Phytochemical and Microbiological investigation on the roots of *Piper chaba* Hunter and *Bombax ceiba* Linn.

A DISSERTATION IN PARTIAL FULFILLMENT FOR THE REQUIREMENTS OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN ORGANIC CHEMISTRY

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DEDICATED
TO
MY PARENTS

LATE Dr. SURENDRA NATH ROY
And
LATE GOURI RANI ROY

ACKNOWLEDGEMENT

At first, I would like to express my gratitude to almighty Allah, who blessed me and given me the capability of completing the research successfully as well as to complete my Ph.D. Degree.

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List of Contents

PART-A

List of Tables (Part-A)

List of the figures (Part-A)

PART-B

Page No.

List of Tables (Part-B)

List of the figures (Part-B)

ABSTRACT

Piper chaba H. belonging to the Piperaceae family and *Bombax ceiba* L. belonging to the Bombacaceae family has been investigated for isolation of secondary metabolites and evaluation of their biological activities. The ethyl acetate extracts from the roots of *Piper chaba* and *Bombax ceiba* was subjected to different chromatographic technique to isolate secondary metabolites and a total of twelve (12) compounds were isolated. From the roots of *Piper chaba* the isolated Five (5) compounds were stigmasterol, Piperine, 5, 6-dihydroxy- 7, 8-dimethyl isoflavan-4-one, two new compound [1-(4"-chlorophenyl)-3-(4'-methoxyphenyl) prop-2-en-1-one] and 1-(4"-chlorophenyl)-3-(p-tolyl)prop-2-en-1-one and *Bombax ceiba* isolated seven (7) compounds were isohemigossypol-1-methyl ether, isohemigossylic acid lactone-2-methyl ether, 2, 7-dihydroxy-5-isopropyl-3-methyl-2H-naphtho[1,8-bc]furan-2-one, protocatechuic acid, Scoplotein, Vasicine and Lupeol. All the isolated compounds were identified by extensive analyses of their high resolution ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz) and 2D NMR (HSQC and HMBC) spectroscopic data.

The compound piperine and all the roots extract of *P. chaba* were found to have mild antioxidant activity. The isolated compounds stigmasterol, Piperine, [1-(4"-chlorophenyl)-3-(4'-methoxyphenyl)prop-2-en-1-one] and the all extracts showed moderate antimicrobial activity.

The methanol extract of the roots of *Bombax ceiba* and different partitionate of the crude methanol extracts i.e. n-hexane (HEX), dichloromethane (DCM), ethyl acetate (EAC) and aqueous soluble partitionate (AQP) were subjected to various biological screenings such as thrombolytic, antioxidant, brine shrimp lethality, antimicrobial, antidiarrheal and central analgesic activities.

Thrombolytic activity of all the extractives of *B. ceiba* was evaluated as a part of discovery of cardioprotective drugs. Among different fractionates of *B. ceiba,* the highest percentage of clot lysis was exhibited as 44.55% by AQP. The antioxidant activity was evaluated in terms of free radical scavenging activity (DPPH assay). Among all extractives of *B. ceiba* the highest free radical scavenging activity showed by AQP with IC_{50} value 3.30 µg/mL. The highest lethality revealed by HEX having LC_{50} value of 1.02 µg/mL in brine shrimp lethality bioassay of different partitionates of the roots of *B. ceiba.* The maximum zone of inhibition, in the microbiological investigation, was produced by DCM extract of *B. ceiba* against *Shigella dysenteriae* (14 mm).

The methanol extract of the roots of *B. ceiba* at dose of 400 mg/kg exhibited significant antidiarrheal activity with 54.57% reduction of diarrheal feces as compared to the standard, Loperamide (68.21%). Crude methanol extract of *B. ceiba* was subjected to central analgesic activity evaluation test. The methanol extract of *B. ceiba* exhibited significant central analgesic activity at 400 mg/kg doses with 62.76% inhibition of writhing.

INTRODUCTION

1.1 General

Plants play a vital role for the existence of life in the universe. Primitive man started to distinguish nutritional and pharmacologically active plants for their survival. The use of medicinal plants for alleviating diseases had its origin in the activities of the most primitive man of the remote past. Illness, physical discomfort, injuries, wounds and fear of death had forced early man to use any natural substance for relieving the pain and suffering caused by these abnormal conditions and for preserving health against diseases and death. By their experience, this knowledge of herbal remedies was transferred from one generation to another at first orally and later in written from a papyri, backed clay tablets, parchments, manuscripts, pharmacopoeias and other works. Therefore, medicinal plants have been in use for the eradication and human suffering since ancient time.

As far as record goes, it appears that Babylonians (about 3000 years B.C.) were aware of a large number of curative florae and their chattels. Some of them are still in use for the same purpose like henbane (*Hyoscyamus* Spp.), Opium (*papaver somniferum*), Castor oil (*Ricinus communis*), Aloevera (*Aloe spp.*) etc[1].

The Chinese have an effective and unique system of medicine. The earliest known Chinese pharmacopoeia, The Pen Tsao, described over 300 medicinal plants and their uses. Although various of several ancient pharmacopoeia still exist today, the main surviving text book is on herbalism written in the 16th century by the physician Li Shih-Cheu describing almost 250 herbs and 15,000 herbal remedies[2]. Today Chinese herbalism is very much an orthodox form of therapy and preventive treatment in China and it is increasingly practiced in the west. Chinese herbalism can be used for a wide range of ailments, including asthma, skin diseases, menstrual problems, digestive disturbances, migraine and is effective when used on its own or in conjunction with another therapy such as acupuncture.

The fleshly media of the inordinate Greek physician Hippocrates (460-370 B.C.) consists of some 260 to 420 curative florae which comprised opium, mint rosemary, sage and verbena. In the interior era, the great Greek pharmacist-physician Galen (131-200 A.D.) used a huge amount of pharmacological active plants in preparing his recipes. The Arabian Muslim general practitioner like Al-Razi and Ibne Sina (9^{th} to 14^{th} century A.D.) transported about uprising in the linctus by conveying firsthand remedies of plant and mineral origin into

general use origin into general use. Enriching the original Greek system of medicine by introducing these new materials and knowledge they laid down the foundation stone of modern western medicine.

The medicinal use of plants in the Indian subcontinent is the Rig Veda (4500 – 1600 BC), which noted that Indo-Aryans used the Soma plant (*Amanita muscaria)* as a medicinal agent. The Vedas made many references to healing plants including sarpagondha (*Rauvolfia serpentine*), while a comprehensive Indian Herbal, the Charaka Samhita, cities more than 500 medicinal plants[3].

Meanwhile ailment, decline and death have continually co-existed with life, the culture of maladies and their usage must also have been contemporaneous with the dawn of the human intellect. It is specious that whatnot evolution science might have made in the field of medicine over the years, plants still remain the primary source of supply of many important painkillers castoff in new prescription. Indeed, the budding of gaining new drugs from herb foundations is so great that thousands of substances of plant origin are now being studied for activity against such formidable foes as heart diseases, cancer, diabetes and AIDS. This type of study is sure to bring fruitful results, because of the fact that the plant kingdom represents a virtually untapped reservoir of new chemical compounds and it has been estimated that only 5-15% of the approximately 2,50,000-5,00,000 species of higher plants of which more than 80,000 are medicinal has been investigated pharmacologically. Thus there are considerable chances of finding new natural compounds with pharmacological activities, useful for the enlargement of new drugs.

Researchers are now occupied composed to find out new drug for incurable diseases. Taxonomist, Chemist, Biochemist, Pharmacologist and Pharmacist are working under collaborative sequencer for making a plant product (s) into a commercial drug[2].

1.2 Medicinal importance of plant materials

Plants not only provide man with food, shelter and medicine, but also the supporting oxygen. From ancient time to modern age the human has been successfully used plants and plant products as effective therapeutic tools for fighting against diseases and various health hazards. Although with the advent of synthetic drugs the use and procurement of plant derived drugs have declined to a large extent, a large number of drugs of modern medicine are obtained from plant sources. According to some generous estimates, almost 80% of the present day medicines are directly or indirectly obtained from plants[3]. As therapeutic use of plants continued with the progress of civilization and development of human knowledge,

scientists endeavored to isolate different chemical constituents from plants, put them to biological and pharmacological tests and thus have been able to identify and isolate therapeutically active compounds, which have been used to prepare modern medicines. In course of time their synthetic analogues have also been prepared. In this way, the discovery of vincristine was done from *Catharanthus roseus*, which is used in the treatment of cancer[5].

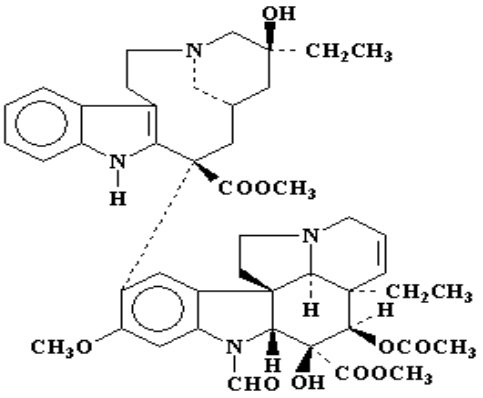

Figure: 1.1 Vincristine

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

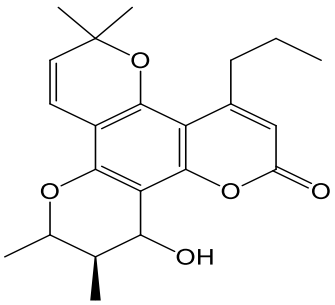

Figure: 1.2 Calanolide A

Ancient Chinese medical texts written 2000 years ago describe the herb *Artemisia annua* as a remedy for malaria. From this indication, in the late 1960s Chinese researchers initiated evaluation of various extracts of this herb. Bioassay guided isolation yielded the new

antimalarial compound artemisinin which is effective in treating chloroquine resistant cases and other severe cases.

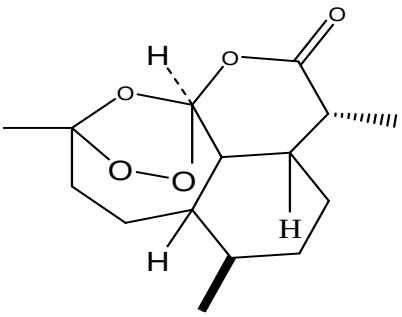

Figure: 1.3 Artemisinin

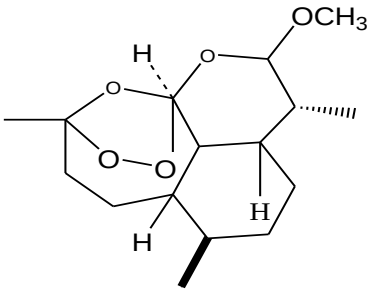

Figure: 1.4 Artem ether

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

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

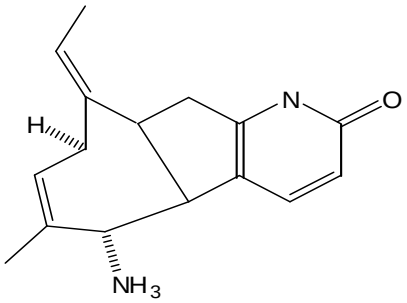

Figure: 1.5 Huperzine

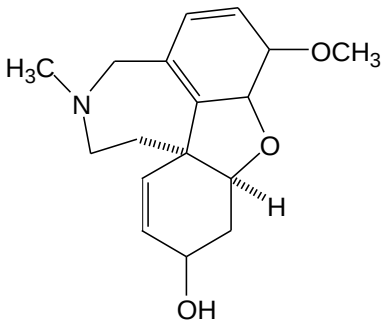

Figure: 1.6 Galathmine

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

In the antidiabetes area, the past decade has witnessed the market introduction of several α-glucosidase inhibitors derived from natural products. A carbose, a complex oligosaccharide one of them, was isolated from *Actinoplanes* sp. At Bayer from a search for α-glucosidase enzyme inhibitors. By inhibiting α-glucosidase, acarbose decreases the release of glucose from ingested carbohydrate and slows the increase of food-induced blood glucose levels. A carbose is now approved in Germany, Japan, the US and other countries and has been used as adjuvant therapy in diabetes.

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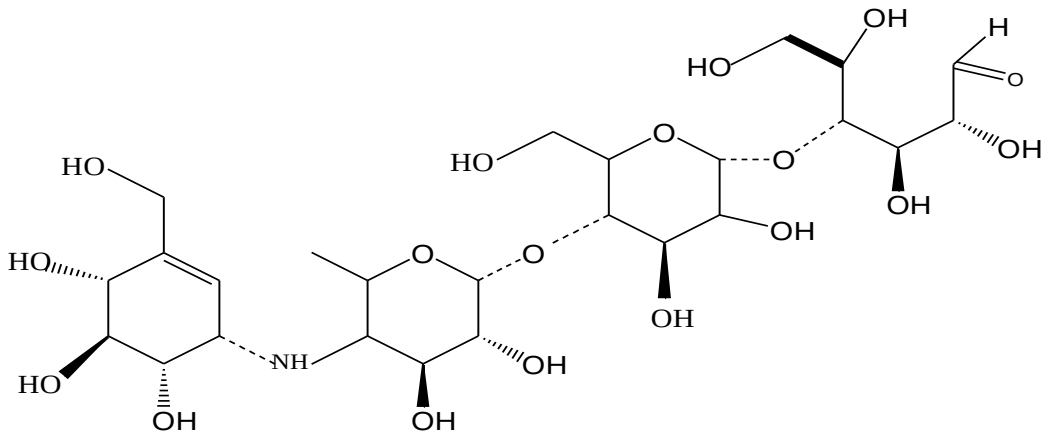


Figure: 1.7 A carbose

Colorist is a diterpene regular creation insulated from the Indian shrub *Coleus forskohlii* at Hoechsts exploration labs in India and has blood weight dropping and cardio active stuffs. Later Forskolin was found as a potent adenylate cyclase activator. Colforsin daproate (NHK-478) is a semi synthetic invention of forskolin imitative and was then carried into phase III irrefutable hearings in Japan for conduct of cardiac inadequacy and phase II ranges for handling of asthma.

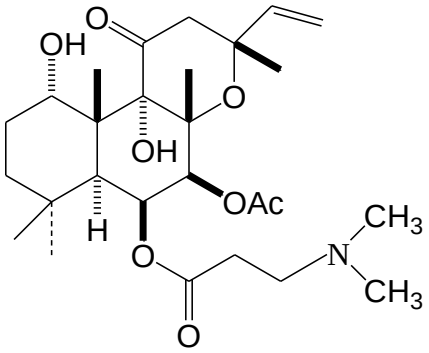


Figure: 1.8 Colforsin daproate

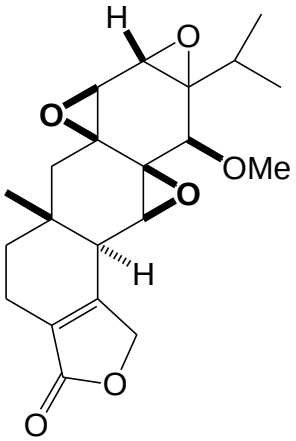


Figure: 1.9 Triptolide

Triptolide (**9**) is an active component isolated from the Chinese plant *Tripterygium wilfordii*, a plant traditionally used for the treatment of rheumatoid arthritis. Variety of formulations were developed in Mainland China and are shown to be effective in the treatment of inflammatory and autoimmune diseases. Triptolide was demonstrated to significantly inhibit arthritis in animal modal and have potent cytotoxicity[19].

disorders[7].

