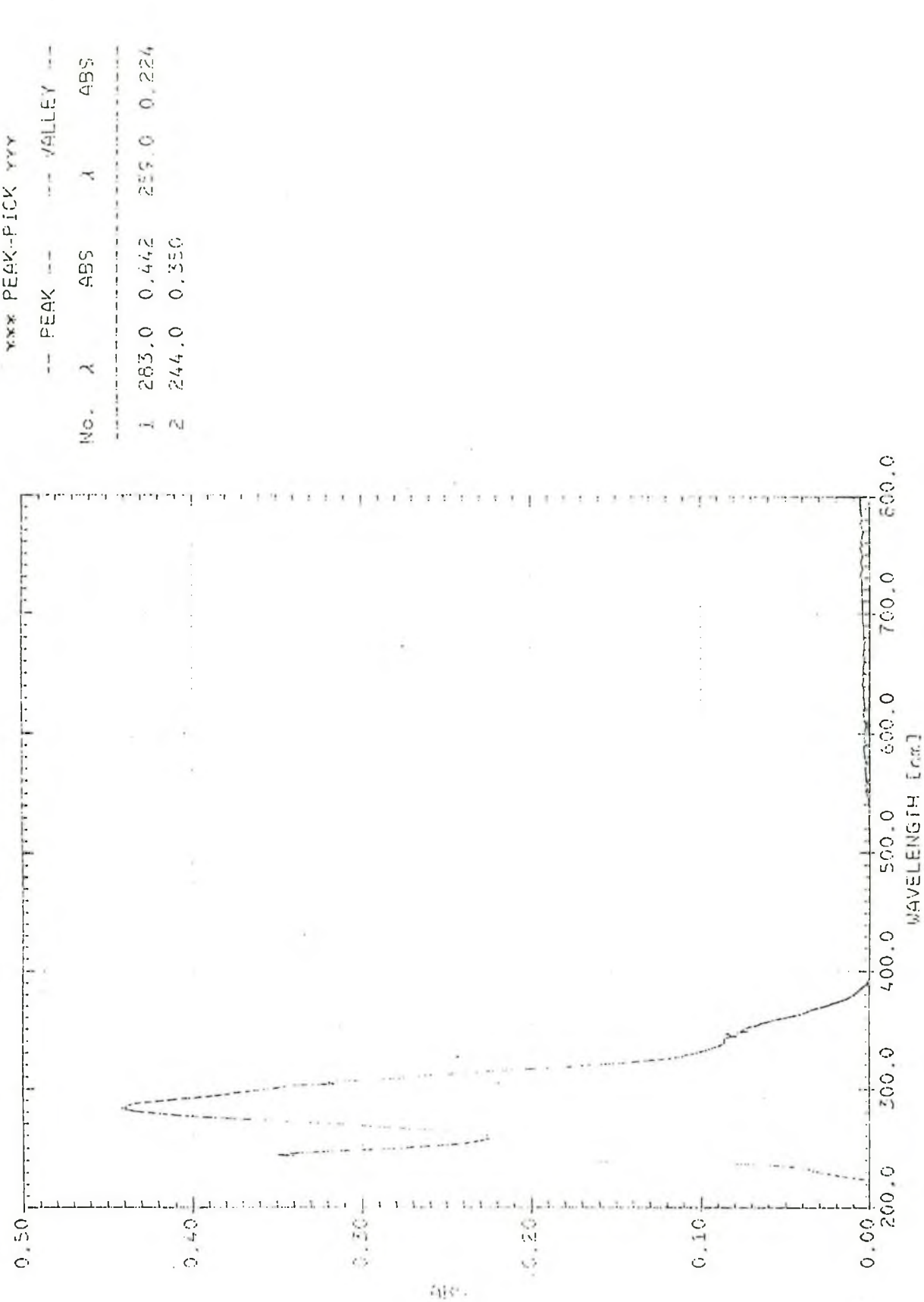


Figure No – 11 Ultraviolet spectrum of compound – II

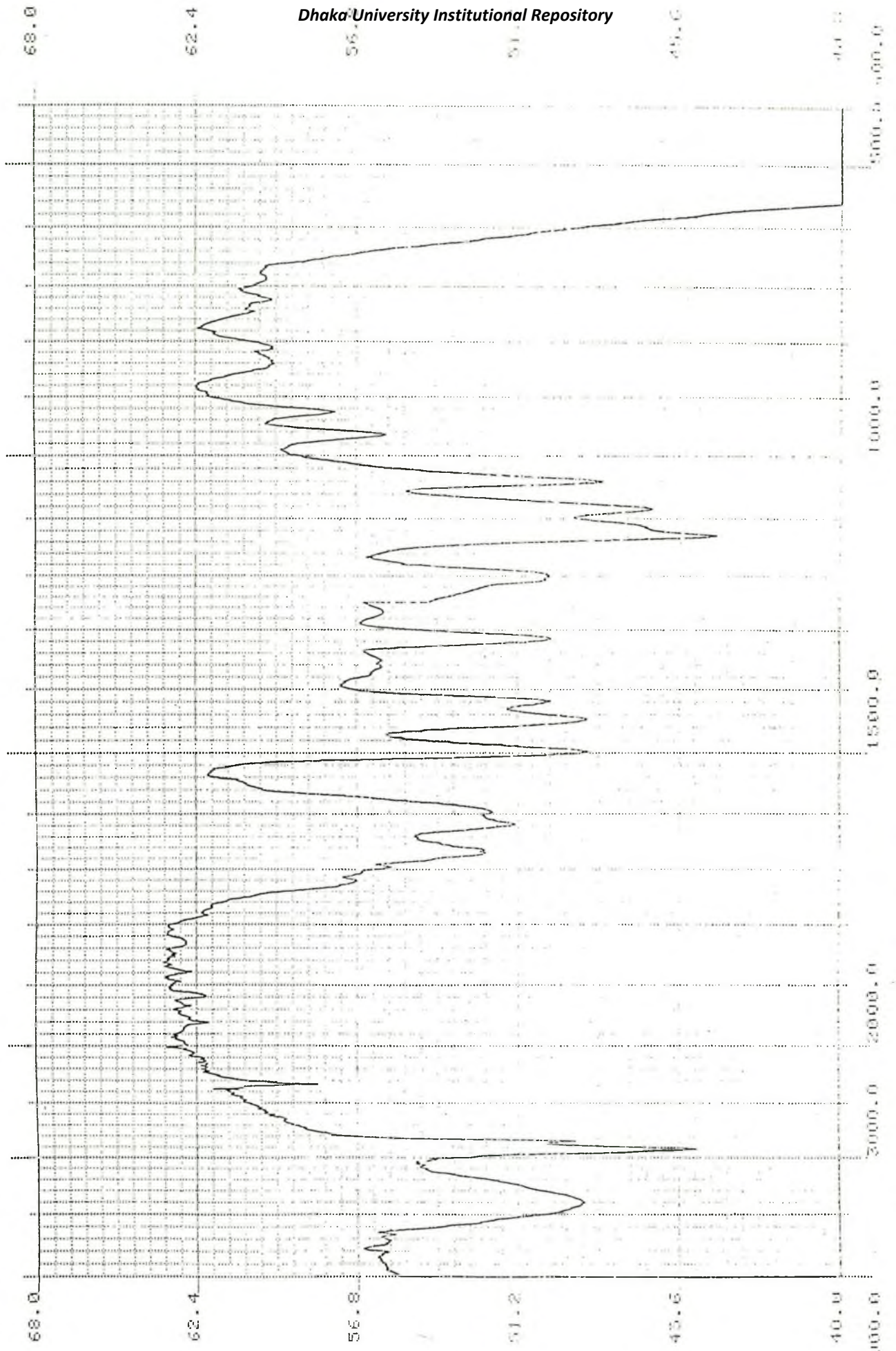


Figure No – 12 IR Spectrum of Compound – II

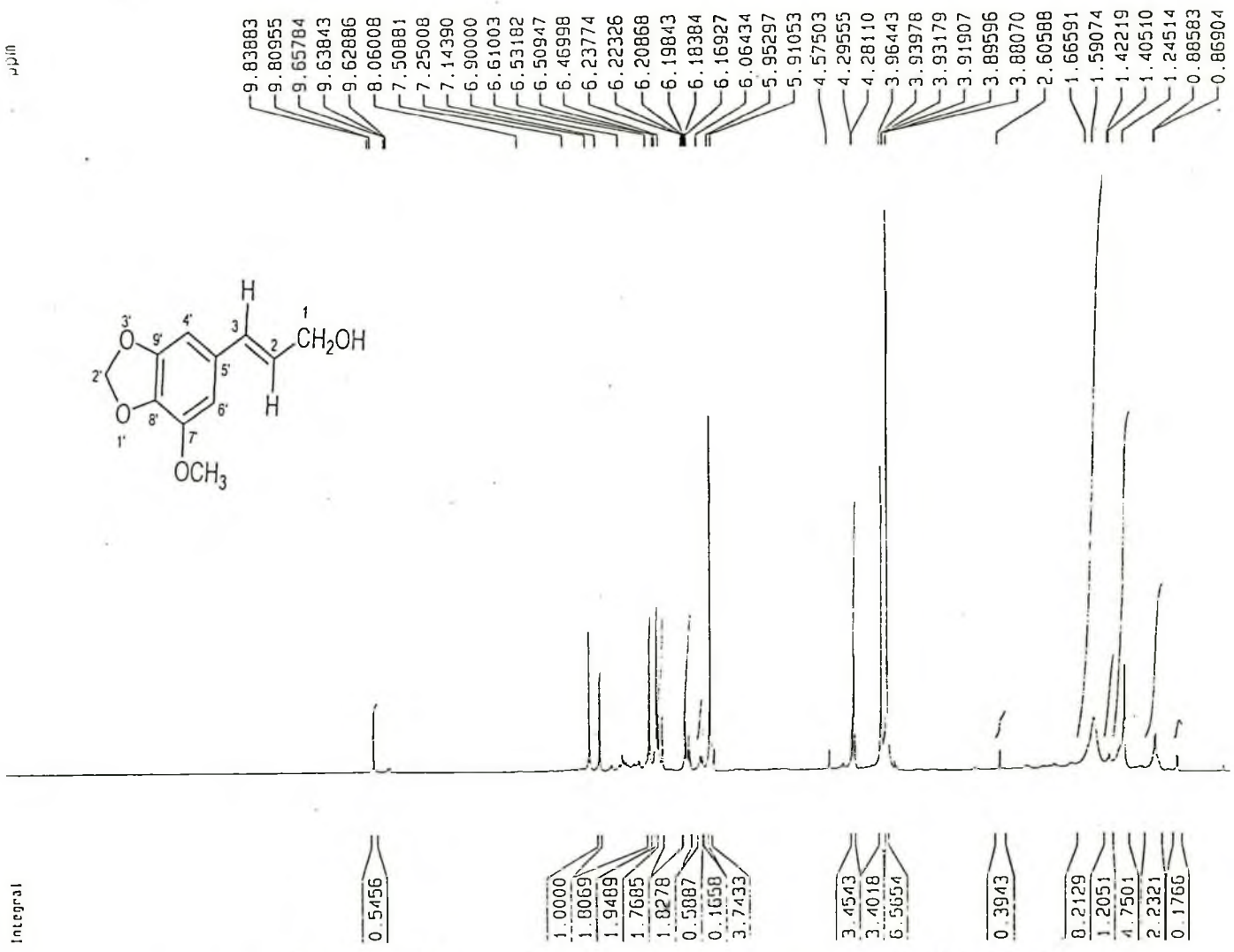


Figure No. 13 : ^{1}H NMR Spectrum of Compound -II (Expanded)

```
Current Data Parameters
NAME                  A3367
EXPNO                    7
PROCNO                   1

F2 - Acquisition Parameters
Date_              20070426
Time                 18.44
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                    362
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            400.1400124 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.40

1D NMR plot parameters
CX                 20.00 cm
F1P               14.049 ppm
F1                5621.76 Hz
F2P               -0.027 ppm
F2                -10.89 Hz
PPMCM          0.70333 ppm/cm
HZCM           281.63205 Hz/cm
```

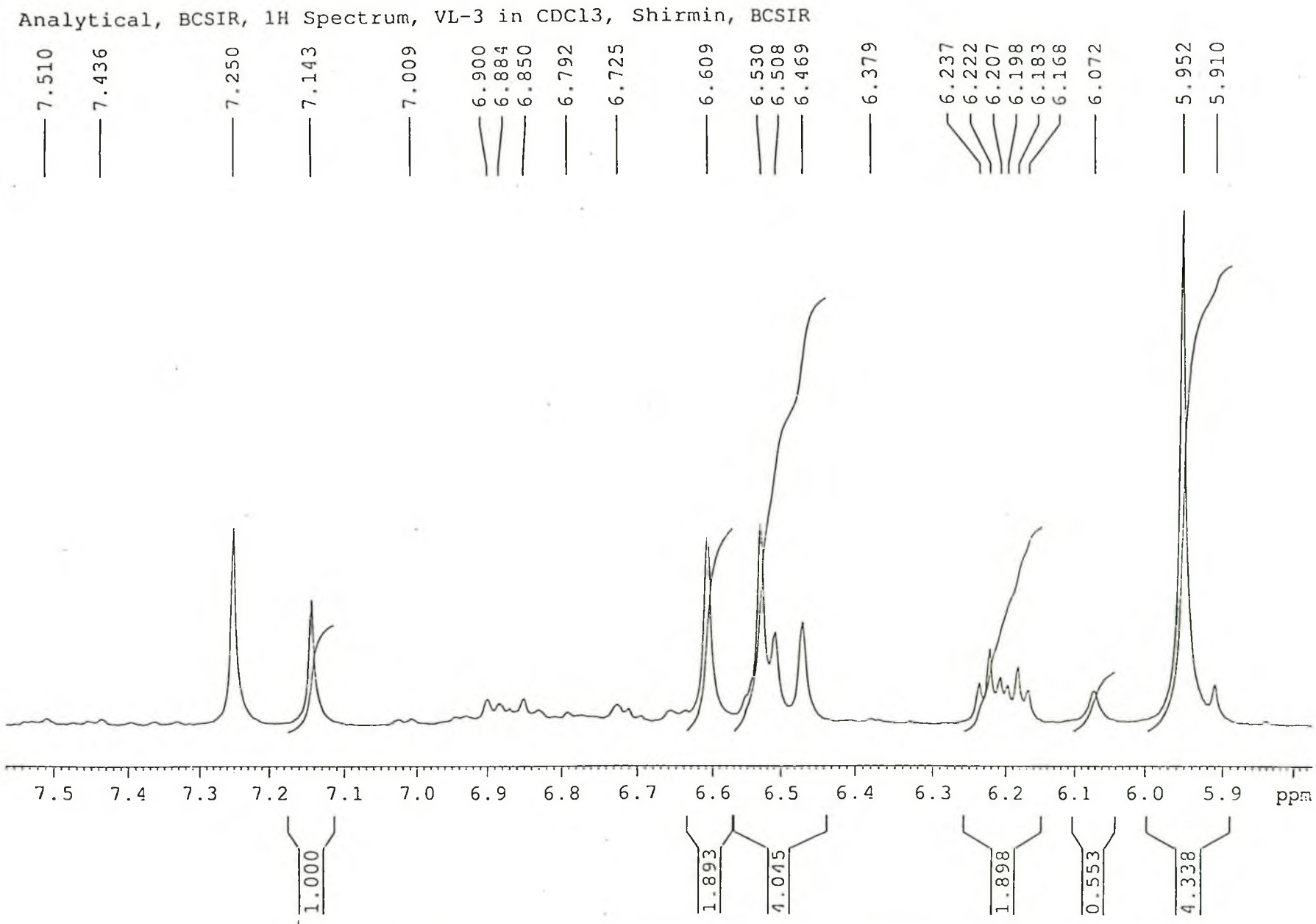


Figure No. -14: ^{1}H NMR Spectrum of Compound -II (Expanded)

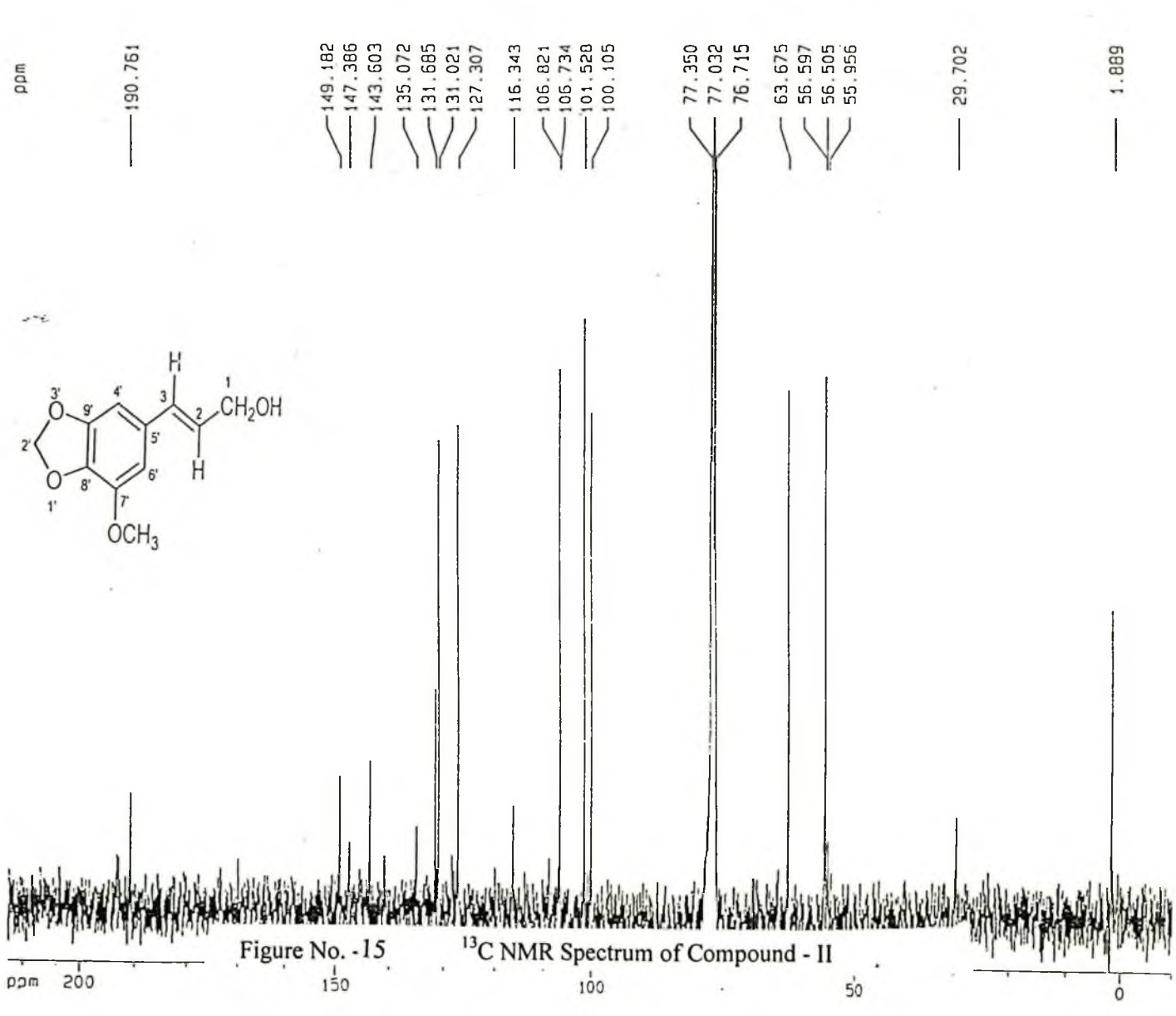


Figure No. -15 ^{13}C NMR Spectrum of Compound - II

```
Current Data Parameters
NAME                  A3367
EXPNO                    2
PROCNO                   1

F2 - Acquisition Parameters
Date_              20070317
Time                  16.59
INSTRUM               cpx400
PROBHD     5 mm Multinuc
PULPROG               zgpg30
TD                    32768
SOLVENT               Aceton
NS                     8325
DS                        2
SWH               24154.590 Hz
FIDRES             0.737140 Hz
AQ                 0.6783476 sec
RG                    16384
DW                   20.700 usec
DE                    6.00 usec
TE                   300.0 K
D1                1.50000000 sec
d11               0.03000000 sec
d12               0.00002000 sec

********** CHANNEL f1 **********
NUC1                     13C
P1                    8.30 usec
PL1                  -6.00 dB
SFO1            100.6253045 MHz

********** CHANNEL f2 **********
CPDPRG2             waltz16
NUC2                     1H
PCPD2                80.00 usec
PL2                  -6.00 dB
PL12                 16.00 dB
PL13                120.00 dB
SFO2            400.1400000 MHz

F2 - Processing parameters
SI                       32768
SF              100.6152823 MHz
WDW                      EM
SSB                       0
LB                     2.50 Hz
GB                        0
PC                     1.40

1D NMR plot parameters
CX                    20.00 cm
F1P                 212.807 ppm
F1                21411.61 Hz
F2P                -10.042 ppm
F2                -1010.37 Hz
PPMCM              11.14243 ppm/cm
HZCM             1121.09600 Hz/cm
```