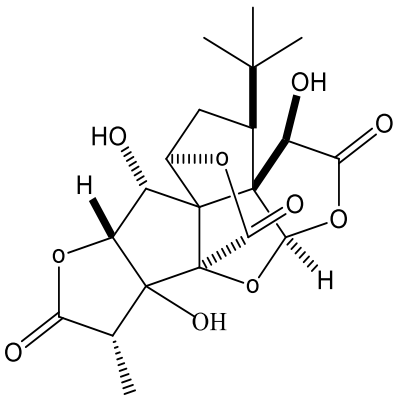


Figure: 1.10 Ginkgolide B

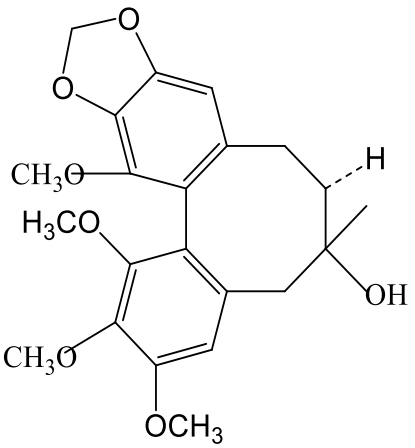


Figure: 1.11 Gomisin A

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

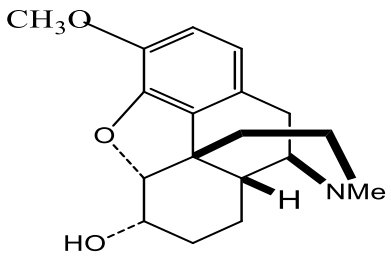
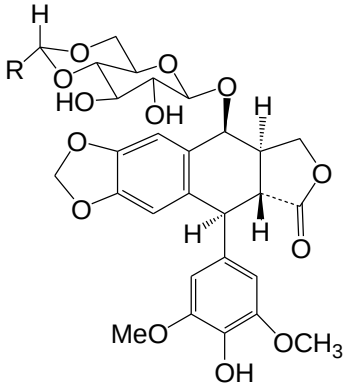


Figure: 1.12 Dextromethorphan

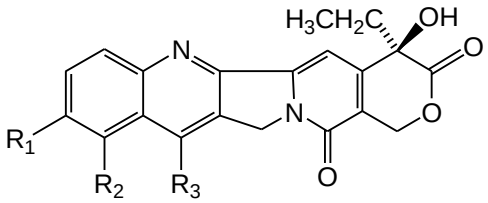
Morphine was first isolated by Serturner in 1806 followed by Codeine in 1832 by Robiquet and then the non-morphine alkaloid papaverine by Merck in 1848 from the seeds of *Poppy*. Dextromethorphan is a semisythentic product of morphine used in most cough syrup today.

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



R = Me; Etoposide

R =  ; Teniposide



Camptothecin $R_1 = R_2 = R_3 = H$;

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

Figure: 1.13 Camptothecin and Toptican Camptothectin

Camptothecin and Toptican Camptothectin was isolated from the Chinese ornamental tree *Camptotheca acuminata* by Wani and Wall. It was advanced to clinical trial by NCI in the 1970s but was dropped because of severe bladder toxicity. But toptican is a modified camptothecin was approved for use in the USA in 1996. The discovery of quinine was done from *Cinchona* bark, which is, used in the treatment of malaria by French scientist Caventon and Pelletier[9].

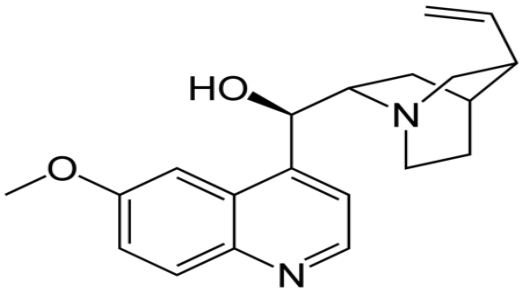


Figure: 1.14 Quinine

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

Plants will endure toward remain enormously important as basis of novel drugs as disclosed by fresh supports in the United States of several new plant derived drugs based on the secondary metabolites of plants. For example, in the treatment of refractory ovarian cancer, new drug has recently stayed accepted in the Joint Positions from taxol, an anti cancer taxane diterpenoid derived from the relativity scarce pacific western Yew tree, *Taxus brevifolia* Nutt. A somewhat firsthand semi-synthetic antineoplastic agent based on podophylotoxin is etoposide a component of the Mayapple *Podophyllum petatum*, which is convenient in the chemotherapeutic usage of stubborn testicular carcinomas, lesser lockup lung carcinomas, non-Hodgkin's lymphoma and non-lymphocytic leukemia.

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

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

Drug/Chemical	Action/Clinical use	Plant source
Betulinic acid	Anticancerous	*Betula alba*
Camptothecin	Anticancerous	*Camptotheca acuminata*
Chymopapain	Proteolytic, mucolytic	*Carica papaya*
Cissampeline	Skeletal muscle relaxant	*Cissampelos pareira*
Colchiceine amide	Antitumor agent	*Colchicum autumnale*
Colchicine	Antitumor agent, anti-gout	*Colchicum autumnale*
Curcumin	Choleretic	*Curcuma longa*
Cynarin	Choleretic	*Cynara scolymus*
Danthron	Laxative	*Cassia species*
L-Dopa	Anti-parkinsonism	*Mucuna sp*
Etoposide	Antitumor agent	*Podophyllum peltatum*
Glaucarubin	Amoebicide	*Simarouba glauca*
Glycyrrhizin	Sweetener, Addison's disease	*Glycyrrhiza glabra*
Hesperidin	Capillary fragility	*Citrus species*
Irinotecan	Anticancer, antitumor agent	*Camptotheca acuminata*
Lapachol	Anticancer, antitumor	*Tabebuia species*
Menthol	Rubefacient	*Mentha species*
Papain	Proteolytic, mucolytic	*Carica papaya*
Pilocarpine	Parasympathomimetic	*Pilocarpus jaborandi*
Podophyllotoxin	Antitumor anticancer agent	*Podophyllum peltatum*
Quinidine	Antiarrhythmic	*Cinchona ledgeriana*
Quinine	Antimalarial, antipyretic	*Cinchona ledgeriana*
Rutin	Capillary fragility	*Citrus species*
Sennosides A, B	Laxative	*Cassia species*
Stevioside	Sweetner	*Stevia rebaudiana*
Taxol	Antitumor agent	*Taxus brevifolia*
Teniposide	Antitumor agent	*Podophyllum peltatum*
Theobromine	Diuretic, vasodilator	*Theobroma cacao*

1.3 Status of medicinal plants in Bangladesh

The number of medicinal herbs included in the *Materia medica* of traditional medicine in this subcontinent at present stands at about 2000. About 460 to 550 of such therapeutic basils has so distant been recruited as mounting or obtainable in Bangladesh. Almost 85% of bucolic inhabitants is reliant on on remedial shrubberies for their chief well-being care. Herbal drug is extensively charity now the country in old-style healthcare arrangement like that as Ayurvedic, Unani, Hekimi and other form of vernacular usages.

1.4 Description of the *Piperaceae* family

The Piperaceae family is typically thymes or trees including about 280 types and 360 sorts, counting winding systems. The herbal *Piper chaba* is a herbaceous herb. It cultivates incompetently virtually all the region of Bangladesh. Besides Bangladesh, it grows through the bigger portion of India, such as the sub-Himalayan tract Chota Nagpur, Central India, central provinces parts of the Bombay and Madaas etc. The *Piper* species examined by us called *Piper chaba*. Its roots and stem bargain copious claims in drug and are chiefly beneficial in asthma, bronchitis, fever and pain abdomen. The marker compound Piperine is the distinctive compound of the sort and it was first isolated 1819. It is a sreeper that spreads on the ground or way take support of other trees. The leaves are simple, 2.5 to 5 inch long, conflicting and decussate; stipules are absent. The older greeneries are demtate shady in colour and formed. The floras are bisexual, zygomorphic and frequently are allied with noticeable, repeatedly lustrously painted bracts. The calyx is typically profoundly 3-5 lobed or sometimes is highly abridged with more frequent miniature fangs. The corolla regularly 4-merous, habitually, zygomorphic and generally 3 lipped. An annular liquid floppy is frequently found everywhere the base of the ovary. The fruit is normally an elastically dehiscent locolicidal capsule. The seed stalk or funiculars of each seed is altered into a hook shaped ejaculator or retinaculum that purposes in hurling out the germs through dehiscence.

1.5 Description of *Piper chaba* Hunter.

Piper chaba Hunter belongs to Piperaceae family. It has about 450 species[2] and are native throughout the tropics and subtropics, with a few species in Eurasia. The herb *Piper chaba* Hunter (Bengali name: Chui Jhal) is an important medicinal plant of Bangladesh and throughout the world as well. Piperine has been isolated from the roots of this plant[6]. In pipernonaline, guineensine, studies have shown that chemicals isolated from the *Piper*

chaba plant have potential anti-inflammatory, antibacterial, antifungal and analgesic activities. Leaves and bark of *Piper chaba* are used for diabetes, malaria and jaundice. The bark is used for making an external application for pain and chest. The literature survey showed the isolation of some active compounds from the plants of *Piper chaba* genus. Since the plants *Piper chaba* is being used extensively in our country as an herbal medicine, it is necessary to have knowledge of the constituents of the plant of our native species.

Kingdom : Plantae

Division : Magnoliphyta

Class : Eudicots

Sub-class : Asterids

Order : Piperales

Family : Piperaceae

Genus : *Piper*

Species : *Piper chaba*

1.5.1 Scientific classification

Binomial name : *Piper chaba* Hunter.

Other names: Chui Jhal (both Bengali and in Hindi), Chobica (West bengal)

1.6 Photographs of *Piper Chaba* Hunter

Figure 1.15: Stem, roots and whole plant of *Piper chaba* Hunter

1.7 General description and chemical investigation of *Piper chaba* Hunter

The compound piperine are called the king of alkaloid. In traditional Chinese, Southeast Asian and Indian herb and used for periods in Ayurveda medication. The sage has been admired for giving communicable syndromes and extremely observed also as having a pre-emptive effect from numerous sicknesses, due to its influential resistant consolidation welfares. The worldwide influenza rampant of 1919 was one of the greatest overwhelming transferable outbursts in earth antiquity, murder lots universal, in numerous kingdoms. Though, in India, the astonishing prophylactic welfares of piperine and guineensine were accredited with discontinuing the lethal worm. It is a powerful stimulator of the protected arrangement by binary shortest conducts:

(1) Antigen- specific response: antibodies are made to counteract invading microbes,

(2) Non-specific immune response: macrophage cells scavenger and destroy invaders. King of Bitters activates both responses, making it effective against a variety of infections and oncogenic, cancer-causing agents.

Constituents: flavonoids, diterpenoid, steroid, alkaloid and alkaloid derivatives, paniculides, farnesols, polyphenols, arabinogalactan protein. According to the literature survey the already isolated compounds are as follows:

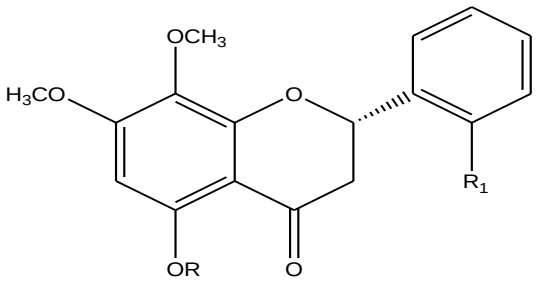


Figure: 1.16 5-hydroxy-7, 8-dimethoxyflavone

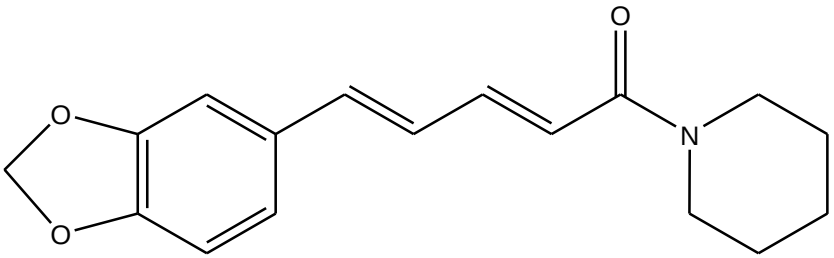


Figure: 1.17 Piperine

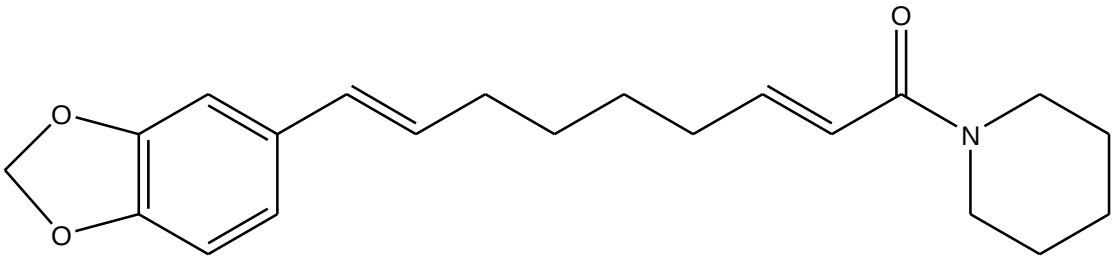


Figure: 1.18 Pipernoaline

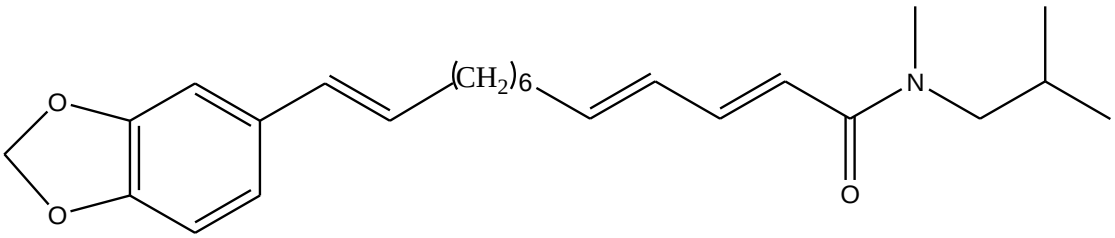


Figure: 1.19 Guineensine

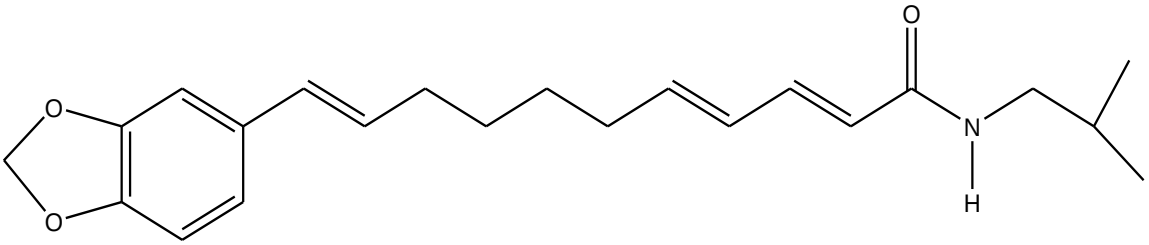


Figure: 1.20 Isobutylamide

Figure: 1.21: Phytoconstituents of *Piper chaba* Hunter

Actions: It has numerous bioactive compound and display the bioactivity against antigens. A daptogen (helps to normalise a physical function, depending on what the individual needs, e.g. it will lower high blood pressure, but raise low blood pressure; Antiseptic (fights bacterial activity); Antibiotic; Analgesic (pain reliever); Anti-inflammatory (reduces swelling); Antioxidant (helps in protecting the body from free radical damage); Antidiabetic; Antiacne (protects skin from pimples); Anti-carcinogenic (activity against different types of cancer, and leukemia; Antithrombotic (blood clot preventative); Antiviral (inhibits viral activity); Antimicrobial (significant activity in fighting the common cold, flu, respiratory infections); Antiperiodic (counteracts periodic/intermittent diseases such as malaria); Antipyretic (reduces fever, typically began by several impurities or toxins); Bitter tonic; Blood sterilizer; Cardio-protective (protects heart muscles); Choleretic (alters the things and flow of bile); Gastric (promotes absorption); Depurative (acts to clean and purify

the body, predominantly the blood); Expectorant (promotes mucus discharge from respiratory system); Hepato-protective (helps to guard liver and gall bladder occupations); Hypoglycemic (blood sugar reducer); Immuno-stimulant; Laxative; Prophylactic (helps prevent disease); Sedative; Thrombolytic (blood clot buster); Vermicides (used to murder colonic caterpillars and benefits care the entrails).

1.8 Medicinal importance of *Piper chaba* Hunter.

Nowadays, research on medicinal plants has attracted a lot of attention globally. A number of evidence has been accumulated to demonstrate promising potential of medicinal plants used in various traditional, complementary, and alternative systems. In recent years, a medicinal plant, *Piper chaba* Hunter, and its major active phytochemicals have been extensively studied for several pharmacological activities. To understand the mechanism of action, researches have to be carried out at molecular levels. The present review aims at compiling consequential compendium of pharmacological benefits of health on this plant and its major alkaloids constituent piperine that have been tested in various experimental models using modern scientific methodologies.

Piper chaba Hunter is a herbaceous plant, normally notorious as "Monarch of Bitters," in the domestic Piperaceae. It is extensively refined in Southern Asia. Typically the greeneries and origins have been conventionally recycled over the periods for dissimilar healing drives in Asia and Europe as a folklore medicine for a extensive range of illnesses or as an herbal addition for métier upgrade. The *Indian Pharmacopoeia* tells that it is a principal component of at least 28 Ayurvedic formulations.[10,11] In outmoded Chinese drug, it is an significant "taciturn stuff" rosemary used to free the form of warmth, as in temperatures, and to dismiss poisons from the physique.[12] In Scandinavian republics, it is usually rummage-sale to stop Extensive research has revealed that *Piper chaba* has a surprisingly broad range of pharmacological effects and some of them are extremely beneficial, such as anti-inflammatory,[11] antidiarrheal, antiviral, antimalarial,[16] hepatoprotective,[22] cardio-vascular,[30] anticancer,[31] and immunostimulatory[40] activities. On the other hand, male reproductive toxicity[45] and cytotoxicity[47] of this plant have been reported as well.

1.9 Morphology, Chemistry and biotransformation

Piper chaba Hunter is a yearly plant, tremendously vicious in palate in each fragment of the vegetable frame. It cultivates straight to a tallness of 28-49 cm in humid dappled spaces through glabrous verdures and white floras with rose-purple adverts lard the petals. The stalk is shadowy lime, 0.6-12 in elevation, 3–12 mn in distance, quadrangular by longitudinal grooves and sections on the perspectives of the newer buckets, somewhat distended at the bulges; shrubberies glabrous, up to 8 cm extended and 5.5 cm wide-ranging, lanceolate, pinnate; plants minor, in negligent dispersal axillary besides fatal racemes; pills linear-oblong, severe at together trimmings, 1.8 cm x 0.4 cm; kernels frequent, deputize quadrate, yellowish brunette. It produces plentifully in southeastern Asia. i.e., India, Sri Lanka, Pakistan, Java, Malaysia, and Indonesia, though it refined extensively in India, China, and Thailand[47].

The aerial parts of the plant (stem) are used to extract the active phytochemicals. Previous investigations oil the chemical composition of *Piper chaba* showed that it is a rich source of piperine (**Fig: 1.17**) and piperine derivatives such as pipernoaline (**Fig: 1.18**), guineensine (**Fig: 1.19**), and isobutylamide (**Fig: 1.20).**[48-49] The primary bioactive component of the medicinal plant *Piper chaba is* a Piperine.[50-51] Pipernoaline is a colorless crystalline bicyclic alkaloid derivatives and has a very bitter taste. It presents in all parts of the plant, maximally in the leaves (> 2%).

When orally consumed, piperine appears to accumulate in organs throughout the viscera. Pharmacokinetic studies showed that pipernoaline is quickly absorbed and extensively metabolized in rats and hunians.[53] Ninety percent is eliminated within 48 hr. piperine metabolites are mainly identified as sulfonic acid adducts and make sulfate compounds, as well as glucuronide conjugations. Ten metabolites of this plant guineensine as sulfonates, sulfate ester compounds, and piperine analogues were isolated from rat urine, feces, and the contents of the small intestine after the drug was orally administrated to rats.[54-56] While those metabolites isolated from human urine were as sulfates, cysteine S-conjugate, and glucuronide conjugates.[57] One of the metabolites. Isobutylamide was reported to be identical to the anti-inflammatory drug. Lian-bi-zhi, which is being clinically used in China.[11]

1.10 Pharmacological Potential

1.10.1 Hepatoprotective Activity

In Ayurveda drug, there are 25 dissimilar therapies covering *Piper chaba* used to extravagance liver syndromes. Management of *Piper chaba* Hunter proscribed hexachlorocyclohexane induced increase in the happenings of glut amyl trans peptidase. Glutathione-S-transferees and lipid peroxidation in mouse liver, an suggestion of potential antioxidant and hepatoprotective belongings of *Piper chaba* Hunter.[59] Sprig quotation of *Piper chaba* and piperine were originate to be actual in avoiding carbon tetrachloride encouraged liver injury in rats and mice[60]. The Piperine also displayed major hepatoprotective consequence alongside countless types of liver injury encouraged through galactosamine,[61,62] paracetamol,[63] were a complex capacity than a classical antioxidant silymarin in stopping a reduction of bitterness manufacture persuaded through paracetamol.[64] In other educations, piperine and pipernoaline was optional to show a part as a robust stimulator of gall-bladder purpose by creating a important escalation in vitriol stream, spleen salts, and irritability acid in mindful mice and anaesthetized guinea sheep. This was noticeable development, *i.e.*, development of hunger and liver purpose examinations, slow retrieval from jaundice, subsiding of temperature, in the mainstream of infectious hepatitis patients afterward incessant behavior through *Piper chaba* Hunter.[67]

1.10.2 Immunological Potential

Modern investigation consumes thrillingly designated that excerpts of *Piper chaba* might had the possible aimed at nosy with the feasibility of the humanoid immune-deficiency virus (HIV) and advised that *Piper chaba* could combine with contemporary drugs beside learned immune-deficiency conditions (AIDS).[68] *Piper chaba* contains materials, one of which is piperine which episodic or adapted the cellular signal transduction trail of the worm, subsequent in nosy the important enzymes and viral imitation consequently.[69,70] It was future by way of a strong stimulator of resistant system by two methods. The chief antigen-specific reply; antibodies remained made to counteract entering microorganisms and the second was a generic resistant reply: macrophage lockups hunted and devastated attackers. Meanwhile *Piper chaba* triggered both responses; it may be actual in contradiction of a diversity of communicable and oncogenic mediators.

1.10.3 Anti-inflammatory Activity

Folk remedial remedy *Piper chaba* has also used for infection, discomfort discount, and ailments of the duodenal territory. *Piper chaba* has an ability of lower infection and established self-sufficiently in several reports. It has exposed that the piperine shaped by diverse fever-inducing accents, such as microbial endotoxins, pneumococcus, hemolytic streptococcus, typhoid, paratyphoid and 2, 4-dinitrophenol. The painkilling movement of pipernoaline was scrawnier than aspirin while anti-pyretic activity was analogous to that of as pirin[66, 67]. An anti-inflammatory advantage of piperine by reduction of inducible nitric oxide synthase (iNOS) protein countenance concluded preclusion of the *de novo* protein synthesis and lessening the protein steadiness via a post-transcriptional mechanism have been reliant[78]

1.10.4 Respiratory System Benefits

The medicinal source of the herb *piper chaba* has been studied to be larger to palliative in the easing the personal indications of simple upper breathing area contagion (URI) and existence initial sign of a occurrence consequence.[73] There was sensibly robust indication after scientific prosecutions to propose that *Piper chaba* was current in plummeting the harshness and the extent of URI once usage stayed ongoing inside the first 36-48 hrs. warnings[74]. Pre-emption of the common emotionless with *Piper chaba* was deliberate in an initial double-blind revision. There was a important reduction in the occurrence and strength of the warnings in the straightforward shared frosts, compared to the palliative after the *Piper chaba* regimen intakc[75]. The comparative danger of infectious a cold designated that the defensive effect could be due to resistant stimulatory possessions of guineensine. Additionally, the plant *Piper chaba* quicker convalescence of common cold patients with symptoms counting adenoidal release, adenoidal staidness, sore gullet, earache, cough, fever, headache and malaisc.[75] The treatment of URI remains unclear to date its the mechanism of action of *Piper chaba* Hunter. The seeming efficiency of the herb could be founded anti-inflammatory possessions or on its immune modulatory things. Moreover research is required both to clarify whether *Piper chaba* is an operative in handling of URI and to clarify the apparatus through which this assistance is facilitated.

1.10.5 Antimalarial Activity

In the subtropical countries malaria is still a predominant syndrome. *Piper chaba* were originate to noticeably obstruct the multiplication of *Piper betle.* The protective exploit of *Piper chaba* is future to be for recurrence of the crucial antioxidant enzyme superoxide dismutase. Fashionable pooch, *Piper chaba* cuttings successfully slaughtered filaria that blocked lymph canals, subsequently leading to elephantiasis[63]. Another study also informed anti-malarial effect of *Piper chaba* in contradiction of *Plasmodium falciparum.*[65]

1.10.6 Antidiarrheal and Intestinal Effects

Worldwide diarrhea is unique of the highest ten reasons of passing and is a foremost cause of expiration in children in emergent republics; expressly beneath five ages of stage. Sundry recent tablets recycled toward discharge the indicators. i.e., kaolin-pectin, bismuth, lope amide, has unwanted lateral properties. It has been supposed that *Piper chaba* was actual touching microscopic dysentery and diarrhea, but how it was accomplished takes stood undecided up to date. Quotations of *Piper chaba* have been presented significant anti-diarrheal bustle beside *Escherichia coli* connected diarrhea, while piperine exhibited similar movement to lope amide the most common anti-diarrheal remedy. In a binary unseeing homework, patients with severe diarrhea and bacillary dysentery responded constructively to *Piper chaba.*[71]

1.10.7 Cardiovascular Activity

The properties of *Piper chaba* in an atherosclerotic bunny model disclosed upsurges of the nitric oxide, cyclic guanosine monophosphate, and movement of superoxide dismutase per drops of lipid bleach and endothelia. This comments optional the conceivable of *Piper chaba* is an antioxidant to reservation endothelial function, subsequent in upkeep of the equilibrium of nitric oxidefendothelin.[72] In another study, The hypotensive effects of the *Piper chaba* crude extract occurred in the nonattendance of noteworthy change in heart rate, representative more influence of the hypotensive reply on circulatory bustle than a straight exploit on the emotion, and were lastly future to be arbitrated finished adrenoceptors, autonomic cyst and histaminergic receptors. However that, Piperine remained not the hypotensive active compound of *Piper chaba* it remained unhurried.

1.10.8 Psycho-pharmacological Activity

Psycho-pharmacological revisions existed conducted per an excerpt of *Piper chaba*. It was superficial that the excerpt had a powerful central panicky system sedative feat as indicated by its entrancing potentiation conclusion; it was being produced hypothermia and demonstrated an pain-relieving action beside CH_3COOH induced twisting, By applying same device as reserpine and chlorpromazine.[72] Moreover, discount in investigative deeds with the extract was in conformism with alike actions bent by other relaxing drug[73]. The cutting also revealed substantial motor inco-ordination and sway relaxant activity.

1.10.9 Hypoglycemic Activity

Suggestively prohibited education of hyperglycemia induced through uttered direction of glucose in rabbits a water extract of *Piper chaba*, but it failed to do adrenaline-induced hyperglycemia. Furthermore, lasting direction (6 weeks) of *Piper chaba was* unable to validate abstaining blood darling dropping effect. Hence, might preclude glucose fascination from gut[74].

1.10.10 Antifertility Activity

Piper chaba H. possessed anti-fertility and pregnancy-terminating effects and stopped spermatogenesis in male rats. None of the female mice that daily consumed *Piper chaba* mixed food became pregnant when mated with the male of potential fertility who did not receive the treatment.[61] The observations suggested an anti-spermatogenic or anti-androgenic abilities as well as ovulation preventive effect of the plant. Hence, using of the herb during pregnancy should be avoided. *Piper chaba* proved to affect spermatogenesis in rats by preventing cytokinesis of the dividing spermatogenic cell lines with appearances of sertoli cell damage and a spermatotoxic effect.. Changes in the biochemical parameters in rats, such as significant decreases in protein content, but marked increases in cholesterol, acid phosphatase, and alkaline phosphatase levels with appearance of fructose in the reproductive system, suggested antifertility effects of the piperine.[61]

1.11 Aim of the work

Bangladesh is a good repository of medicinal plants belonging to various families, including Piperaceae and Bombacaceae. The Piperaceous and Bombacaceous plants contain wide range of chemical and unique pharmacologically active compounds including antimicrobial, antifungal, antioxidant, anticancer, antiseptic, astringent, stomachic, anti-inflammatory, antirheumatic, antidiarrhea and antiemetic activities. *Piper chaba* and *Bombax ceiba* is one of the important members of the Piperaceae family and Bombacaceae family and well known for its medicinal properties, its different parts including flowers, roots, stem barks and leaves etc. have been used in traditional medicine for a long time. Since this both plants is available in Bangladesh and a lot of herbal health centers and herbal industries are using such related herbal plants for treatments, so if the biological activity of this plant can be studied thoroughly, this may be a cost- effective treatment. So, the objective of the study is isolation and structural elucidation of the bioactive compounds by chemical and spectroscopic methods [UV, FTIR, ^{1}H-NMR, ^{13}C-NMR, DEPT-135 and 2D-NMR (HSQC and HMBC) etc.] and to explore the possibility of developing new drug candidates from this plants for the treatment of various diseases.

1.12 Present study protocol

The present study will be designed to carry out phytochemical investigation on the extracts of chosen plant materials to isolate pure compounds as well as to observe biological activities of the isolated pure compounds. Crude extracts and their different fractions will also be studied for biological activity. The study protocol consist of the following steps:

1. The roots of the plant of *Piper chaba* (Locally known as Chui Jhal) will be collected from Katalbari Upazila in the district of Kurigram and *Bombax ceiba* (Locally known as Shemul) from Boraigram Upazila in the district of Nator, Bangladesh.
2. Taxonomic identification of the plants will be confirmed consulting with at the Department of Botany and also at National Herbarium's Botanist.
3. Roots of the plants will be separated from stem and cleaned to remove mud and dust particles. Root materials will be dried at room temperature under a shade for three weeks and finally in an oven at 37^{o}C.
4. The dried root will be turned into powder and will be subjected to cold extraction with methanol.
5. The methanol extract will be concentrated to a thick mass using rotary evaporator and will be partitioned with n- hexane followed by EtOAc using a separatory funnel.
6. The partitioned EtOAc extract will be fractionated by column chromatography using different solvent systems.
7. Pure compounds will be isolated and purified from different column fractions following various chromatographic methods and recrystallization.
8. Structure of the isolated compounds will be determined with the help of chemical and spectroscopic methods (IR, 1H-NMR, ^{13}C-NMR, DEPT, HSQC and HMBC etc.).
9. *In vitro* biological activities (antibacterial, antifungal, antidiarrheal, thrombolytic and analgesic) of crude extracts, column fractions and pure compounds will be investigated.
10. Antioxidant property of crude extracts, column fractions and pure compounds will be investigated.