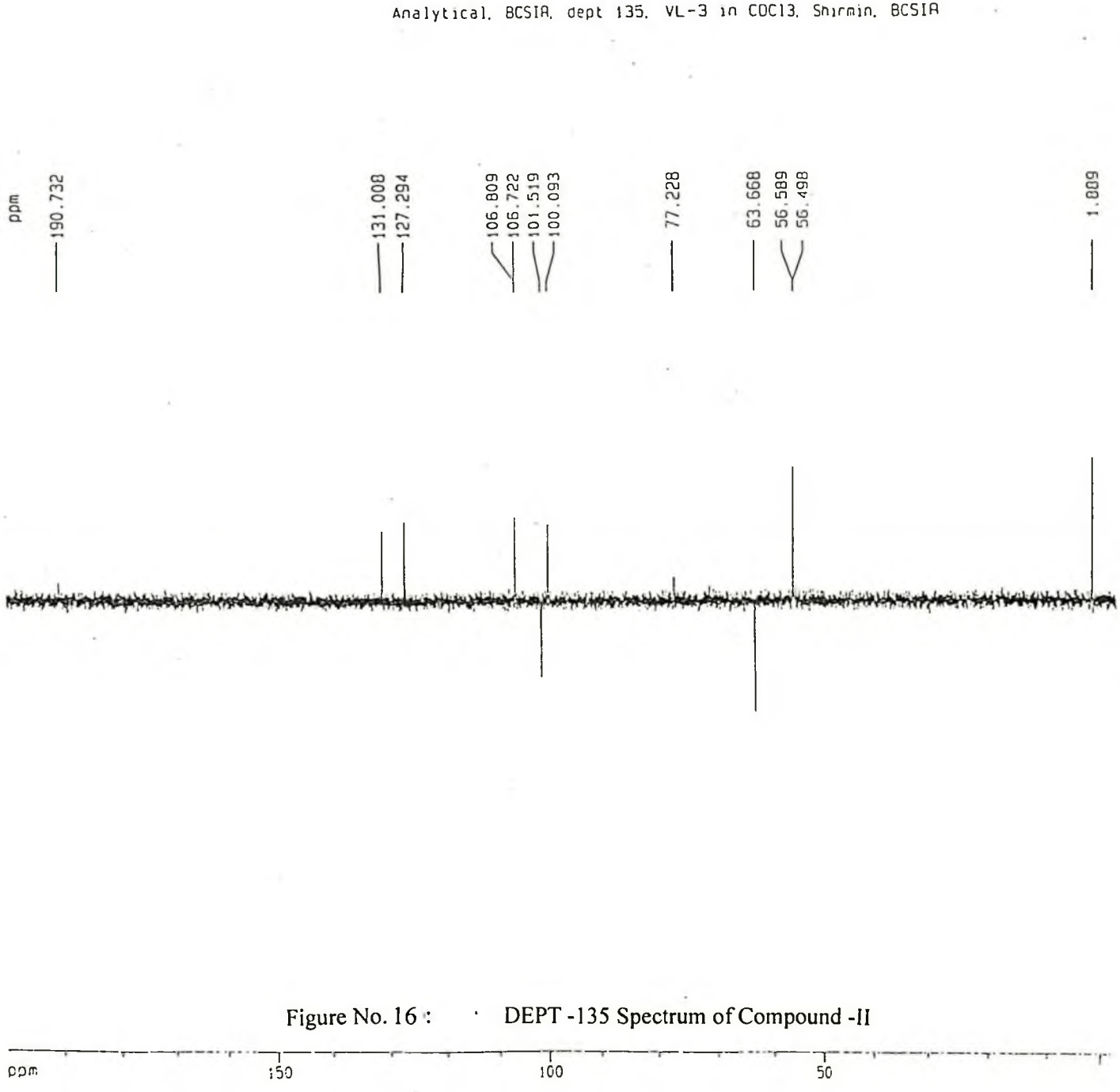


Figure No. 16 : DEPT -135 Spectrum of Compound -II

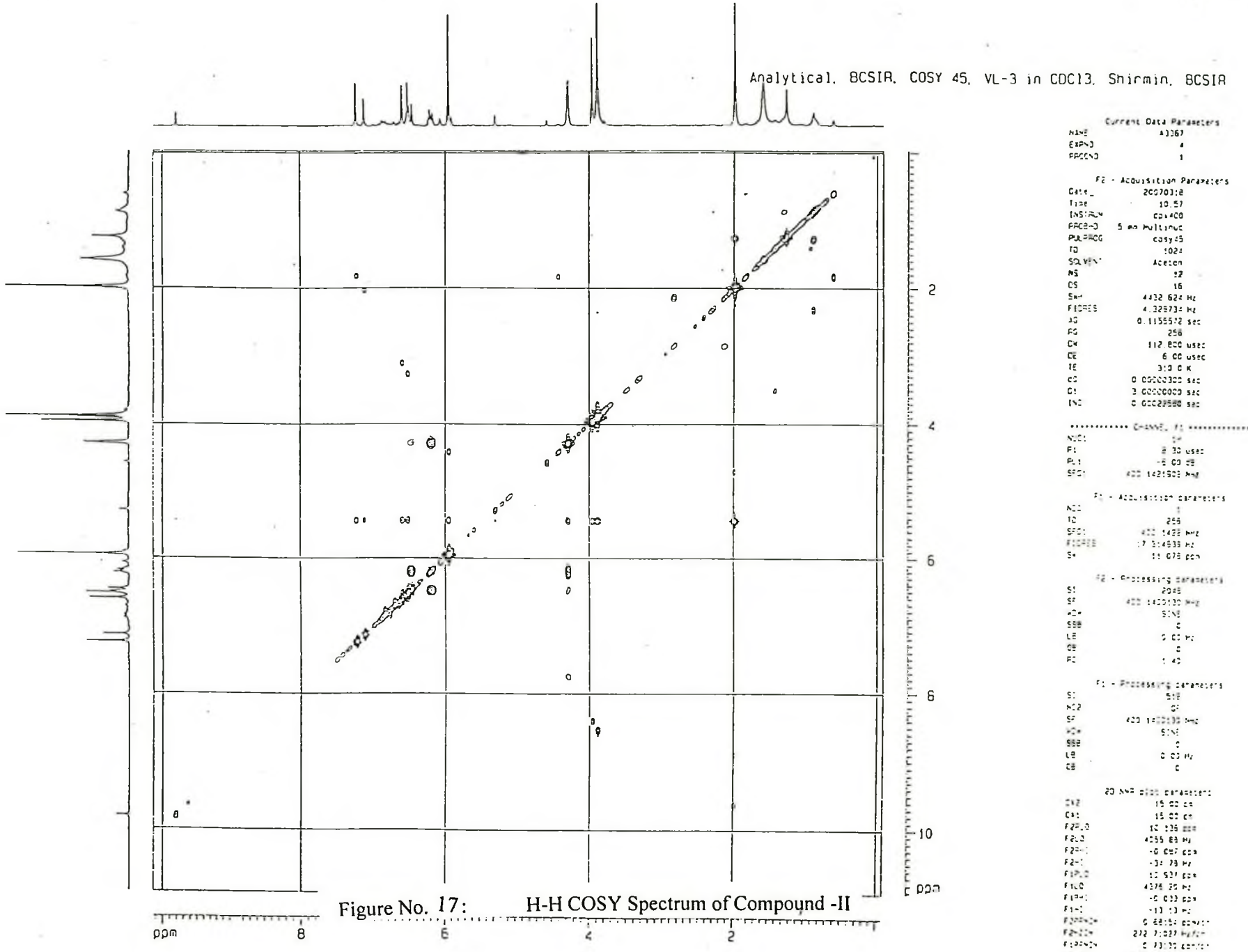


Figure No. 17: H-H COSY Spectrum of Compound -II

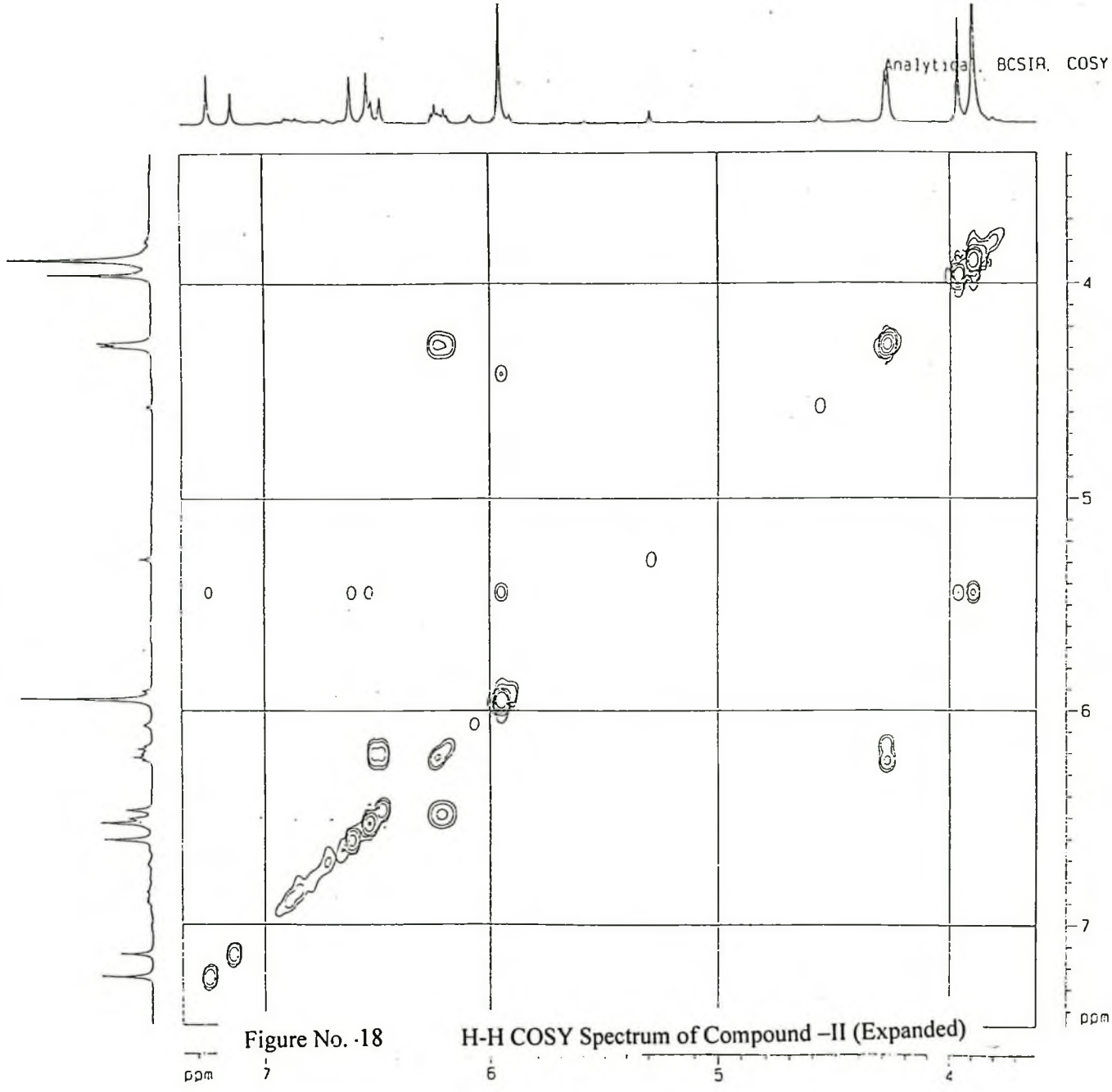


Figure No. -18 H-H COSY Spectrum of Compound –II (Expanded)