EXPERIMENTAL

2.0 General methods

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

2.1 Collection of the *Piper chaba* H. plant

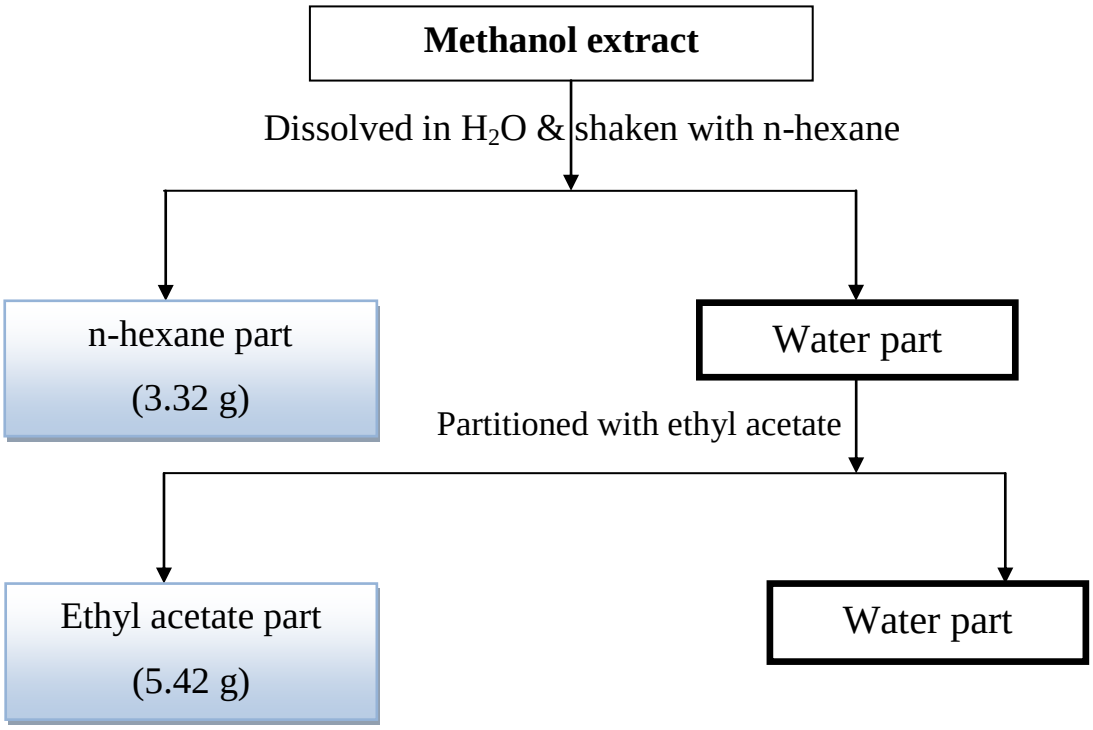
The roots of the plant of *Piper chaba* (Locally known as Chui Jhal) has been collected from Katalbari Upazila in the district of Kurigram, Bangladesh.

2.2 Identification of species

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

2.3 Extraction, partition and isolation of the compounds from the roots of *Piper chaba*

The collected roots of the plant *Piper chaba* were cleaned to remove mud and dust particles and dried in open air followed by drying in an oven at 37^{o}C. Afterwards it was powdered (~200 mesh) using a grinding machine. This root powder of *Piper chaba* (~568 g) was used throughout this investigation. The root powder was extracted with methanol. The extract *Piper chaba* was concentrated to dry mass (18.74 g) using rotary evaporator. The methanol extract was then partitioned by separatory funnel by using n-hexane, ethyl acetate and water successively. All the crude extracts were also subjected to antimicrobial tests.



Scheme 2.1: Partition of methanol extract

2.4 Phytochemical screening (Edeoga H.O. *et. al*., 2005) **of the roots of *Piper chaba* H.** Chemical tests for different class of compounds were carried out on the aqueous extract and on the powdered specimens using standard procedures to identify the phytochemical constituents and the result are given in Table 2.1

a) Test for tannins: The dried root powder (~0.5 g) was boiled in water (20 ml) in a test tube and then filtered. A few drops of ferric chloride solution (0.1% w/v) were added to the filtrate and a brownish green coloration was observed which indicated the presence of tannins.

b) Test for Alkaloids: Extracted 2g of root powder by warming for two minutes with 20 mL 1% H_2SO_4 in a 50 mL conical flask on a water bath with intermittent shaking centrifuge. Supernatant was pipetted into a small conical flask and the test was carried out taken 0.1 mL of the sample solution in a semi-micro tube and one drop of Meyer's reagent. It gives a cream precipitate. A positive test indicates the presence of alkaloids.

c) Test for saponin: The powdered sample (~2 g) was boiled in distilled water (20 ml) in a test tube and filtered. The filtrate (10 ml) was mixed with distilled water (5 ml) and shaken vigorously for a stable persistent froth. The frothing was mixed with olive oil (3 drops) and shaken vigorously, No specific change was found.

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

i) Dilute ammonia solution (5 mL) was added to a portion of the aqueous extract of the plant followed by addition of concentrated H_2SO_4, a yellow colour was observed in extract. The selected plants were indicated the presence of flavonoids.

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

f) Test for terpenoids (Salkowski test): The water extract (5 ml) was mixed in chloroform (2 ml), and concentrated H_2SO_4 acid (3 ml) was carefully added to form a layer. A reddish brown coloration at the interface was formed indicating the positive test for the presence of terpenoids.

g) Test for cardiac glycosides (Keller-Killani test): The water extract (5 mL) was treated with glacial acetic acid (2 ml) containing ferric chloride solution (1 drop). This was underplayed with concentrated H_2SO_4 acid (1 mL). No specific change was found.

h) Test of carbohydrates

0.5 mL of aqueous extract was added to 5 mL of benedict's solution and boiled for 5 min. A coloured precipitate was formed. The selected plants were indicated the presence of carbohydrates.

Table 2.1: Result of qualitative analysis for phytochemical screening of roots of *Piper chaba* H.

Tannin	Carbohydrates	Alkaloid	Saponin	Flavonoid	Steroid	Terpenoid	Cardiac Glycoside
+	+	+	-	+	+	+	_

- The positive sign (+) indicates the presence of phytochemicals
- The negative sign (–) indicates the absence of phytochemicals

2.5 Investigation of the EtOAc extract

2.5.1 Thin layer chromatography (TLC)

The crude EtOAc extract was subjected to TLC screening to see the type of compounds present in the extract. TLC analysis of the EtOAc extract showed several spots in iodine chamber and vanillin-sulfuric acid spray on TLC plate. TLC pattern gives an idea about the polarity of the compounds, which are present in the extract.

2.5.2 Fractionation of the EtOAc extract by column chromatography.

The ethyl acetate extract was concentrated to dry mass (5.42g) using rotary evaporator. The dry mass of ethyl acetate extract was absorbed by the column grade silica gel. This sample was placed on the top of the bed of column packed with TLC grade silica gel. The column was first eluted with 100% n-hexane and then eluted with mixtures of n-hexane and ethylacetate increasing the polarity of the solvents and finally with the mixtures of ethyl acetate methanol. The eluents were collected in an amount of100 mL in a series of test tube. Solvent systems used as mobile phases in the analysis of ethyl acetate extract were listed in table 2.2

Table-2.2: Fractions collected from column chromatography of EtOAc extract (5.42 g) using different solvent systems.

Fraction no.	Solvent system	Volume
1 - 10	n-hexane (100%)	200 mL
11 - 16	n- hexane : dichloromethane (90:10)	120 mL
17 - 22	n- hexane : dichloromethane (80:20)	120 mL
23 - 27	n- hexane : dichloromethane (70:30)	100 mL
28 - 32	n- hexane : dichloromethane (60:40)	100 mL
33 - 38	n- hexane : dichloromethane (50:50)	100 mL

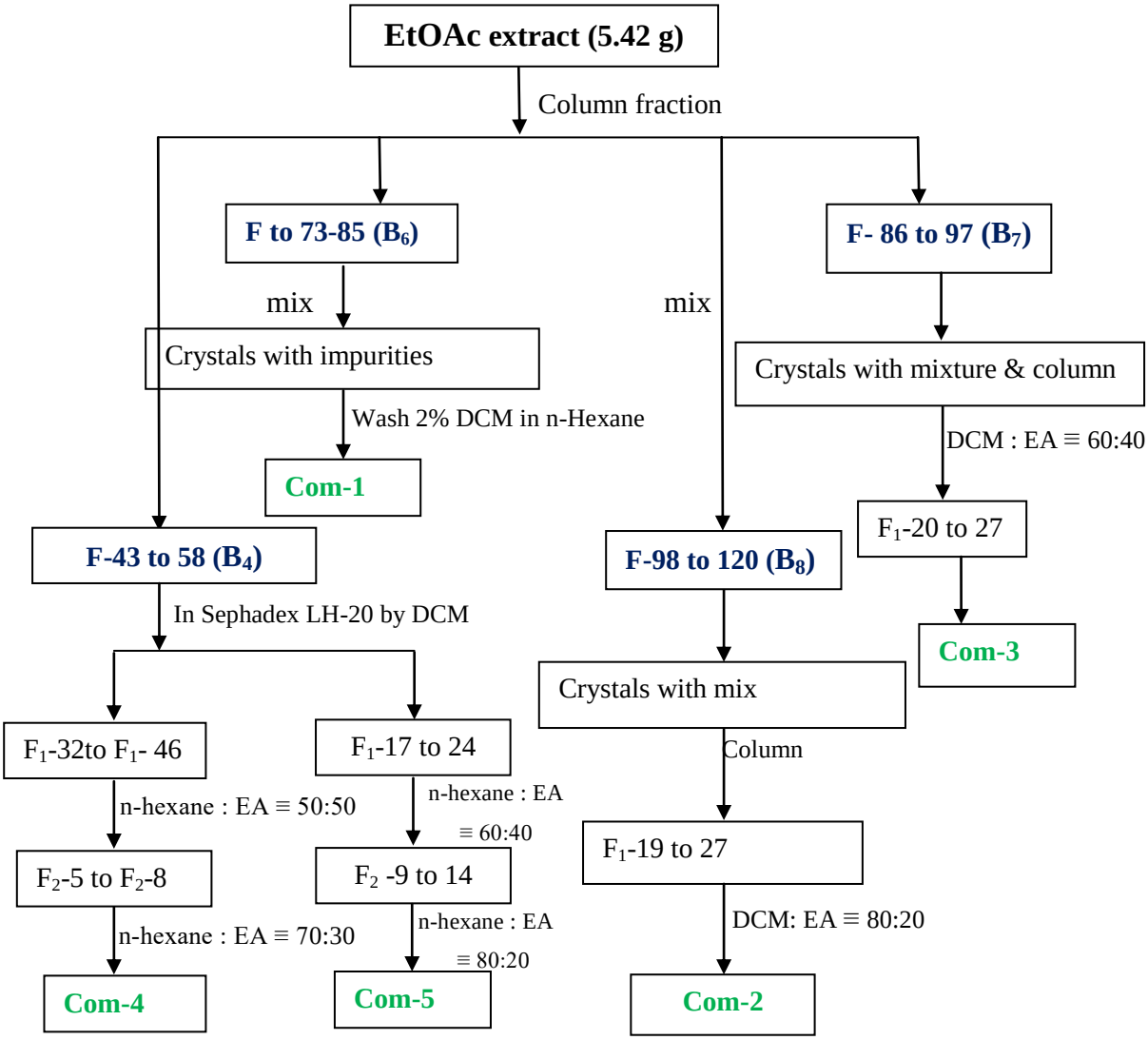
39 - 43	n- hexane : dichloromethane (40:60)	100 mL
44 - 48	n- hexane : dichloromethane (30:70)	100 mL
49 - 53	n- hexane : dichloromethane (20:80)	100 mL
54 - 58	n- hexane : dichloromethane (10:90)	100 mL
59 - 63	dichloromethane (100%)	100 mL
64 - 68	dichloromethane : ethyl acetate (97.5: 2.5)	100 mL
69 - 73	dichloromethane : ethyl acetate (95:5)	100 mL
74 - 80	dichloromethane : ethyl acetate (90:10)	150 mL
81 - 85	dichloromethane : ethyl acetate (87.5:12.5)	100 mL
86 - 93	dichloromethane: ethyl acetate (85:15)	130 mL
94 - 105	dichloromethane: ethyl acetate (80:20)	220 mL
106 - 111	dichloromethane: ethyl acetate (70:30)	100 mL
112 - 117	dichloromethane: ethyl acetate (60:40)	100 mL
118 - 125	dichloromethane: ethyl acetate (50:50)	140 mL
126 - 130	dichloromethane: ethyl acetate (40:60)	100 mL
131 - 136	dichloromethane: ethyl acetate (20:80)	100 mL
137 - 144	ethyl acetate (100%)	130 mL
145 - 149	ethyl acetate : methanol (90:10)	100 mL
150 - 155	ethyl acetate : methanol (80:20)	100 mL
156 - 161	ethyl acetate : methanol (70:30)	100 mL
162 - 167	ethyl acetate : methanol (60:40)	100 mL
168 - 173	ethyl acetate : methanol (50:50)	100 mL
174 – 179	ethyl acetate : methanol (40:60)	100 mL
180 - 185	ethyl acetate : methanol (50:50)	100 mL
186-191	ethyl acetate : methanol (70:30)	100 mL
192-200	Methanol (100%)	150 mL

2.5.2.1. Analysis of EtOAc Fractions by TLC

All the column fractions were examined by TLC under UV light and by spraying with vanillin-sulphuric acid reagent. Depending on the TLC behavior similar fractions were combined together in a beaker and these were marked as B_1, B_2, B_3, B_4, B_5, B_6, B_7, B_8, B_9, B_{10}, B_{11}, B_{12} and B_{13} (**Table-2.3).**

Table 2.3: Screening of the fractions by similar TLC pattern.

Fraction No.	Solvent system for TLC	No. of spots (After spraying with vanillin sulfuric acid & heating at 105^0C)
B_1 (1-10)	90:10 (Hex:DCM)	No Spot was found
B_2 (11-24)	20:80 (DCM : Hex)	No Spot was found
B_3 (25-42)	30:70 (DCM : Hex)	No spot was found
***B_4** (43-58)	**80:20 (DCM : Hex)**	**Three spot with mild tailing**
B_5 (59-72)	90: 10 (DCM : Hex)	No prominent spot
***B_6** (73-85)	**95:5 (DCM : EtOAc)**	**Single spot with tailing**
***B_7** (86-97)	**90: 10 (DCM : EtOAc)**	**Four spots were found**
***B_8** (98-120)	**80: 20 (DCM : EtOAc)**	**Single spots with tailing**
B_9 (121-140)	60:40 (DCM : EtOAc)	Two spots with long tailing
B_{10} (142-161)	60:40 (DCM : EtOAc)	Spots merged into tailing
B_{11} (162-173)	20:80 (DCM:EtOAc)	Two spots with tailing
B_{12} (174-185)	100% EtOAc	Spots merged into tailing
B_{13} (186-200)	95:5 (EtOAc:MeOH)	Spots merged into tailing



Scheme 2.3: Scheme of isolating different compounds from the ethyl acetate extract of the root.

2.5.2.2. Purification of the Fractions B_4 (F- 43 to 58) by Column Chromatography

The dry mass of the fraction B_4 (225 mg) was mixed with column grade silica gel. This sample was placed on the top of the bed of column; packed with column grade silica gel. The column was first eluted with 100% hexane and then eluted with mixtures of hexane with increasing amount of dichloromethane, ethyl acetate and finally with methanol. The eluents were collected in an amount of about 50 mL in a series of test tubes. Solvent systems used as mobile phases in the analysis of fraction were B_4 listed in **Table-2.4**.

Table 2.4: Number of fractions collected in test tubes from column chromatography of fraction **B_4** using different solvent systems.

Solvent system			Amount of solvent (mL)	Number of test tube
Hexane %	**DCM %**	**EtOAc %**		
100	0	0	200	Beaker-1
50	50	0	100	Beaker-2
40	60	0	50	1-5
20	80	0	50	6-8
10	90	0	50	9-11
4	96	0	50	12-13
0	100	0	100	14-19
0	90	10	100	20-37
0	80	20	100	Beaker-3
0	70	30	100	Beaker-4

2.5.2.2.1 Screening of the Fractions

Each of the fractions was monitored by TLC and the fractions of similar behaviors were combined together and marked as T_1, T_2, T_3, T_4, T_5 and T_6 (**Table-2.5**)

Table 2.5: Screening of the fractions by similar TLC pattern

No. of test tubes	Fraction No.	Solvent system for TLC	No. of spots (After spraying with vanillin sulfuric acid & heating at 105^0C)
1-7	T_1	100 % (hexane)	No spot was observed
8-14	T_2	50: 50 (DCM : hexane)	No spot was observed
*15-21	**T_3**	95:5 (DCM : EtOAc)	**Single spot was observed**
*22-28	**T_4**	90:10 (DCM : EtOAc)	**Single spot was observed**
29-37	T_5	80:20 (DCM : EtOAc)	Three spots merged with tailing
Conical flax-1	T_6	75:25(DCM : EtOAc)	Three spots merged with tailing

2.5.2.2.2 Analysis of the Fractions by TLC

TLC analysis of the fraction was further carried out and attempt was taken to characterize the fraction **T_3** and **T_4** as the fraction contains compounds which could be easily separable (according to TLC pattern). The rest of the fractions were mixture of compounds.

2.5.2.2.3 Analysis of the Fraction T_3

The fraction S_3 was left undisturbed at room temperature for several days. When a white crystalline solid was obtained, a portion was dissolved in dichloromethane and it was checked by TLC. A Single spot was observed. The fraction **T_3** was renamed as **Compound-4.**

2.5.2.2.4 Analysis of the Fraction T_4

The fraction S_4 was left undisturbed at room temperature for several days. When a white crystalline solid was obtained, a portion was dissolved in dichloromethane and it was checked by TLC. A single spot was observed. The fraction **T_4** was renamed as **Compound-5.**

2.5.2.3 Purification of the Fractions B_6 (F- 73 to 85) by Column Chromatography

The dry mass of the fraction B_6 (485 mg) was mixed with column grade silica gel. This sample was placed on the top of the bed of column; packed with column grade silica gel. The column was first eluted with 100% hexane and then eluted with mixtures of hexane with increasing amount of dichloromethane, ethyl acetate and finally with methanol. The eluents were collected in an amount of about 50 mL in a series of test tubes. Solvent systems used as mobile phases in the analysis of fraction were **B_6 (**Beaker-6) listed in **Table-2.6**

Table 2.6: Number of fractions collected in test tubes from column chromatography of fraction **B_6** using different solvent systems

Solvent system			Amount of solvent (mL)	Number of test tube
Hexane %	DCM %	EtOAc %		
100	0	0	200	1-10
50	50	0	100	12-16
20	80	0	50	17-18
10	90	0	100	19-23
4	96	0	100	24-30
0	100	0	100	31-35
0	90	10	100	36-40
0	80	20	100	41-46
0	0	100	150	Beaker

2.5.2.3.1 Screening of the Fractions

Each of the fractions was monitored by TLC and the fractions of similar behaviors were combined together and marked as T_1 (A), T_2 (A), T_3 (A), T_4 (A), T_5 (A) and T_6 (A)

Table 2.7: Screening of the fractions by similar TLC pattern

No. of test tubes	Fraction No.	Solvent system for TLC	No. of spots (After spraying with vanillin sulfuric acid & heating at 105^0C)
1-14	T_1 (A)	100 % (hexane)	No spot was observed
15-24	T_2 (A)	50: 50 (DCM : hexane)	No spot was observed
25-33	T_3 (A)	80:20 (DCM : hexane)	Two spots merged with tailing
***34-40**	T_4 (A)	**90:10 (DCM : EtOAc)**	**Single spot was observed**
41-46	T_5 (A)	80:20 (DCM : EtOAc)	Three spots merged with tailing
Beaker-1	T_6 (A)	70:30 (DCM : EtOAc)	No spot was observed

2.5.2.3.2 Analysis of the Fractions by TLC

TLC analysis of the fraction was further carried out and attempt was taken to characterize the fraction **T_4 (A)** as the fraction contains compounds which could be easily separable (according to TLC pattern). The rest of the fractions are a pure compound.

2.5.2.3.3 Analysis of the Fraction T_4 (A)

The fraction **T_4 (A)** was left undisturbed at room temperature for several days. When a white crystalline solid was obtained, a portion was dissolved in dichloromethane and it was checked by TLC. A single spot was observed. The fraction **T_4 (A)** was renamed as **compound-1.**

2.5.2.4 Purification of the Fractions B_7 (F-86 to 97) by Column Chromatography

The dry mass of the fraction B_7 (~634 mg) was mixed with column grade silica gel. This sample was placed on the top of the bed of column; packed with column grade silica gel. The column was first eluted with 100% hexane and then eluted with mixtures of hexane with increasing amount of dichloromethane, ethylacetate and finally with methanol. The eluents were collected in an amount of about 50 mL in a series of test tubes.

Table 2.8: Number of fractions collected in test tubes from column chromatography of fraction **B_7** (Beaker-7) using different solvent systems.

Solvent system			Amount of solvent (mL)	Number of Fractions
Hexane %	DCM %	EtOAc %		
100	0	0	220	Conical flask-1
50	50	0	200	Conical flask-2
20	80	0	150	Conical flask-3
10	90	0	100	1-5
5	95	0	100	6-10
0	100	0	100	11-15
0	90	10	100	16-20
0	80	20	150	Beaker-1
0	0	100	200	Beaker-2

2.5.2.4.1. Screening of the Fractions

Each of the fractions was monitored by TLC and the fractions of similar behaviors were combined together and marked as D_1, D_2, D_3, D_4, D_5, D_6 and D_7 (**Table-2.9**)

Table 2.9: Screening of the fractions by similar TLC pattern

No. of beaker & Conical flax	Fraction No.	Solvent system for TLC	No. of spots (After spraying with vanillin sulfuric acid & heating at 105^0C)
Conical flask-1	D_1	100 % (hexane)	No spot was observed
Conical flask-2	D_2	50 : 50 (DCM : hexane)	No spot was observed
Conical flask-3	D_3	80 :20 (DCM : hexane)	Two spots merged with tailing
Beaker-1	D_4	90 :10 (DCM : EtOAc)	Single spot was observed
***Beaker-2**	**D_5**	**70 :30 (DCM : EtOAc)**	**Single spot was observed**
Beaker-3	D_6	60 :40 (DCM : EtOAc)	Single spot was observed
Beaker-4	D_7	30 :70 (DCM : EtOAc)	Spots merged with tailing

2.5.2.4.2 Analysis of the Fractions by TLC

TLC analysis of the fraction was further carried out and attempt was taken to characterize the fraction **D_5** as the fraction contains compounds which could be easily separable (according to TLC pattern). The rest of the fractions were pure compounds.

2.5.2.4.3. Analysis of the Fraction D_5

The fraction **D_5** was left undisturbed at room temperature for several days. When a white crystalline solid was obtained, a portion was dissolved in dichloromethane and it was checked by TLC. The fraction **D_5** was renamed as **compound-3.**

2.5.2.5 Purification of the Fractions B_8 (F-98 to 120) by Column Chromatography

The dry mass of the fraction B_8 (~940 mg) was mixed with column grade silica gel. This sample was placed on the top of the bed of column; packed with column grade silica gel. The column was first eluted with 100% hexane and then eluted with mixtures of hexane with increasing amount of dichloromethane, ethylacetate and finally with methanol. The eluents were collected in an amount of about 50 mL in a series of test tubes.

Table 2.10: Number of fractions collected in test tubes from column chromatography of fraction **B_8** using different solvent systems.

Solvent system			Amount of solvent (mL)	Number of Beaker
Hexane %	DCM %	EtOAc %		
100	0	0	150	Beaker-1
60	40	0	200	Beaker-2
20	80	0	150	Beaker-3
10	90	0	100	Beaker-4
5	95	0	100	Beaker-5
0	100	0	100	Beaker-6
0	90	10	100	Beaker-7
0	80	20	150	Beaker-8
0	0	100	200	Beaker-9

2.5.2.5.1 Screening of the Fractions

Each of the fractions was monitored by TLC and the fractions of similar behaviors were combined together and marked as S_1, S_2, S_3, S_4, S_5, S_6 and S_7 (**Table-2.11**)

Table 2.11: Screening of the fractions by similar TLC pattern

No. of Conical flax	Fraction No	Solvent system for TLC	No. of spots (After spraying with vanillin sulfuric acid & heating at 105^0C)
Conical flask-1	S_1	100 % (hexane)	No spot was observed
Conical flask-2	S_2	50 : 50 (DCM : hexane)	No spot was observed
Conical flask-3	S_3	80 :20 (DCM : hexane)	Two spots merged with tailing
Conical flask-4	S_4	90 :10 (DCM : EtOAc)	Single spot was observed
Conical flask-5	***S_5**	**80 :20 (DCM : EtOAc)**	**Single spot was observed**
Conical flask-6	S_6	60 :40 (DCM : EtOAc)	Single spot was observed
Conical flask-7	S_7	30 :70 (DCM : EtOAc)	Spots merged with tailing

2.5.2.5.2 Analysis of the Fractions by TLC

TLC analysis of the fraction was further carried out and attempt was taken to characterize the fraction **S_5** as the fraction contains compounds which could be easily separable (according to TLC pattern). The rest of the fractions were pure compounds.

2.5.2.5.3 Analysis of the Fraction S_5

The fraction **S_5** was left undisturbed at room temperature for several days. When a white crystalline solid was obtained, a portion was dissolved in dichloromethane and it was checked by TLC. The fraction **S_5** was renamed as **Compound-2.**

2.6 Characterization of isolated compounds.

2.6.1 Physical properties of compound-1

Physical state : A white crystalline solid

Solubility : Soluble in dichloromethane, ethyl acetate, methanol and ethanol

Melting point : 138-140^{o}C

R_f value : R_f value 0.79 (n-hexan: EA ≡ 50:50)

Amount : ~8.0 mg.

2.6.1.1 ^{1}H-NMR spectroscopy of compound-1

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

2.6.1.2 ^{13}C-NMR spectroscopy of compound-1

The ^{13}C-NMR spectrum (100 MHz) in $CDCl_3$ of the compound-1 has signals at δ_C (ppm) 37.30, 31.72, 71.85, 42.36, 140.81,121.74, 31.96, 31.96,50.2, 36.56, 21.13, 39.83, 40.50, 56.82, 24.34, 28.28, 56.12, 12.08, 19.43, 36.19, 18.82, 138.33, 129.31, 45.9, 29.23, 19.84, 19.08, 23.13, 12.26.

2.6.2. Physical properties of compound-2

Physical state : A white crystalline solid

Solubility : Soluble in dichloromethane, ethyl acetate and methanol

Melting point : 128-130^{o}C

R_f value : R_f value 0.63 (20 % in EtOAc in dichloromethane)

Amount : ~24.0 mg.

2.6.2.1 ^{1}H-NMR spectroscopy of compound-2

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of the compound-2 has signals at δ_H (ppm) 7.36 (1H, d), 6.95 (1H, d), 6.86 (1H, d), 5.95 (1H, s), 3.56 (1H, brs), 1.64 (2H, m), 1.57 (2H, m), 1.54 (1H, m).

2.6.2.2 ^{13}C-NMR spectroscopy of compound-2

The ^{13}C-NMR spectrum (100 MHz, $CDCl_3$) of the compound-2 has signals at δ_C (ppm) 165.6, 119.7, 142.8, 125.37, 138.31, 131.04, 105.6, 148.21, 108.49, 122.50, 101.27, 45.3, 26.18, 24.66, 25.36, 46.9

2.6.3 Physical properties of compound-3

Physical state : A white crystalline solid

Solubility : Soluble in dichloromethane, ethyl acetate and methanol

Melting point : 142-143^{o}C

R_f value : R_f value 0.36 (40% EtOAC in dichloromethane)

Amount : ~7.0 mg.

2.6.3.1 ^{1}H-NMR spectroscopy of compound-3

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

2.6.3.2 ^{13}C-NMR spectroscopy of compound-3

The ^{13}C-NMR spectrum (100 MHz, $CDCl_3$) of the compound-3 has signals at δ_C (ppm) 60.66, 51.64, 178.48, 130.27, 129.83, 129.14, 128.88, 165.31, 147.96,104.32, 127.84, 140.59, 138.07, 133.01, 127.66, 18.61, 13.92.

2.6.4 Physical properties of compound-4

Physical state : A white crystalline solid

Solubility : Soluble in dichloromethane, ethyl acetate and methanol

Melting point : 112-113^{o}C

R_f value : R_f value 0.51 (5 % EtOAc in dichloromethane)

Amount : ~7.5 mg.

Molecular Formula : $C_{16}H_{13}O_2Cl$

Molecular Weight : (M^+ 273)

2.6.4.1 ^{1}H-NMR spectroscopy of compound-4

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of the compound-4 has signals at δ_H (ppm) 7.37 (d), 7.78 (d), 7.60 (d), 6.93 (d), 7.95 (d), 7.46 (d), 3.85 (s)

2.6.4.2 ^{13}C-NMR spectroscopy of compound-4

The ^{13}C-NMR spectrum (100 MHz, $CDCl_3$) of the compound-4 has signals at δ_C (ppm) 189.29, 119.17, 145.29, 127.45, 130.39, 114.50, 161.88, 136.82, 129.87, 128.91, 138.99, 55.48

2.6.5 Physical properties of compound-5

Physical state : A white crystalline solid

Solubility : Soluble in dichloromethane, ethyl acetate and methanol

Melting point : 117-118 ^{o}C

R_f value : R_f value 0.4 (10% EtOAc in dichloromethane)

Amount : ~ 6.5 mg.

Molecular Formula : $C_{16}H_{13}OCl$

Molecular Weight : (M^+ 257)

2.6.5.1 ^{1}H-NMR spectroscopy of compound-5

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of the compound-5 has signals at δ_H (ppm) 7.44 (d), 7.79 (d), 7.54 (d), 7.95 (d), 7.95 (d), 7.47 (d), 2.39 (s)

2.6.5.2 ^{13}C-NMR spectroscopy of compound-5 The ^{13}C-NMR spectrum (100 MHz, $CDCl_3$) of the compound-5 has signals at δ_C (ppm) 189.35, 120.51, 145.52, 131.98, 128.60, 128.95, 141.41, 136.68, 129.92, 129.80, 139.12, 21.61.

RESULTS AND DISCUSSION

3.0 Preliminary investigation of the plant material

The roots of the plant of *Piper chaba* (Locally known as Chui Jhal) was collected from Katalbari Upajalla in the district of Kurigram. The collected roots were cleaned to remove mud and dust particles and was dried at room temperature (25^0C) under a shade for three weeks and finally in an oven at 37^oC. The dried root was powdered and subjected to cold extraction with methanol. The methanol extract was concentrated to a thick mass using rotary evaporator. This methanol extract was partitioned with n- hexane followed by EtOAc using a separatory funnel. The partitioned EtOAc extract was fractionated by column chromatography using different solvent system as mobile phase and silica gel (60–120 mesh) as stationary phase. Eluents were collected in 25 mL aliquots and TLC was used to monitor the fractions.

More than 200 fractions of the column eluent were collected. These collected fractions were subjected to TLC analysis and similar fractions were mixed together based on their TLC patterns and were further fractionated by preparative TLC using different solvent systems. Efforts also were given to isolate pure compound through crystallization/re-crystallization of the isolated fractions.

Out of those successive separation affords five compounds was isolated from this EtOAc extract of the roots of *Piper chaba*. The structure of all the isolated compounds has been established and characterized by IR, ^{1}H-NMR, ^{13}C-NMR, HSQC, HMBC and DEPT-135.

3.1 Characterization of isolated compounds from the roots of *Piper chaba* H.

3.1.1 Characterization of compound-1

The compound-1 (~8.0 mg) was a colorless crystalline solid having R_f value: 0.79 (n-hexane: EtOAc = 50:50) and its melting point was found to be 138-140^{o}C. It was soluble in dichloromethane. On spraying with vanillin-sulfuric acid spray reagent, followed by heating at 105°C for several minutes, purple color appeared.

The IR spectroscopic analysis (in KBr) of the compound-1 showed the absorption at 3238 cm^{-1} for O-H stretching, at 2958 cm^{-1} due to cyclic olefinic >C = CH- stretching, at 2933 cm^{-1} and 2864 cm^{-1} due to C-H stretching. Other absorption at 1454 cm^{-1} for alkyne (C=C) group.

The ^{1}H-NMR spectrum (400 MHz, in $CDCl_3$) of compound-1 displayed the methylene and methyl protons signals at δ 1.04 - 2.29 ppm and at δ 0.85 ppm. The broad peak at δ 3.51 ppm indicated the presence of oxymethine proton lined with two different methylene groups (-CH_2-CHOH-CH_2-). The other signals at δ 5.14 and 5.01 (J= 6.8 Hz) ppm were assigned to two olefinic protons.