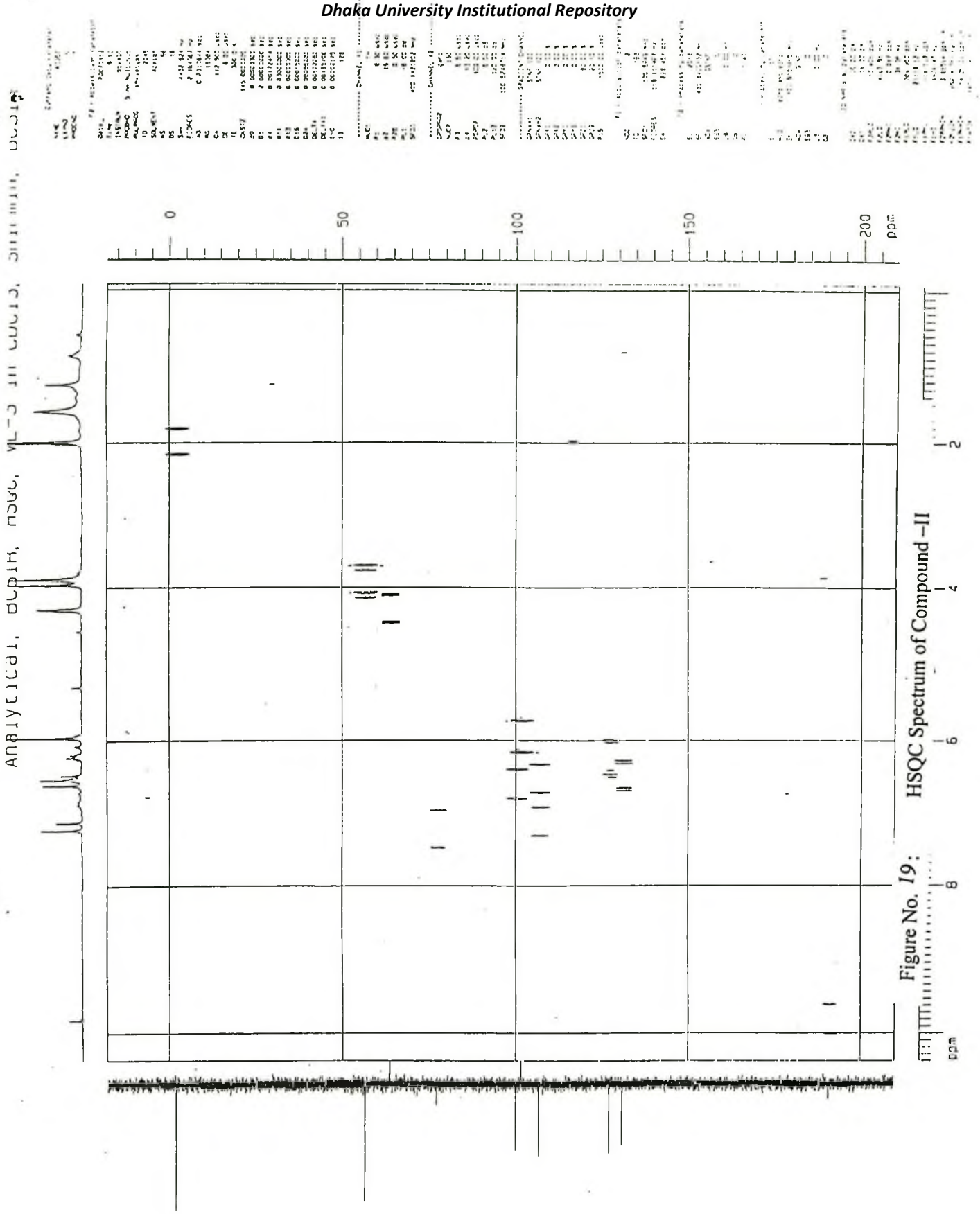


Figure No. 19: HSQC Spectrum of Compound –II

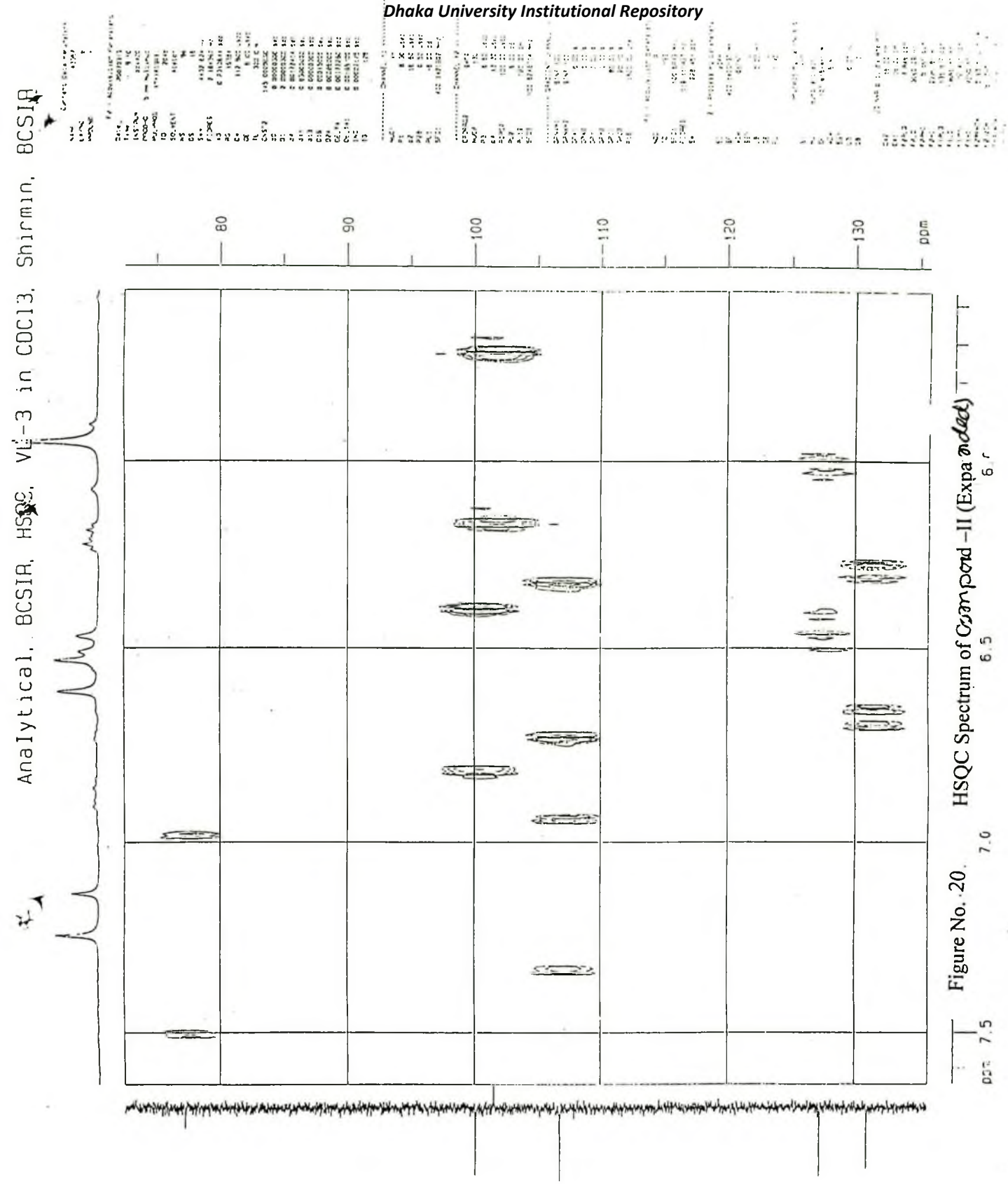


Figure No. 20. HSQC Spectrum of Compound –II (Expanded)

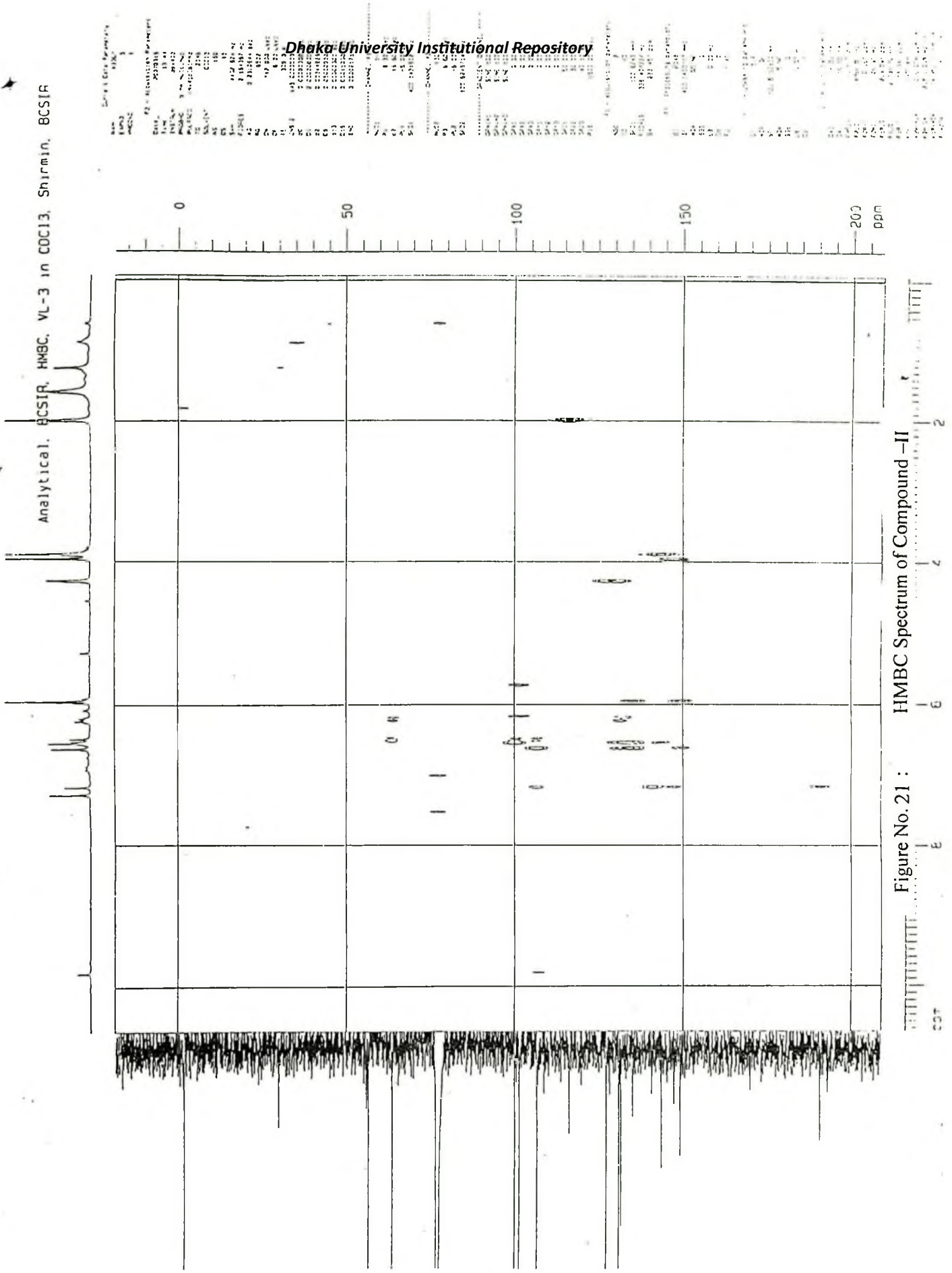


Figure No. 21 : HMBC Spectrum of Compound –II

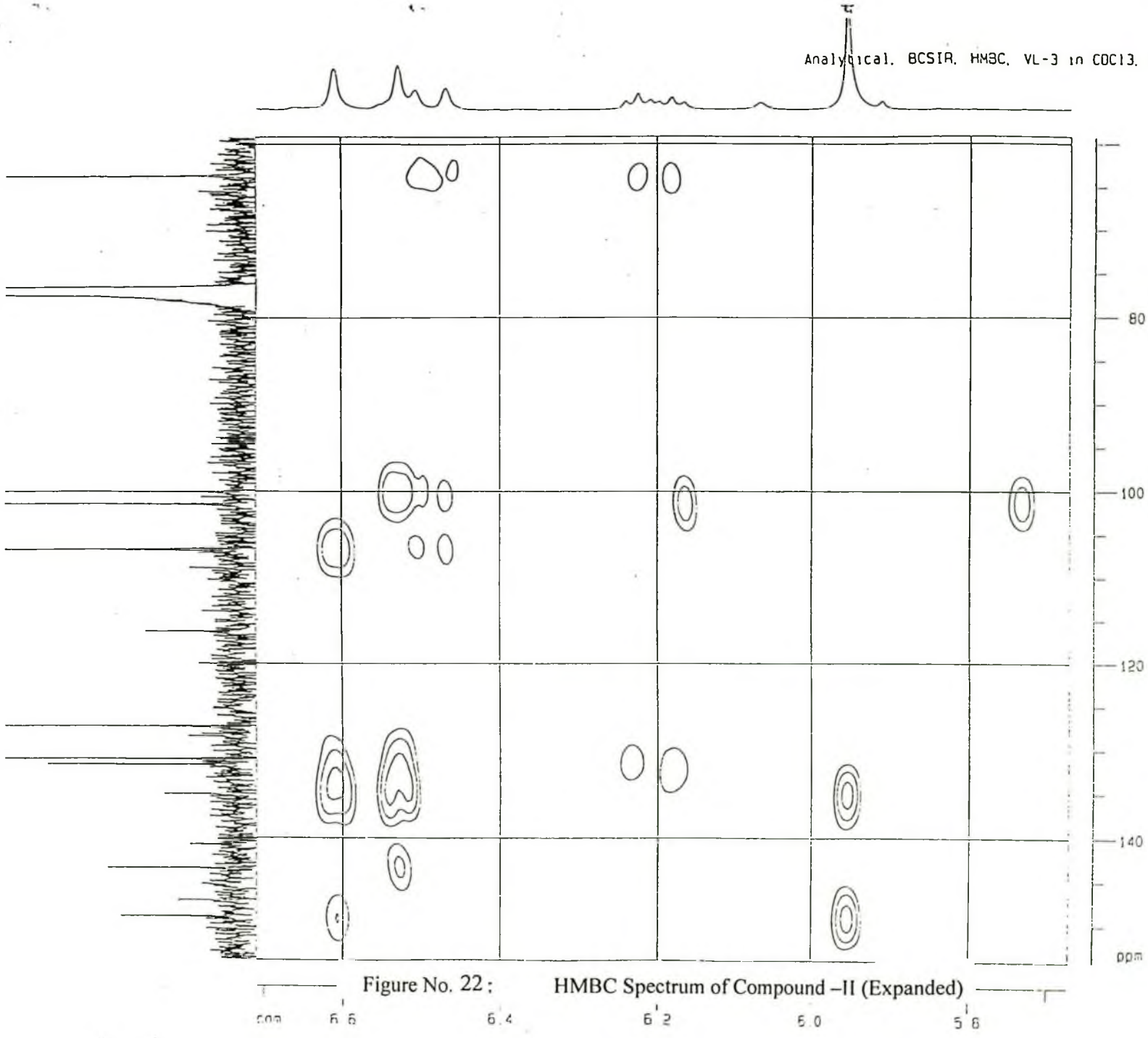


Figure No. 22 : HMBC Spectrum of Compound –II (Expanded)

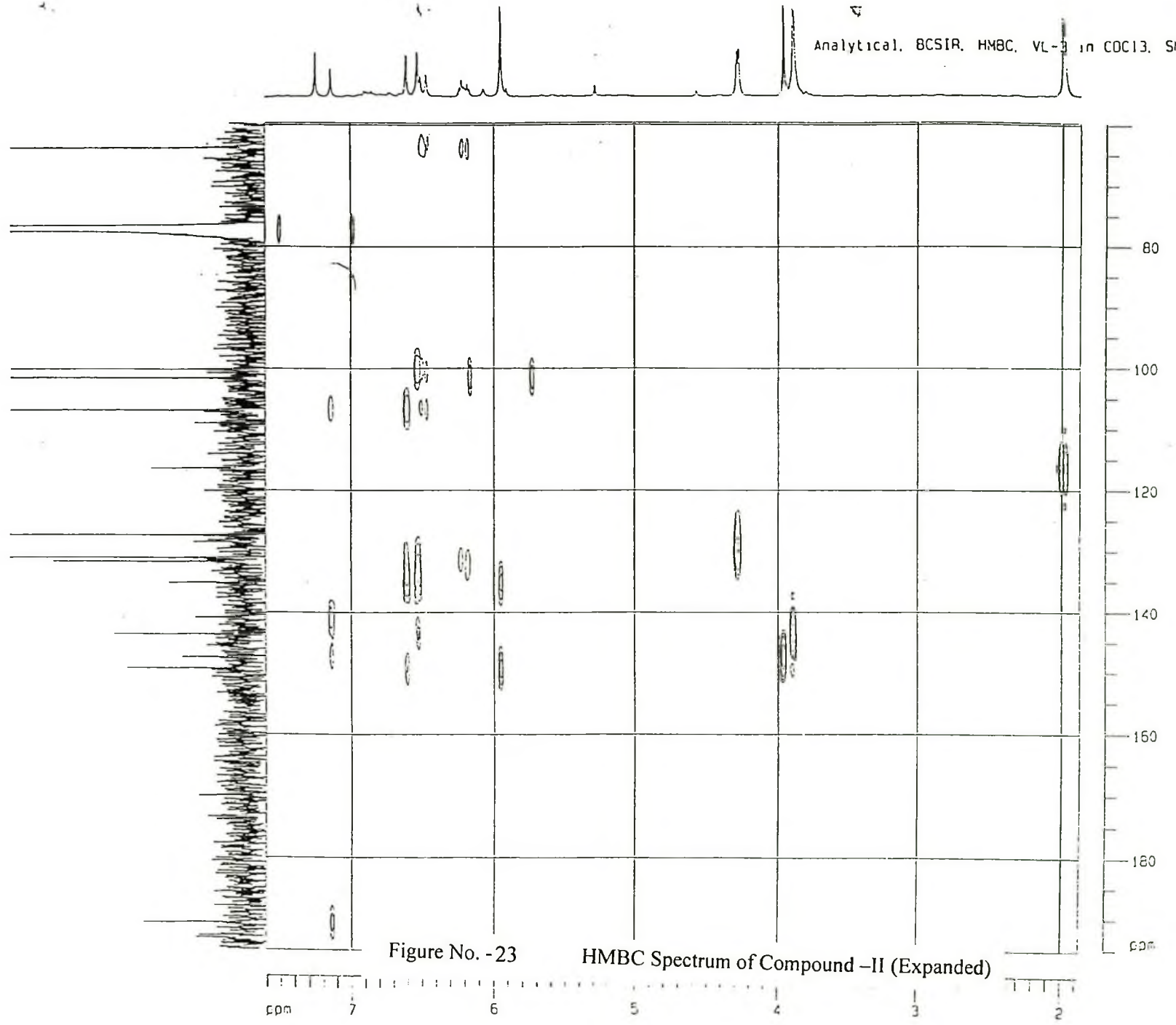


Figure No. -23 HMBC Spectrum of Compound –II (Expanded)

PART B

RESULTS AND DISCUSSION

3. RESULTS AND DISCUSSION

3.1. Collection of seeds

The seeds of the *Myristica fragrans* were bought from local market and crushed to remove the kernel. The kernel free seeds were powdered and dried in an oven at 40^0C.

3.2. Different extractives of seeds of *Myristica fragrans*

The seeds power of *Myristica fragrans* (450 g) was Soaked in pet ether (500 mL×3) for overnight. The pet ether extract free (170 g) solid material was extracted with 80% ethanol in a conical flask. The extract was evaporated in a rotary vacuum evaporator with added water to remove ethanol completely. The resulting aqueous solution was partitioned with hexane to remove residual fat.

The aqueous suspension was partitioned with ethyl acetate using a separating funnel. The ethyl acetate soluble layer was evaporated to dryness and finally dried by high vacuum in a freeze-dryer. The amount of ethyl acetate soluble part was 10.3 g.

The water soluble part was further partitioned with 1-butanol. After evaporation and freeze-drying 3.3 g of 1-butanol soluble and 9.2g of water soluble parts were obtained.

3.3 Fractionation of ethyl acetate soluble part by column chromatography

Ethyl acetate extract of *Myristica fragrans (8.0 g)* was fractionated by column chromatography using hexane, dichloromethane and methanol as mobile phase. The eluted samples were monitored by TLC and finally seven different fractions (Table - 1) were obtained. The fourth fraction (Z_4, 60 g) gave three spots with tailing on TLC. This fraction was further fractionated by silica gel column. Four different fractions

(Table-5) were collected according to the TLC pattern. Among them fractions $Z_{4.1}$ and $Z_{4.3}$ gave single spots. These were marked as compound II and compound III respectively.

3.4. Characterization of compounds

3.4.1. Characterization of compound I as myristicin, 6-Allyl-4-methoxybenzo[d][1,3] dioxol.

Compound I was isolated from the column fraction by elution with hexane 25 -35% dichloromethane. It was obtained as brown gum. It was soluble in chloroform and dichloromethane. Spraying the developed plate with ceric (IV) sulphate-sulphuric acid reagent, followed by heating gave a violet colour.

3.4.1.1. Structure elucidation of compound I by spectroscopic method

i) Infrared spectroscopy

The IR spectrum of compound I showed absorption at 2910 cm^{-1} indicating the presence of C-H in conjugation. Absorption at 1620 cm^{-1}, 1420 cm^{-1} for C=C of phenyl group, Absorption at 915 cm^{-1} and 805 suggested for C-H bending mode of phenyl group.

ii) NMR spectroscopy

The molecular formula of Compound I was deduced as $C_{11}H_{12}O_3$ from its ^1H and ^{13}C NMR spectra and DEPT 135 experimental data. The ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of this compound displayed 11 carbon resonances while the HSQC experiment indicated that 7 out of 11 carbons were attached to protons. ^1H NMR (400 MHz, $CDCl_3$ and DEPT 135 (100 MHz, $CDCl_3$) spectra revealed the presence of one methoxy, three methylenes, three methines and four quaternary carbon atoms. The ^1H NMR and carbon ^{13}NMR Spectral data are shown in Table-9.

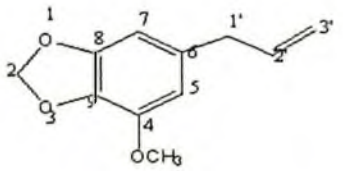
The resonance at δ5.91 ppm in the ^{1}H NMR and at δ137.3 ppm in the ^{13}C NMR spectra, in conjunction with the DEPT 135 spectrum, could be attributed to one olefinic proton. The cross peak in the H-H COSY spectrum between the methylene protons at δ3.28 ppm and loefinic proton at δ5.91 ppm indicated that they were vicinal. The resonance at δ5.07 ppm in the ^{1}H NMR at the δ115.8 ppm in the ^{13}C MR spectra in conjunction with the DEPT 135 spectrum, could be attributed to one terminal olefinic methylene. Again, the cross peak in the COSY spectrum between this methylene protons at δ5.07 ppm and the olefinic proton at δ5.91 ppm indicated that they were vicinal.