The ^{13}C-NMR spectrum (100 MHz, in $CDCl_3$) of the compound -1 showed the presence of oxymethine carbon at δ 71.85 (C-3) ppm. The peaks at δ 138.33 (C-22) and 129.31 (C-23) ppm indicated the presence of two olefinic carbons, at δ 140.81 and 121.74 ppm indicates the presence of double bond in a tertiary carbon and a methylene carbon**.** These ^{1}H NMR and ^{13}C-NMR data are very close agreement with the published data **[Ref. 10].**

The ^{1}H NMR and ^{13}C NMR data of compound-1 were tabulated and compared with the published data of stigmasterol **[Ref. 10]** in **Table 3.0**

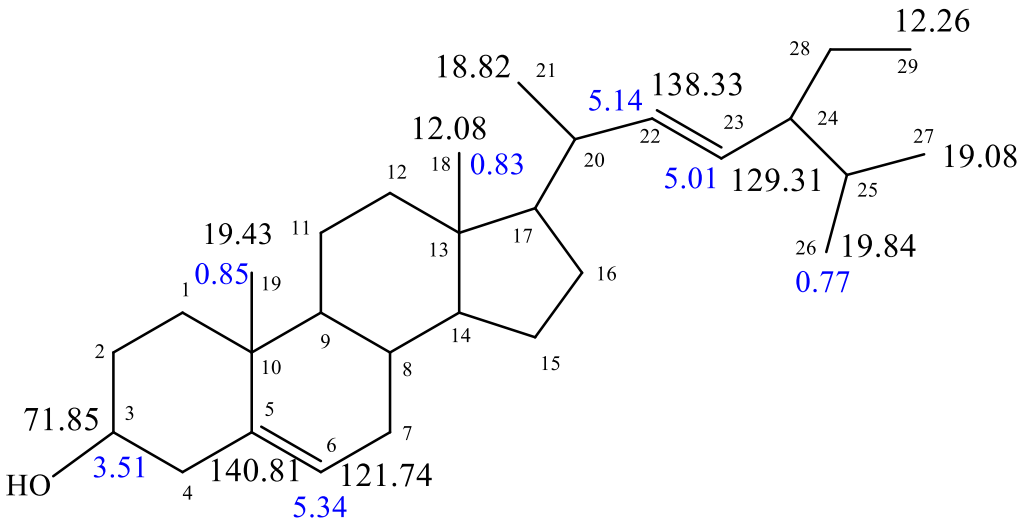


Figure 3.1: Structure of compound-1 showing ^{1}H-NMR ^{13}C NMR signals

Table 3.0: The ^{1}H-NMR and ^{13}C-NMR data of compound-1 and stigmasterol.

Carbon no.	Type of carbon	Compound-1 (Chemical shift in ppm)		stigmasterol (Chemical shift in ppm)	
		δ_H	δ_C	δ_H	δ_C
1	CH_2	-	37.31	-	37.30
2	CH_2	-	31.69	-	31.72
3	CH	3.50 (m)	71.81	3.51 (m)	71.85
4	CH_2	-	42.55	-	42.36
5	C	-	140.81	-	140.41
6	CH	5.346 (br,s)	121.69	5.346 (br,s)	121.74
7	CH_2	-	31.94	-	31.96
8	CH	-	31.94	-	31.96
9	CH	-	50.20	-	50.2
10	C	-	36.56	-	36.56
11	CH_2	-	21.11	-	21.13
12	CH_2	-	39.77	-	39.83
13	C	-	42.35	-	40.5
14	CH	-	56.91	-	56.82
15	CH_2	-	24.39	--	24.34
16	CH_2	-	28.96	-	28.28
17	CH	-	56.02	-	56.12
18	CH_3	0.66 (s)	12.07	0.67 (s)	12.08
19	CH_3	1.01 (s)	19.42	1.01 (s)	19.43
20	CH	-	40.54	-	36.19
21	CH_3	-	21.11	-	18.82
22	CH	5.00 (dd)	138.37	5.01 (dd)	138.33
23	CH	5.33 (dd)	129.69	5.34 (dd)	129.31
24	CH	-	51.29	-	45.9
25	CH	-	31.49	-	29.23
26	CH_3	0.899 (d)	21.26	0.898 (d)	19.84
27	CH_3	0.831(d)	19.02	0.832 (d)	19.08
28	CH_2	-	25.44	-	23.13
29	CH_3	-	12.29	-	12.26