The presence of two ^{1}H signals at δ6.34 and δ6.37 ppm were attributable to two aromatic protons. The presence of these two aromatic singlet indicated that Compound I might carry a tetra substituted benzene ring. The resonance at δ3.87 ppm in the ^{1}H NMR spectrum and the resonance at δ56.5 ppm in the ^{13}C NMR spectrum in conjunction with the DEPT 135 spectrum proved and presence of an *O*-methyl group. A two proton singlet at δ5.91 ppm in the ^{1}H NMR spectrum and the resonance at δ101.2 ppm in the ^{13}C NMR spectrum in conjunction with the DEPT 135 spectrum proved the presence of a methylenedioxy group.

HMBC experiment also showed and correlation of proton at δ5.91 with carbons at δ134.6 (C-9) and δ148.8 (C-8) in the H-H COSY this proton showed no coupling with another proton.

3.4.1.2. Structure of Compound 1

Analysis of one- and two dimensional NMR spectra including H-H COSY, HSQC and HMBC led to the assignment of the structure as shown in fig- in this structure the C1′-C2′-C3′ portion was assigned by tracing of cross peaks in the COSY spectrum. A 4- methoxybenzo[d][1,3] dioxol moiety was disclosed by HMBC correlation (H-2/C-8; H-2/C-9; H-5/C-1; H-5/C-9; H-7/C-5; H-7/C-8; H-7/C-9; H-7/C-1; H-2/C-6, H-4-OCH_3/C-4,



6-Allyl-4-methoxybenzo[d][1,3]dioxol
Compound I

The ^{1}H NMR and ^{13}C NMR spectra data of compound I was found to be identical to those reported for phenyl propanoid, 6-Allyl-4-methoxybenzo[d][1,3]dioxol (myristicin). It was previously isolated from Piper regenellii and also from the plant *Myristica fragrans.*

Table -9 ^{1}H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR data of Compound I

no.	δ_H J in Hz	δc	COSY	HMBC
1	---	---	---	---
2	5.91 (2H, s)	10.2	---	C8, C9
3	---	---	---	---
4	---	143.5	---	C1′,C2, C4, C6, C8, C9
5	6.37 (1H, s)	107.7	---	---
6	---	133.5	---	---
7	5.34 (1H, s)	102.6	---	C1′,C4, C5, C6, C8, C9
8	---	148.8	---	---
9	---	134.6	---	---
1′	3.28 (2H, d, *j= 6.4)*	40.2	H2′	C2′,C3′, C5, C6, C7, C9
2′	5.91 (1H, m)	137.3	H1′;H3′	C1′, C6
3′	5.07 (2H, d,) *j = 9.6*	115.8	H2′	C1′,C2′
4-OCH3	3.87 (3H, s)	56.5	---	C4

The ^{13}C-NMR data were compared with the reported data of myristicin which was fined very well with the established data. The comparison of the ^{13}C-NMR signals of Compound I and reported Compound is given in Table -10.

Table 10: Comparison of ^{13}C-NMR spectral data of Compound I with reported value.

C- Number	Chemical Shift (ppm)	
	Experimental data	Reported data
C-2	101.2	101.0
C-4	143.5	137.6
C-5	107.7	104.9
C-6	133.5	126.0
C-7	102.6	102.7
C-8	148.8	144.3
C-9	134.6	144.5
C-1′	40.2	33.8
C-2′	137.3	137.3
C-3′	115.8	115.5
C(4-OCH_3)	56.5	51.0

3.4.2 Characterization of compound II as anthriscinol, (*E*)-3-(7-methoxy- benzo[1,3]dioxol -5-yl)- prop-2-en-1-ol

Compound II was isolated from the column fraction by elution with 60%-80% DCM in hexane. It was obtained as yellow gum. It was soluble in chloroform and DCM. Spraying the developed plate with ceric (IV) sulphate- sulphuric acid reagent, followed by heating, gave a brown color.

3.4.2.1 Structure elucidation of compound II by spectroscopic method

i)Ultraviolet (UV) spectroscopy

The UV spectrum (Fig-11) of compound II in chloroform showed characteristics absorption maxima, λ_{max} at 244 nm indicating $\pi \rightarrow \pi^*$ transition of phenyl. Another absorption maxima was found at 283 nm.

ii) Infrared spectroscopy

The IR spectrum of compound II showed absorption at 3400 cm^{-1} indicating the presence of –OH group. The spectrum showed frequency at 2910 cm^{-1} indicating the presence of C-H in conjugation. Absorption at 1600, 1500, 1440 and 1420 cm^{-1} for C=C of phenyl. Absorption at 960, 840, 810 and 720 cm^{-1} suggested for C-H bonding of phenyl.

iii) NMR spectroscopy

The molecular formula of compound II was deduced as $C_{11}H_{12}O_4$ from it's ^1H and ^{13}C-NMR spectra and DEPT 135 experimental data. The ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of this compound displayed 11 carbon resonances, while the HSQC experiment indicated 7 out of 11 carbons were attached to protons. ^1H NMR 400 MHz, $CDCl_3$) and DEPT 135 (100 MHz, $CDCl_3$) spectra revealed the presence of four methines, two methylenes and one methoxy carbons. The ^1H NMR and ^{13}C NMR spectral data are shown in **Table11**.

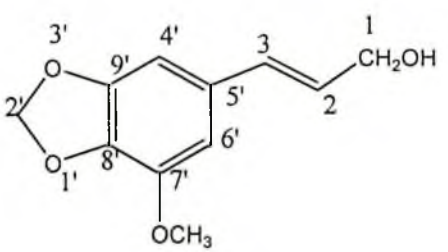
The resonances at δ6.20 and δ6.49 ppm in the ^1H NMR and at δ137.3 and δ131.0 ppm in the ^{13}C NMR spectra, in conjunction with the DEPT 135 spectrum, could be attributed to two olefinic protons. The cross peak in the COSY spectrum between these two protons indicated that they were vicinal. The coupling constant (J= 15.8 Hz) of these olefinic proton indicated their (*E*) configuration. The cross peak in the COSY spectrum between the methylene protons at δ4.29 ppm and olifinic proton at

δ6.20 ppm indicated that they were vicinal. The relatively deshielded value of the mehtylene protons at δ4.29 ppm indicated that methylene group was oxygenated.

The presence of two ^{1}H signals at δ6.63 and δ6.61 ppm were attributed to two aromatic protons. The presence of these two aromatic signals indicated that compound II might carry a tetrasubstituted benzene ring. The resonance at δ3.89 ppm in the ^{1}H NMR spectrum and the resonance at δ56.6 ppm in the ^{13}C NMR spectrum in conjunction with the DEPT 135 spectrum proved the presence of an *O*- methyl group. A two proton signal at δ5.91 ppm in the ^{1}H NMR spectrum and the resonance at δ101.2 ppm in the ^{13}C NMR spectrum in conjunction with the DEPT 135 spectrum proved the presence of amethylenedioxy group.

Structure of Compound II

Analysis of one and two dimensional NMR spectra including H-H COSY, HSQC and HMBC led to the assignment of the structure as shown in fig- in this structure the C1-C2-C3 portion was assigned by tracing of cross peaks in the H-H COSY spectrum. A (7- methoxy-benzo [1,3] dioxol-5-yl) moiety was disclosed by HMBC correlations (H-2/C-5; H-3/C-4′; H-3/C-6′; H-2/C-8′; H-2/C-9′; H-4′/C-6′; H-4′/C-8′; H-6′/C-4′; H-6′/C-8′, H-7′-OC H_3/C-7′.



(*E*)-3-(7-Methoxy-benzo[1,3]dioxol-5-yl)-prop-2-en-1-ol

Compound II

On the basis of these Compound II was characterized as anthriscinol, (*E*)-3-(7-Methoxy-benzo[1,3]dioxol-5-yl)-prop-2-en-1-ol. A literature survey revealed that Compound II was a known phenyl propanoid.

Table 11. ^{1}H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, CDl_3), NMR data of Compound II

no.	δ_H,J in Hz	δc	COSY	HMBC
1	4.29 (2H, d, *J*=5.8)	63.7	H-2	C-2, C-3
2	6.20 (1H, dt, J=15.6, 5.8)	127.3	H-1, H-3	C-1, C-3, C-5′
3	6.49 (1H, d, J=16)	131.1	H-2	C-1, C-4′, C-6′
1′	---	---	---	---
2′	5.95 (2H, s)	101.5	---	C-8′ , C-9′
3′	---	---	---	---
4′	6.61 (1H, s)	100.1	---	C-3, C-5′, C-6′, C-8′, C-C-9′
5′	---	131.7	---	---
6′	6.53 (1H, s)	106.8	---	C-3, C-4′, C-5′, C-8′, C-9′
7′	---	143.6	---	C2′,C3′, C5, C6, C7, C9
8′	---	135.1	---	---
9′	---	149.7	---	---
7′-OCH_3	3.89 (3H, s)	56.6	---	C7

3.4.2.2 Structure of compounds III and IV

^{1}H and ^{13}C NMR spectra of compound III (Fig. 24-25) and compound IV (Fig. 26-27) were recorded. The NMR spectra of the compounds showed that there are mixtures of compounds. As the compounds were mixtures and very small in amount, no further fractions were possible in the present study.