*^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) in $CDCl_3$

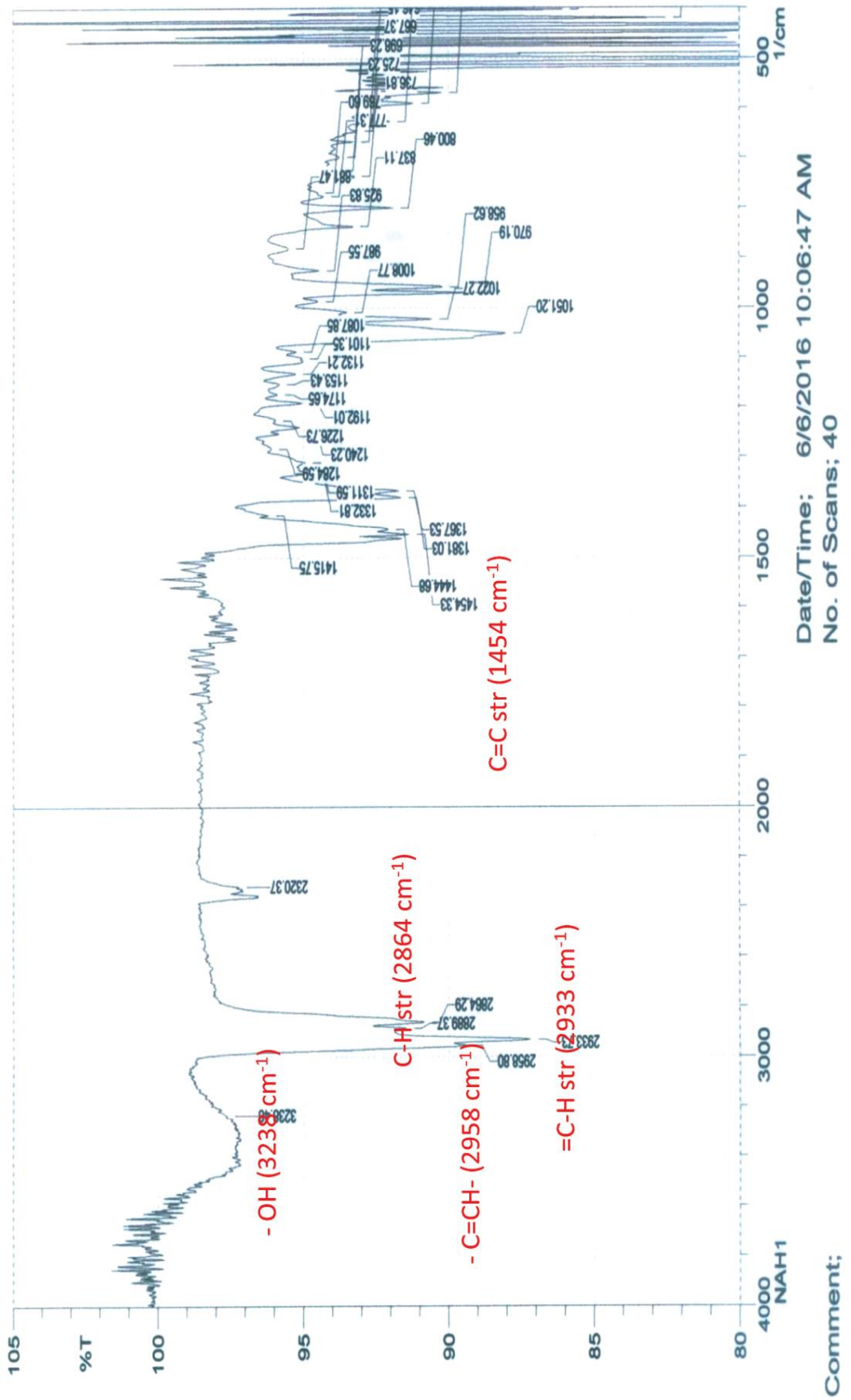


Figure 3.2: IR spectrum of Compound-1

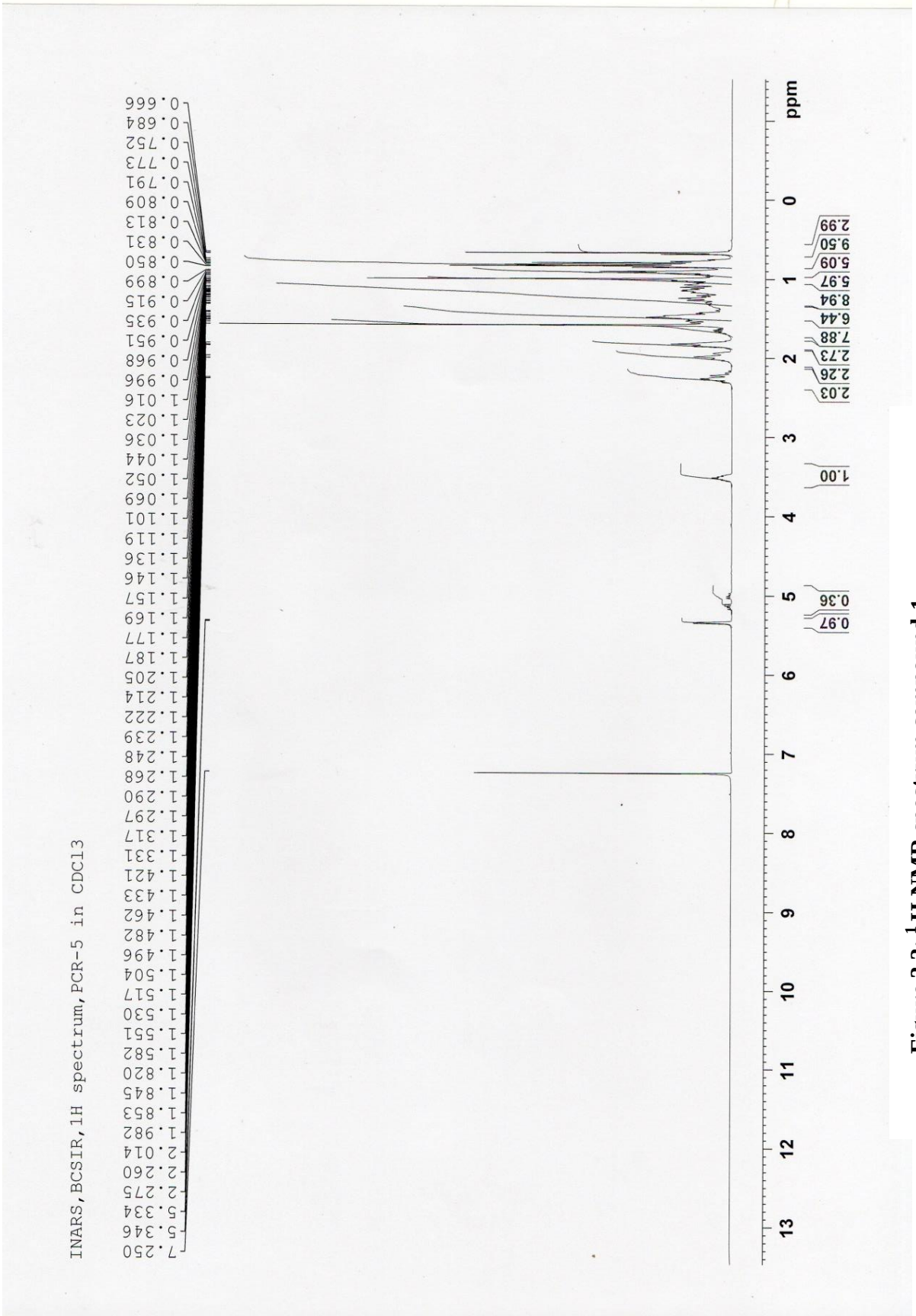


Figure 3.3: ^{1}H NMR spectrum compound-1

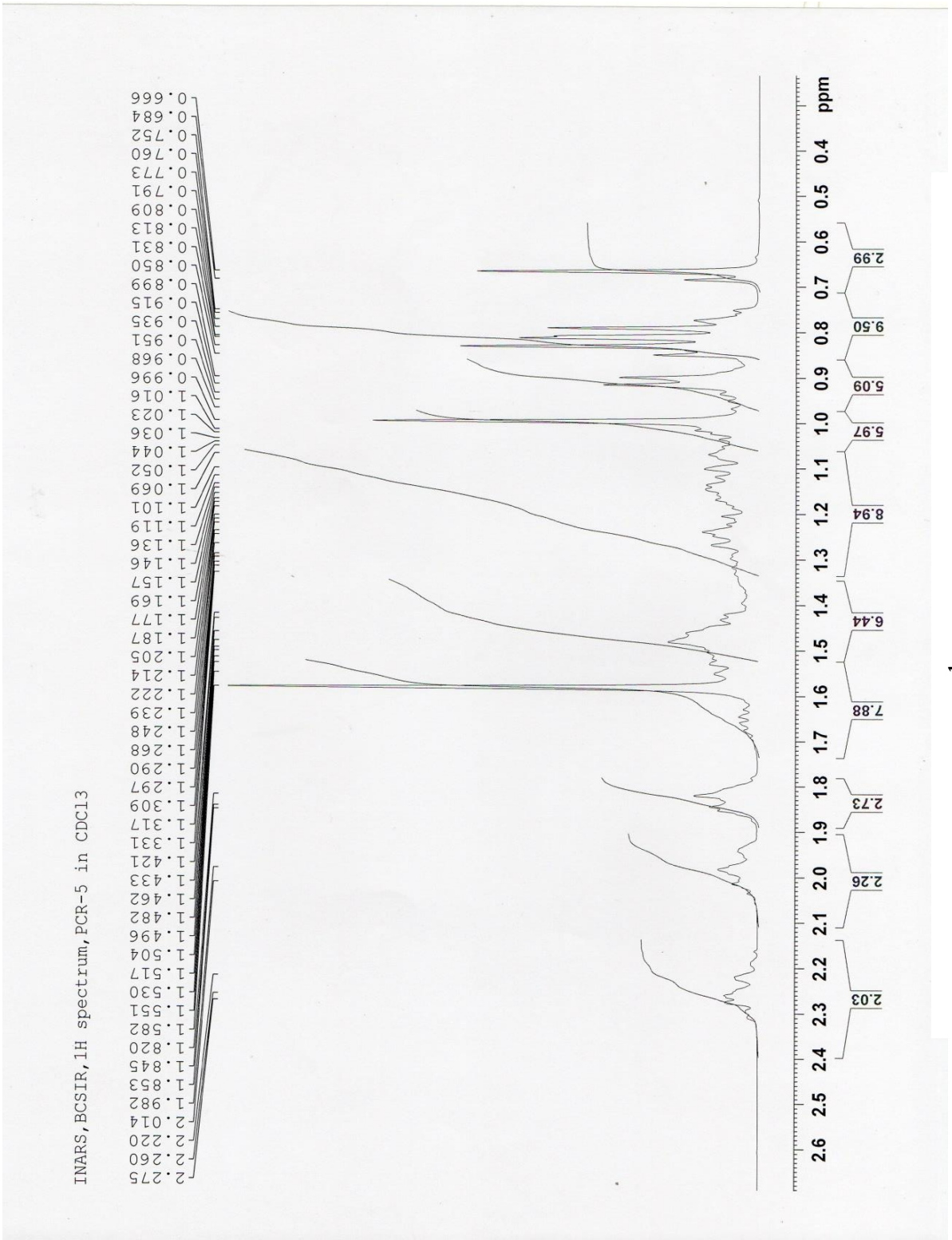


Figure 3.4: Expanded ^{1}H NMR spectrum of compound-1

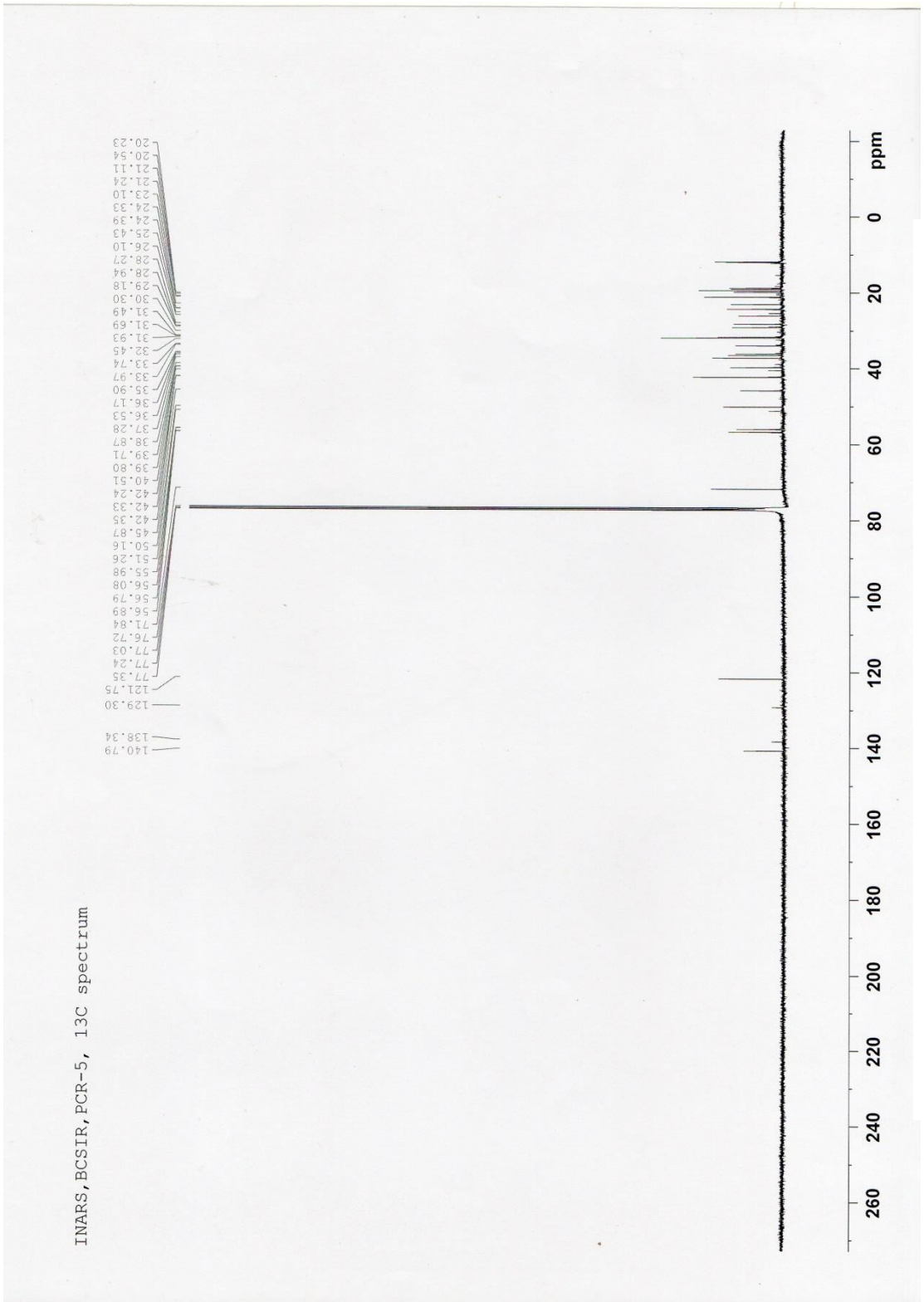


Figure 3.5: ^{13}C NMR spectrum of compound-1

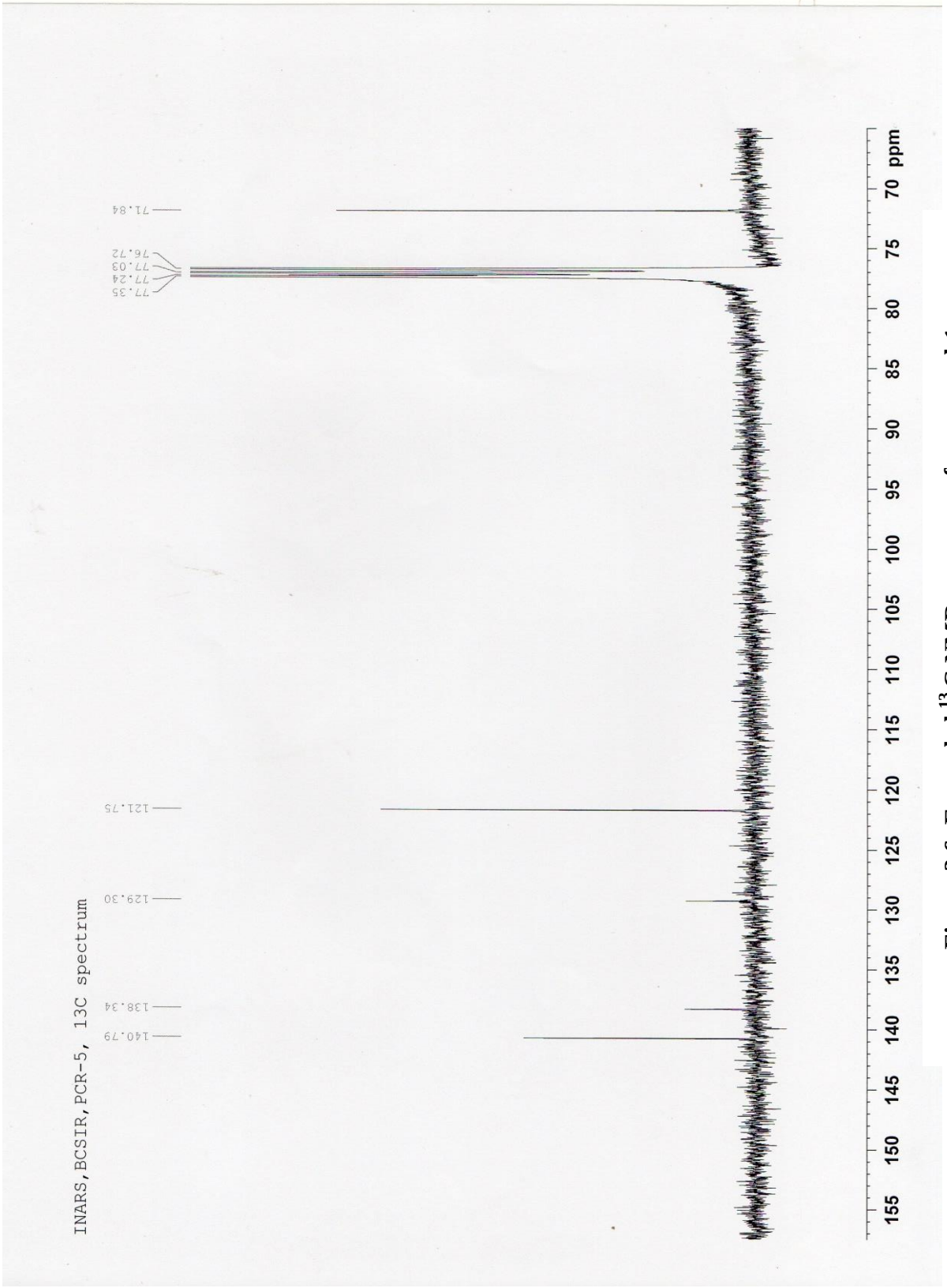


Figure 3.6: Expanded ^{13}C NMR spectrum of compound-1

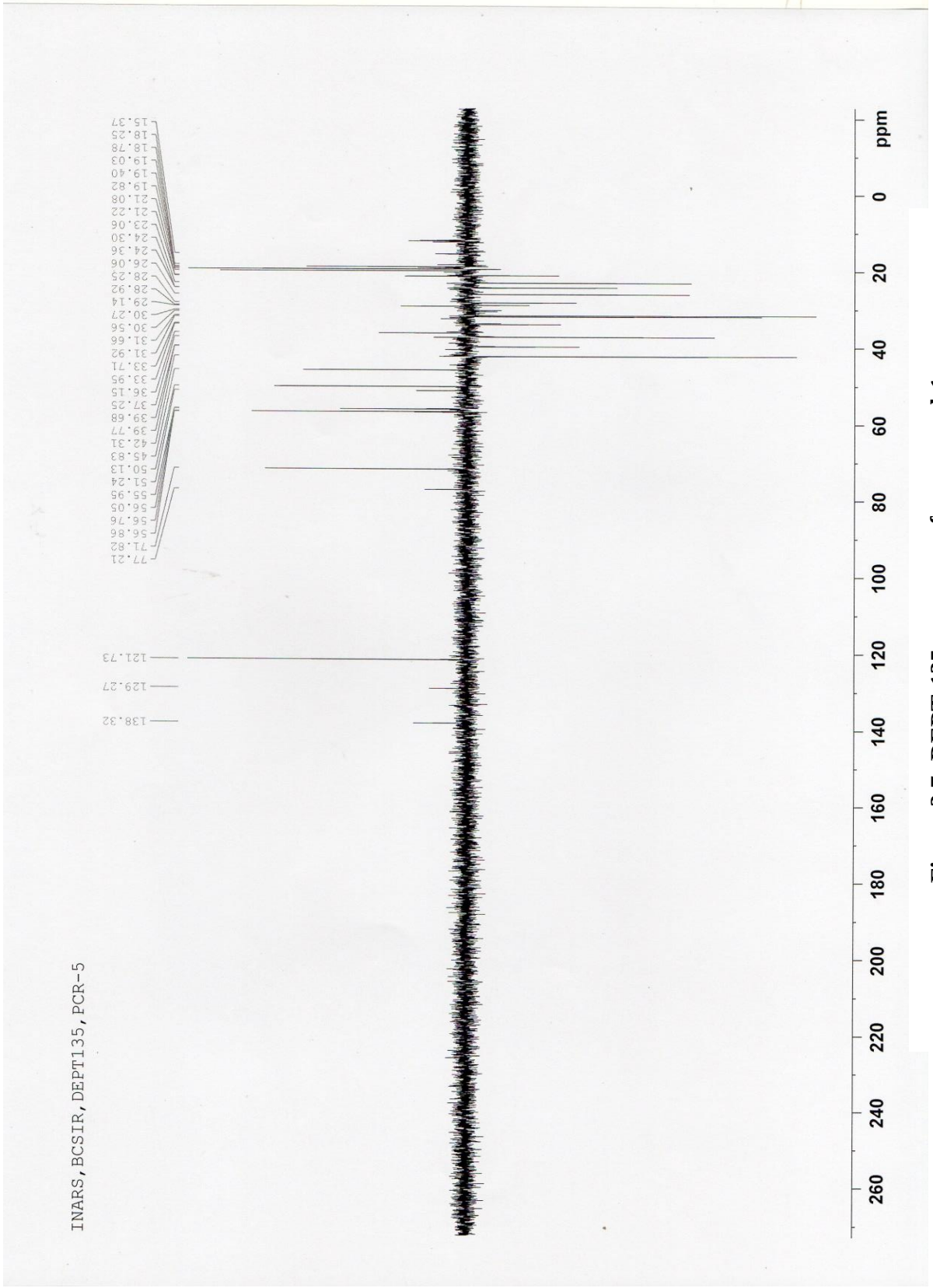


Figure 3.7: DEPT-135 spectrum of compound-1

The IR, ^{1}H NMR and ^{13}C-NMR data of the **compound-1** compared with the published data **[Ref. 10] of stigmasterol,** the structure **compound-1** was suggested as-

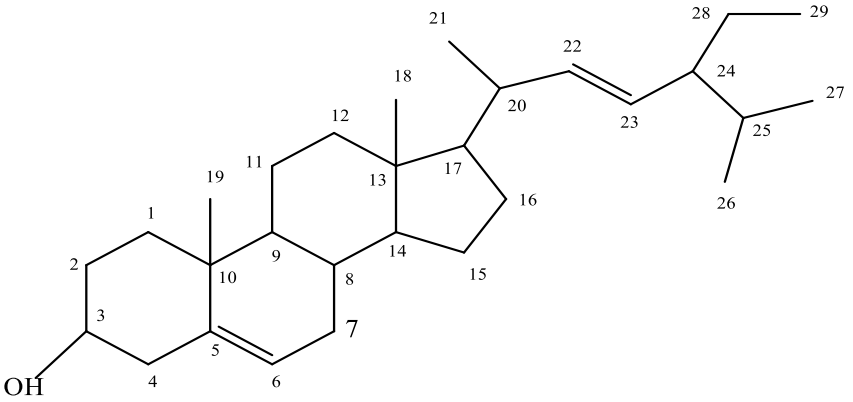


Figure 3.8: Structure of compound-1 (Stigmasterol)

3.1.2. Characterization of compound -2

The compound-2 (~24.0 mg) was a white crystalline solid having R_f value: 0.63 (20% EtOAc in dichloromethane) and its melting point was found to be 128-130^{o}C. It was soluble in dichloromethane. On spraying with vanillin-sulfuric acid spray reagent, followed by heating at 105°C for several minutes, purple color appeared.

The IR spectrum (in KBr) of the compound-2 showed the absorption at 1636 cm^{-1} for the presence of carbonyl group, at 1540 cm^{-1} due to the C=C stretching. The IR spectrum also showed absorption at 2993, 2855 and 1193 cm^{-1} for aromatic C-H stretching and at 922 cm^{-1} for exocyclic methylene.

The ^{1}H-NMR (400 MHz, in $CDCl_3$) spectrum of compound-2 showed different chemical shift for respective protons. The peak at δ 5.9 ppm and at δ 3.5 ppm indicated the presence of a heteroatom bearing methylene and piperidine ring proton. The peak at δ 6.4 (J= 14.7 Hz) and 7.3 ppm (J= 8.3 Hz) for aromatic protons. The other signals at δ 1.5 and at 1.6 and 1.6 ppm for methylene protons.

The ^{13}C-NMR spectrum (100 MHz, in $CDCl_3$) of the compound-2 showed the signal at δ 165.4 ppm due to carbonyl carbon, at δ 105.6, 108.4 and 122.4 ppm due to aromatic carbons. The signal at δ 101.2 ppm confirmed the presence of a methylenedioxy (CH_2-O-CH_2) group in the molecules. The other signals at δ 43.2, 26.7, 24.6, 25.6 and 46.8 ppm suggested the presence of piperdine ring carbons and the signals at δ 119.96, 125.37 and

138.31 for aliphatic methine carbons. These ^{1}H NMR and ^{13}C-NMR data are very close agreement with the published data **[Ref. 16]**

The ^{1}H NMR and ^{13}C NMR data of compound-2 were tabulated and compared with the published data of Piperine **[Ref. 16]** in **Table 3.1**

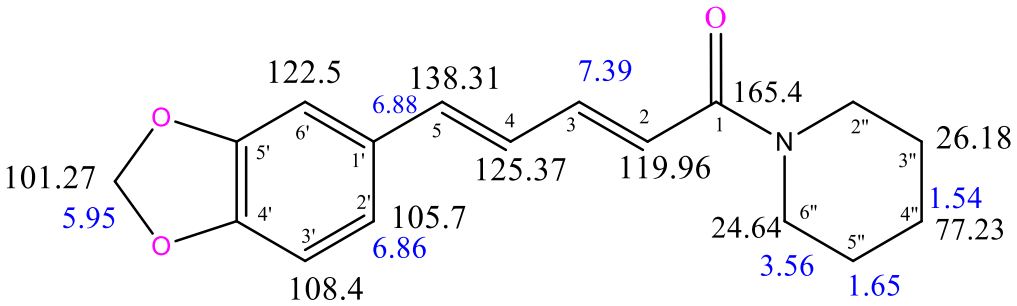


Figure 3.9: Structure of compound-2 showing ^{1}H NMR and ^{13}C NMR signals

Table 3.1: The ^{1}H and NMR ^{13}C-NMR data of compound-2 and Piperine

Position No.	**Chemical shift δ anin ppm***		**Chemical shift δ in ppm of Piperine (**Kirtiikar, K. R., and Basu, R. D. Sharma V. 1994)	
	δ_H (mult., J in Hz)	δ_C	δ_H (mult., J in Hz)	δ_C
1	-	165.47	-	165.57
2	6.38 (1H, d, J= 14.7)	119.71	6.38 (1H, d, J=14.7)	119.72
3	7.36 (1H, ddd, J=14.7, 8.3)	142.82	7.38 (1H, dd)	142.23
4	Overlapping	125.37	Overlapping	125.05
5	Overlapping	138.31	Overlapping	138.13
1′	-	131.04	-	45.82
2′	6.95, d (J=1.5)	105.62	6.95, d (J=1.5)	106.18
3′	-	148.21	-	148.22
4′	-	148.21	-	148.22
5′	Overlapping	108.49	Overlapping)	108.2
6′	6.86, ddd (J=6.0, 1.5)	122.5	6.86, ddd (J=6.0, 1.5)	121.15
methylenedioxy	5.95 s	101.27	5.95 s	101.21
2″	3.56, 1m	45.3	3.56, 1m	45.1
3″	1.54 m	26.18	1.55 m	26.17
4″	1.57 m	75.3	1.57 m	75.5
5″	1.64 m	25.36	1.64 m	25.43
6″	3.56, 2m	46.9	3.57, 2m	46.9