Analytical, BCSIR, 1H Spectrum, Z-8 in CDCl3, Zia, DU

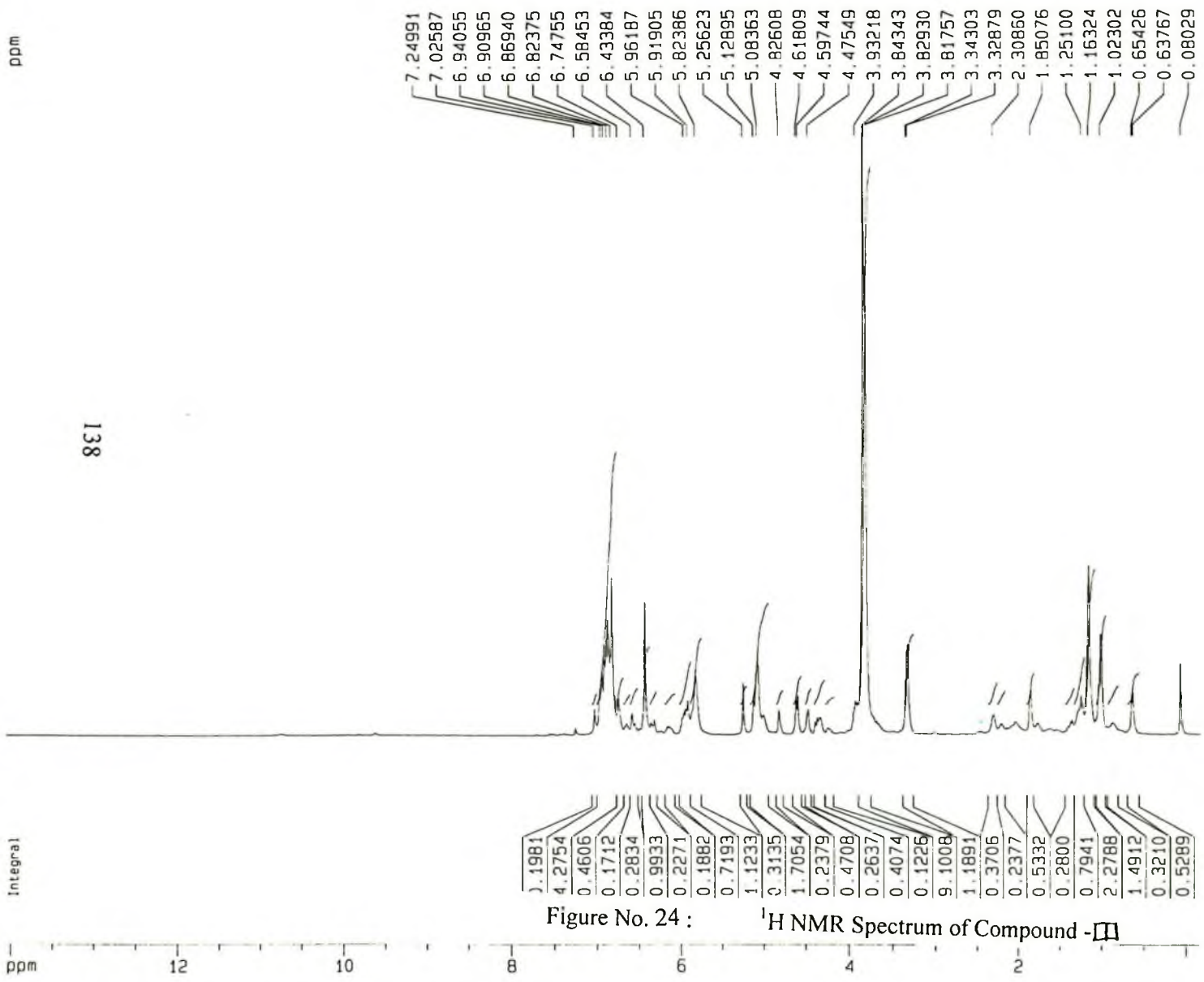


Figure No. 24 : ^{1}H NMR Spectrum of Compound -[illegible]

Current Data Parameters

NAME	A3483
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20070509
Time	15.56
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	40.3
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

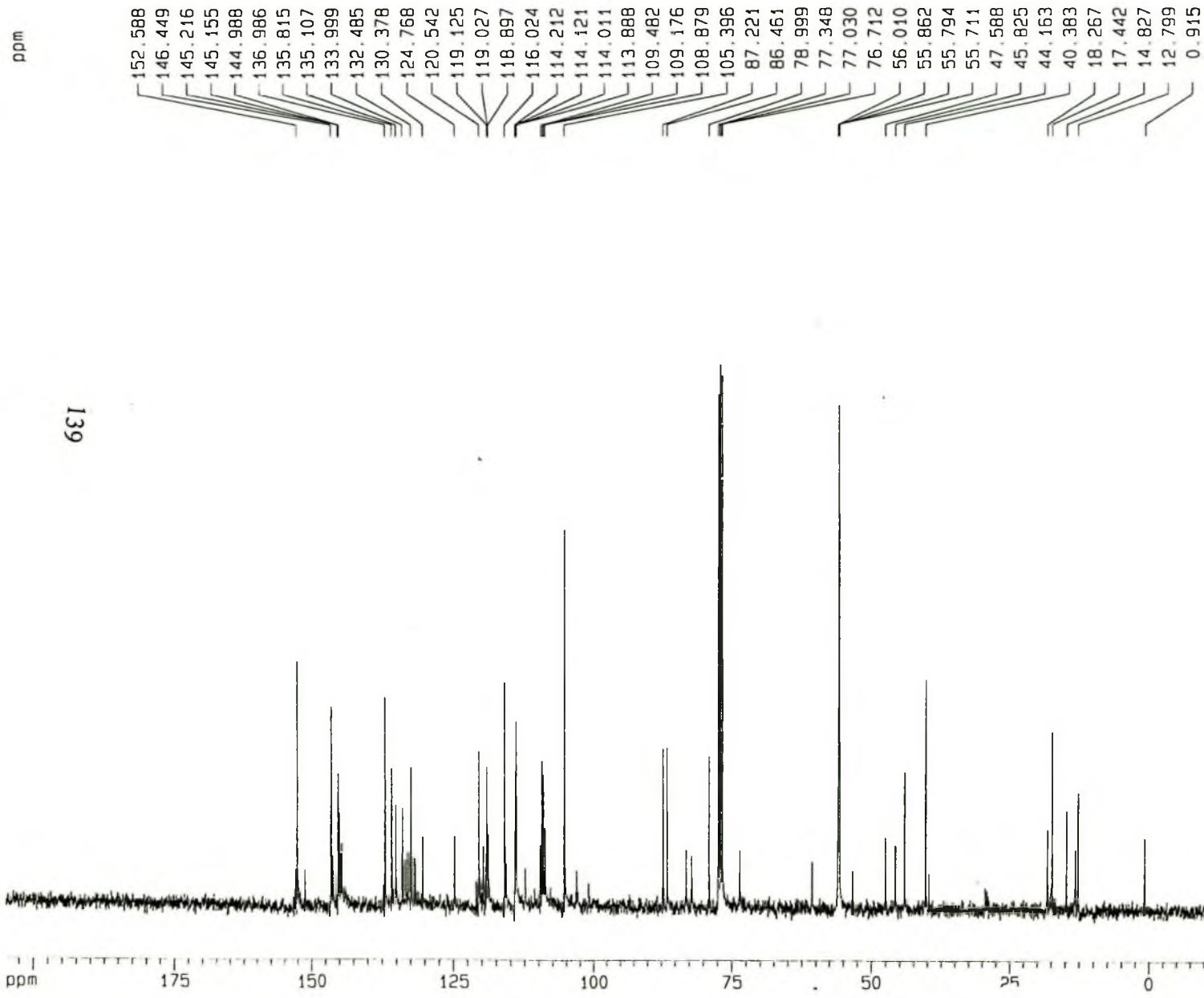
F2 - Processing parameters

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	14.049 ppm
F1	5621.75 Hz
F2P	-0.162 ppm
F2	-64.98 Hz
PPMCM	0.71059 ppm/cm
HZCM	284.33679 Hz/cm

Analytical, BCSIR Lab. Dhaka 13C Spectrum Z-8 in CDCL3, Ziaul, DU.




```
Current Data Parameters
NAME                  A3483
EXPNO                     2
PROCNO                    1

F2 - Acquisition Parameters
Date_              20070514
Time                  11.12
INSTRUM             dpx400
PROBHD     5 mm Multinuc
PULPROG             zgpg30
TD                    32768
SOLVENT               CDCl3
NS                      580
DS                        2
SWH               24154.590 Hz
FIDRES             0.737140 Hz
AQ                 0.6783476 sec
RG                    16384
DW                   20.700 usec
DE                    6.00 usec
TE                   300.0 K
D1                1.50000000 sec
d11               0.03000000 sec
d12               0.00002000 sec

========== CHANNEL f1 ============
NUC1                     13C
P1                    8.30 usec
PL1                  -6.00 dB
SFO1            100.6253045 MHz

========== CHANNEL f2 ============
CPDPRG2              waltz16
NUC2                     1H
PCPD2                80.00 usec
PL2                  -6.00 dB
PL12                 16.00 dB
PL13                120.00 dB
SFO2            400.1400000 MHz

F2 - Processing parameters
SI                    32768
SF              100.6152963 MHz
WDW                      EM
SSB                       0
LB                     2.50 Hz
GB                        0
PC                     1.40

1D NMR plot parameters
CX                   20.00 cm
F1P                 204.817 ppm
F1                20607.74 Hz
F2P                 -13.220 ppm
F2                -1330.13 Hz
PPMCM              10.90186 ppm/cm
HZCM             1096.89343 Hz/cm
```

Figure No. - 25 ^{13}C NMR Spectrum of Compound -III

Analytical, BCSIR, 1H Spectrum, Z-9 in CDCl3, Zia, DU

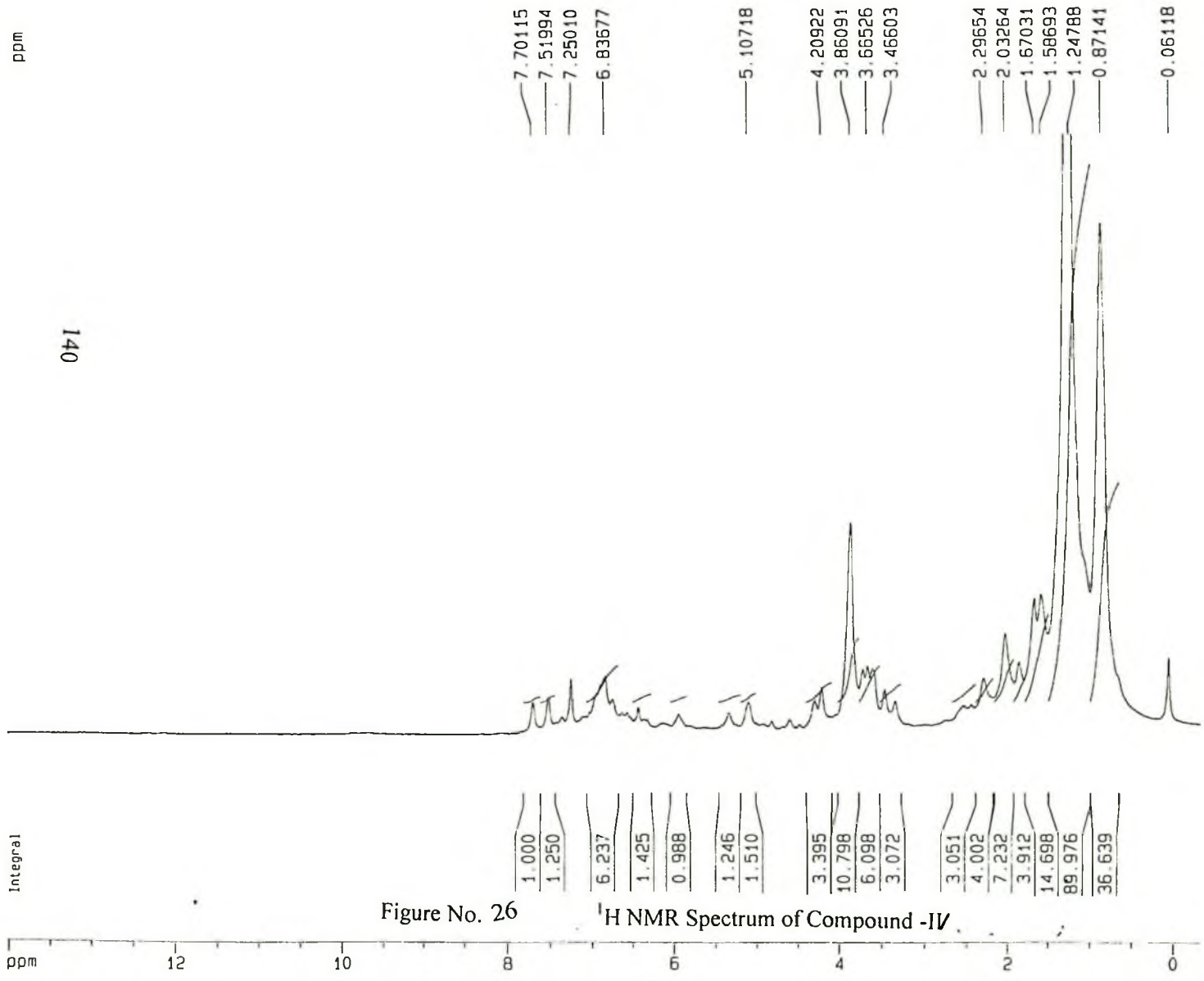


Figure No. 26 ^{1}H NMR Spectrum of Compound -IV

Current Data Parameters

NAME	A3484
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20070509
Time	16.20
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	114
DW	78.000 usec
DE	6.00 usec
TE	310.0 K
D1	1.00000000 sec

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

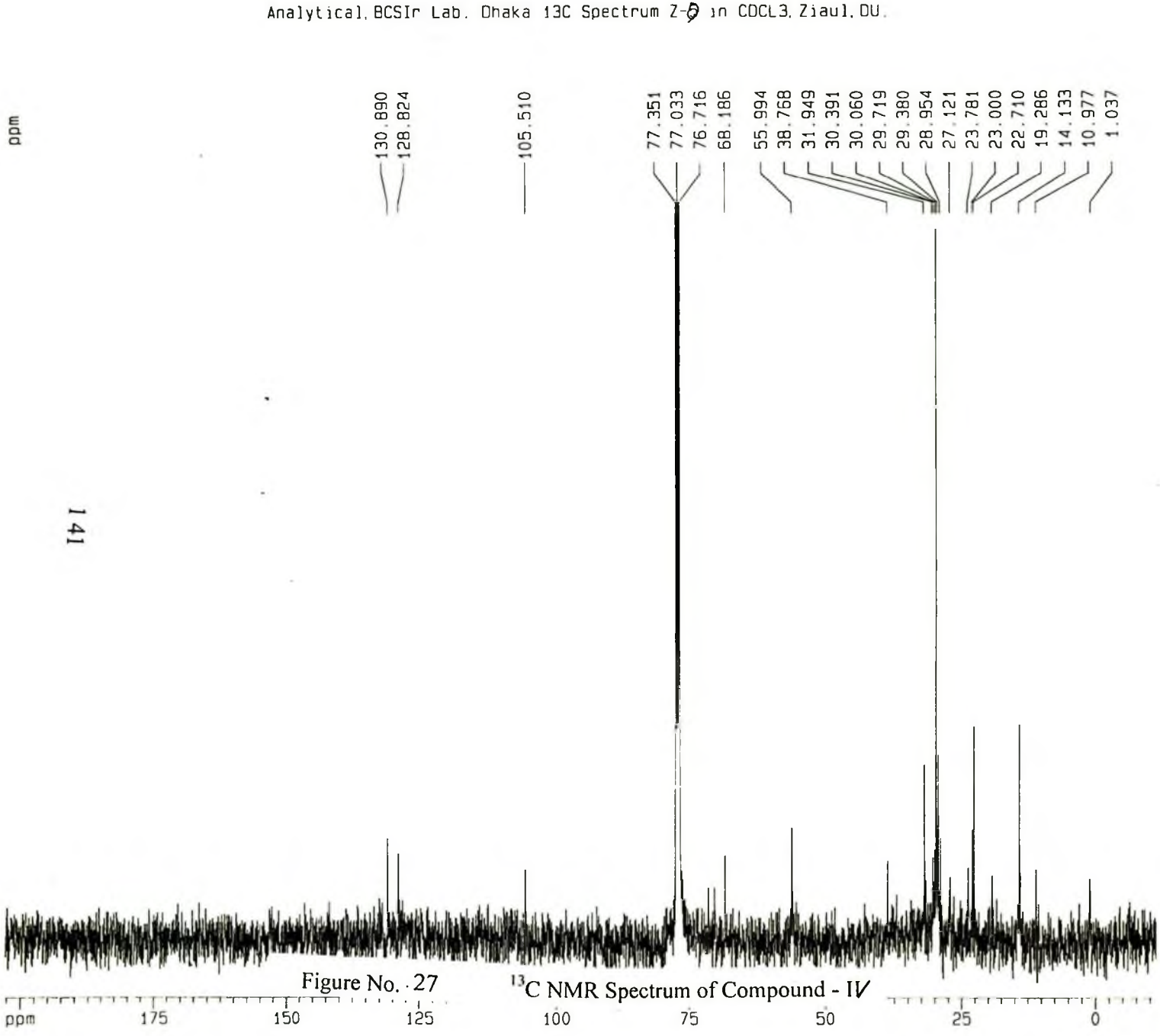
NUC1	1H
P1	8.30 usec
PL1	-6.00 dB
SFO1	400.1428010 MHz

F2 - Processing parameters

SI	32768
SF	400.1400101 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	14.022 ppm
F1	5610.58 Hz
F2P	-0.326 ppm
F2	-130.25 Hz
PPMCM	0.71735 ppm/cm
HZCM	287.04153 Hz/cm



Current Data Parameters

NAME	A3484
EXPNO	2
PROCNO	1

F2 - Acquisition Parameters

Date_	20070514
Time	12.39
INSTRUM	dpx400
PROBHD	5 mm Multinuc
PULPROG	zgpg30
TD	32768
SOLVENT	CDCl3
NS	2017
DS	2
SWH	24154.590 Hz
FIDRES	0.737140 Hz
AQ	0.6783476 sec
RG	16384
DW	20.700 usec
DE	6.00 usec
TE	300.0 K
D1	1.50000000 sec
d11	0.03000000 sec
d12	0.00002000 sec

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

NUC1	13C
P1	8.30 usec
PL1	-6.00 dB
SFO1	100.6253045 MHz

========== CHANNEL f2 ==========

CPDPRG2	waltz16
NUC2	1H
PCPD2	80.00 usec
PL2	-6.00 dB
PL12	16.00 dB
PL13	120.00 dB
SFO2	400.1400000 MHz

F2 - Processing parameters

SI	32768
SF	100.6152815 MHz
WDW	EM
SSB	0
LB	2.50 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	202.685 ppm
F1	20393.16 Hz
F2P	-11.048 ppm
F2	-1111.56 Hz
PPMCM	10.68661 ppm/cm
HZCM	1075.23596 Hz/cm

Figure No. 27 ^{13}C NMR Spectrum of Compound - IV

References

1. Holistic on line com. info@holistion.com
2. Fransworth NR and Morries RW (1988), Higher plant the sleeping Giant of drug development. American Journal of Pharmaceutical Education **148**, 83-97.
3. Rajshekhoran, P.E. Herbal medicine. In world science
4. Daniel Bagozzi, E-mail: bagozzid@who.net.
5. Zutshe U and Rao P G S
6. Daniel Bagozzi, Email: bagozzid@who.net
7. Kronenberg F and Fugh-Berman A. " Complementary and alternative medicine for menopausal symptoms: a review of randomized, controlled trials". *Ann inter Med.* (2002) **137** (10); 805-13.
8. Parkinson, J., Theatrum Botanicum, London, 1640.
9. Li F, Sun S, "Wang W, Foster S and Tyler Ve (1998). *Tyler's Honest Herbal.* A sensible Guide to the Use of herbals and Related Remedies. The Haworth Press New York, USA.
10. Dubeey, N. K., Kumar R. and Tripathi. P., Global Promotion of herbal medicine. India's opportunity. 17. Duke, J. medicinal plant species.
11. Foster S & Tyler Ve (`1998). *Tyler's Honest Herbal. A sensible Guide to the Use of herbals and Related Remedies.* The Howorth Press. New York.
12. Ghosh. D and Landdha, K. S. Herbal safety and GMP: The most debatable argument in the present sesenario, the Pharma review Feb 2006.
13. Varro E., Tyler, Lynn R. Brady and James E.R. Pharmacogonosy (8^{th} edition).
14. Kumar, V. (Review article); IUPAC Workshop and Task group meeting on Development of Methodology and Protocols on Documentation, Evaluation of Safety and Efficacy and Standardization of Herbal Medicine (2006), 6-9 June, LGED and HCDP Bhaban Dhaka, Bangladesh.
15. Choudhury, M I (Review article); IUPAC Workshop and Task group meeting on Development of Methodology and Protocols on Documentation, Evaluation of Safety and Efficacy and Standardization of Herbal Medicine (2006), 6-9 June, LGED and HCDP Bhaban Dhaka, Bangladesh.
16. Smet De PAGM (1995). Health risks of herbal remedies. *Drug Safety,* 13:81-93

17. Kumar V. (Review article); IUPAC Workshop and Task group meeting on Development of Methodology and Protocols on Documentation, Evaluation of Safety and Efficacy and Standardization of Herbal Medicine (2006), 6-9 June, LGED and HCDP Bhaban Dhaka, Bangladesh.

18. Ali, L. (Review article); IUPAC Workshop and Task group meeting on Development of Methodology and Protocols on Documentation, Evaluation of Safety and Efficacy and Standardization of Herbal Medicine (2006), 6-9 June, LGED and HCDP Bhaban Dhaka, Bangladesh.

19. British Medical Association. Complementary medicine. New approaches to good practice. Oxford: Oxford University Press, 1993: 9-36.

20. Muslim, H. M., General Secretary, Unani and Ayurvedic Pharmaceutical Society, Bangladesh.

21. ATSDR- SCEM- chromium (Cr) Toxicity. Clinical Evaluation Sensitivity. 1825 century Blvd, Atlanta. GA 30345.

22. http: // en. wikipedia. Drg/ wiki/ blood. lead- level.

23. http: // en. wikipedia. Drg/ wiki/ blood. Arrinic level.

24. Kirtikar K. R. and Basu B. D. *Indian Medicinal Plants,* 1988, **3**, 2138

25. Monita, Y., Jinno, K., Kawagishi, H., Arimoto, Y., Suganuma, H., Inakuma, T., Sugiyama, K., "Hepatoprotective effect of myristicin form mutmeg (*Myristicafragrans)* on lipopolysacharide/d-galactosamine-induced liver injury". *J. Agric. Food Chem.* 2003, **51**, 1560-1565.

26. Joseph, J. "The Nutmeg: It's botany, agronomy, production, composition and uses,"*J. of Plantation Crops*. 1970, **8**(2), 61-72.

27. Groome, J.R. (1970) A Natural History of the Island of Grenada, W.I., Caribbean Printers Limited, Arma, Trinidad, W.I.

28. Purseglove, J.W., Green, C. L., Brown, D.G. and Robbins, S.R.J. Nutmeg and Mace,' In Spices". 1981, **2**, 174-228, Logmans.

29. Andrew T. Weil, " Nutmeg as a Narcotic"; *Economic botany.* 1965, **19**(3): 194-217.

30. Jansson, J., Lackeman, G.M., Peiters, L.A., Totte, J., Herman, A.G., Vlietinck, A.J., " Nutmeg oil: identification and quantification of its most active constituents as inhibitors of platelet aggregation". *J. Ethnopharmacol.* 1990;**29**, 179-188.

31. Ghani, M. Medicinal plan of Bangladesh with Chemical Constituents and uses. Second edition 2003 Asiatic society of Bangladesh pp 20.

32. Kuo, Y.H., " Studies on several naturally occurring lignans". *Gaoxiong Yi Xue Ke Xue Za Zhi.* 1989,**5**,621-624.

33. Power, F.B., and Salway, A.H., "The constituents of the essential oil of nutmeg" *Jour. Chem Sac.,* 1907; **91**, 2037-2058.