*^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) in $CDCl_3$

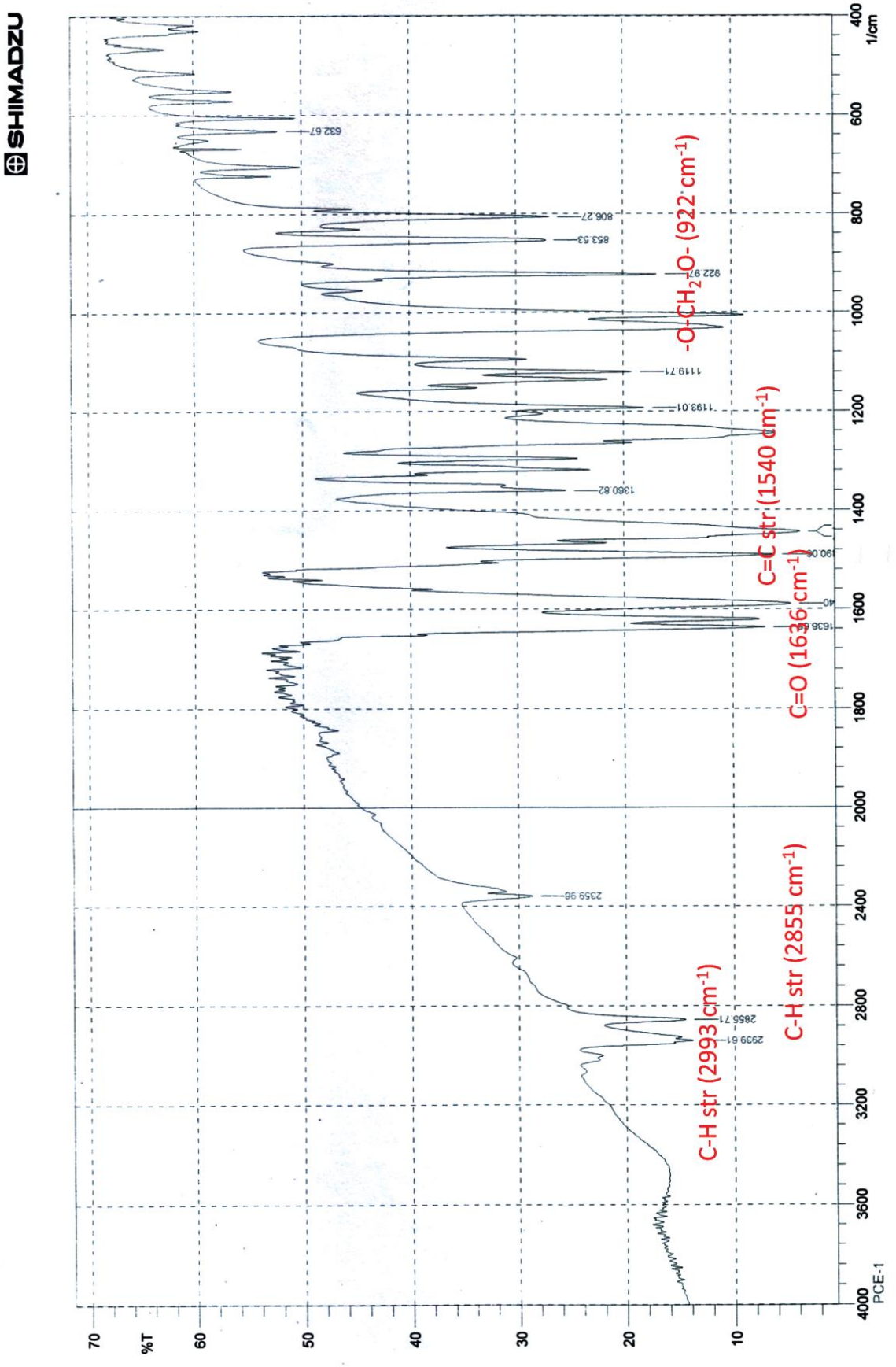


Figure 3.10: IR spectrum of compound-2

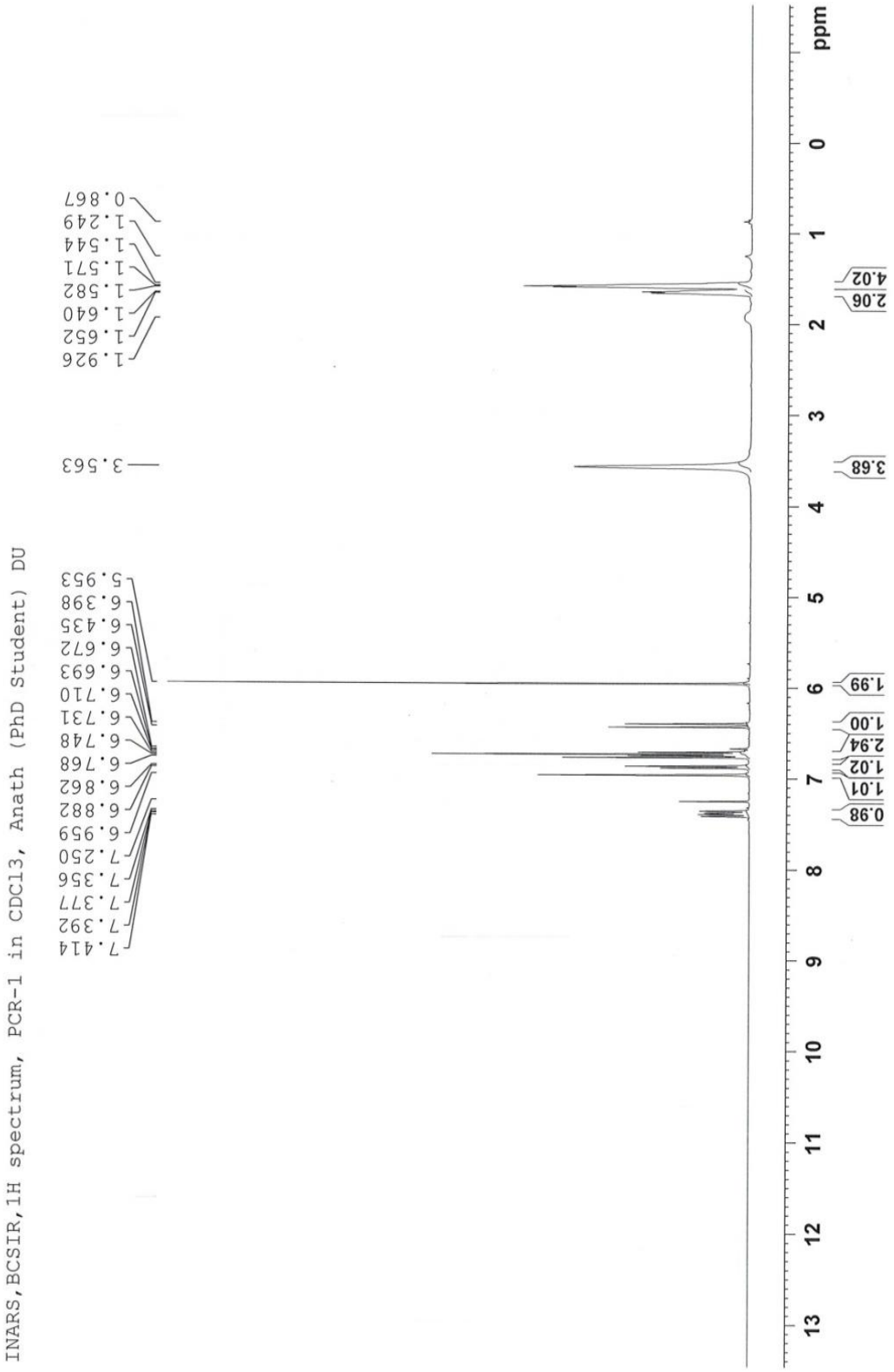


Figure 3.11: ^{1}H NMR spectrum of compound-2

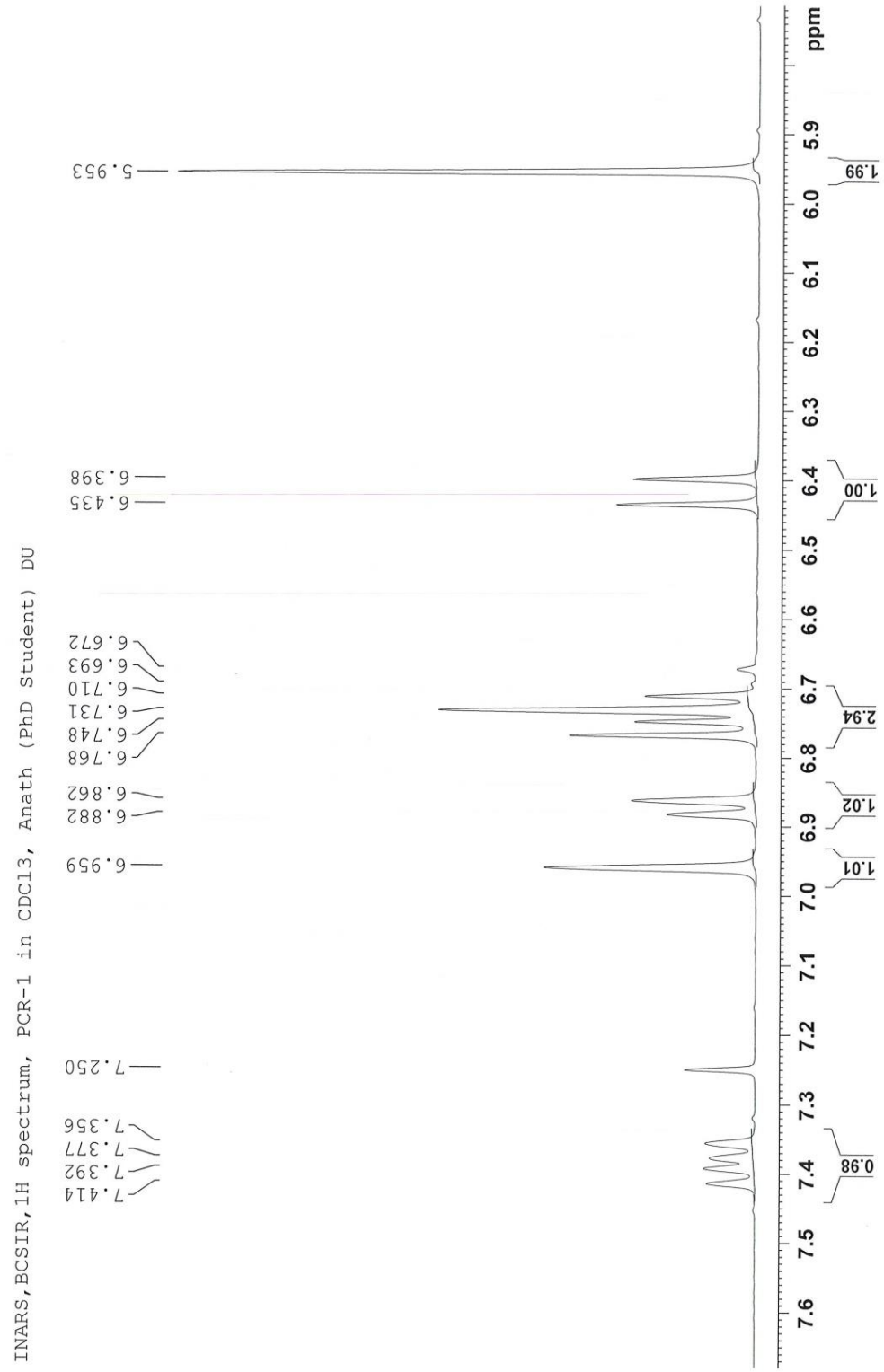


Figure 3.12: Expanded ^{1}H NMR spectrum of compound-2

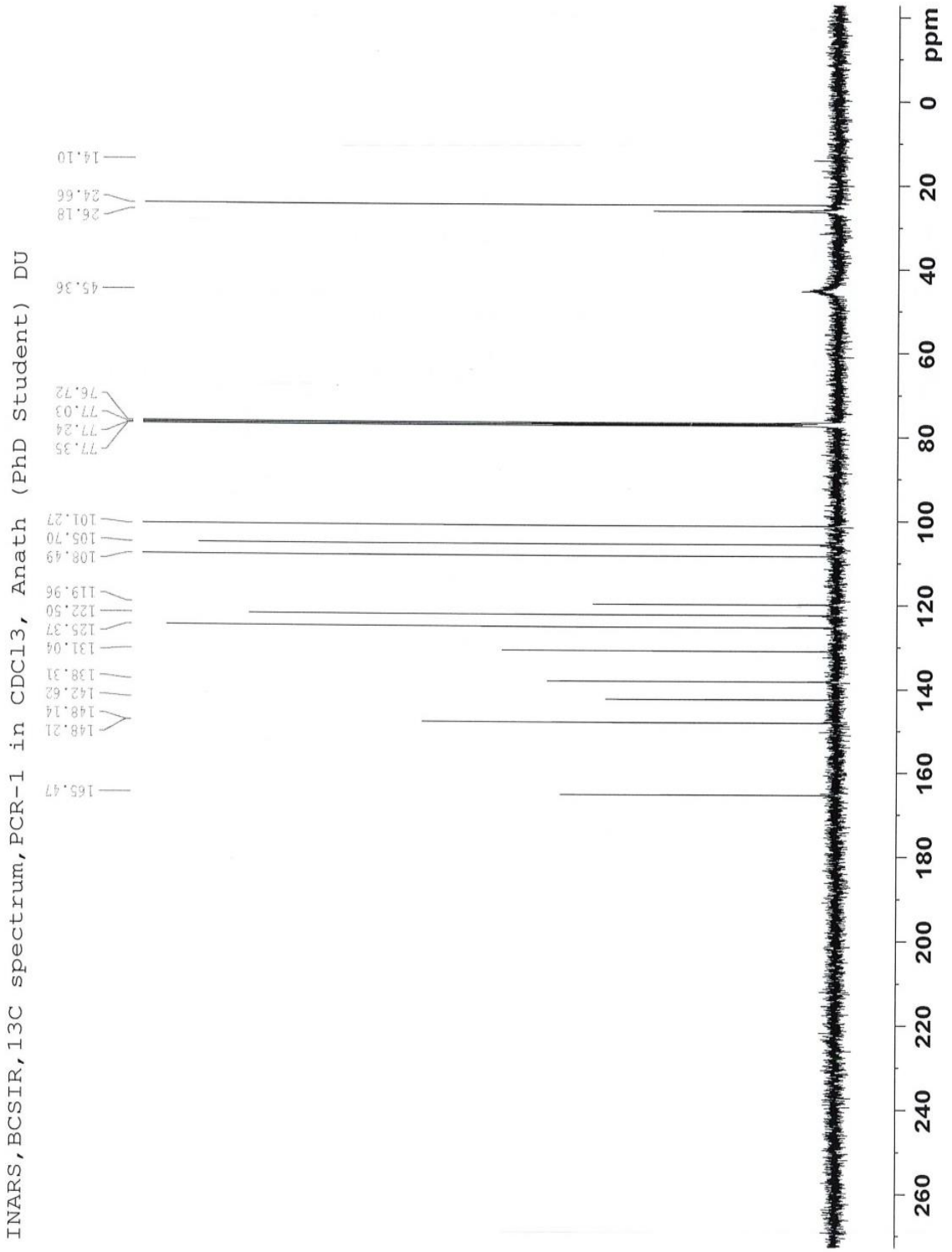


Figure 3.13: ^{13}C NMR spectrum of compound-2

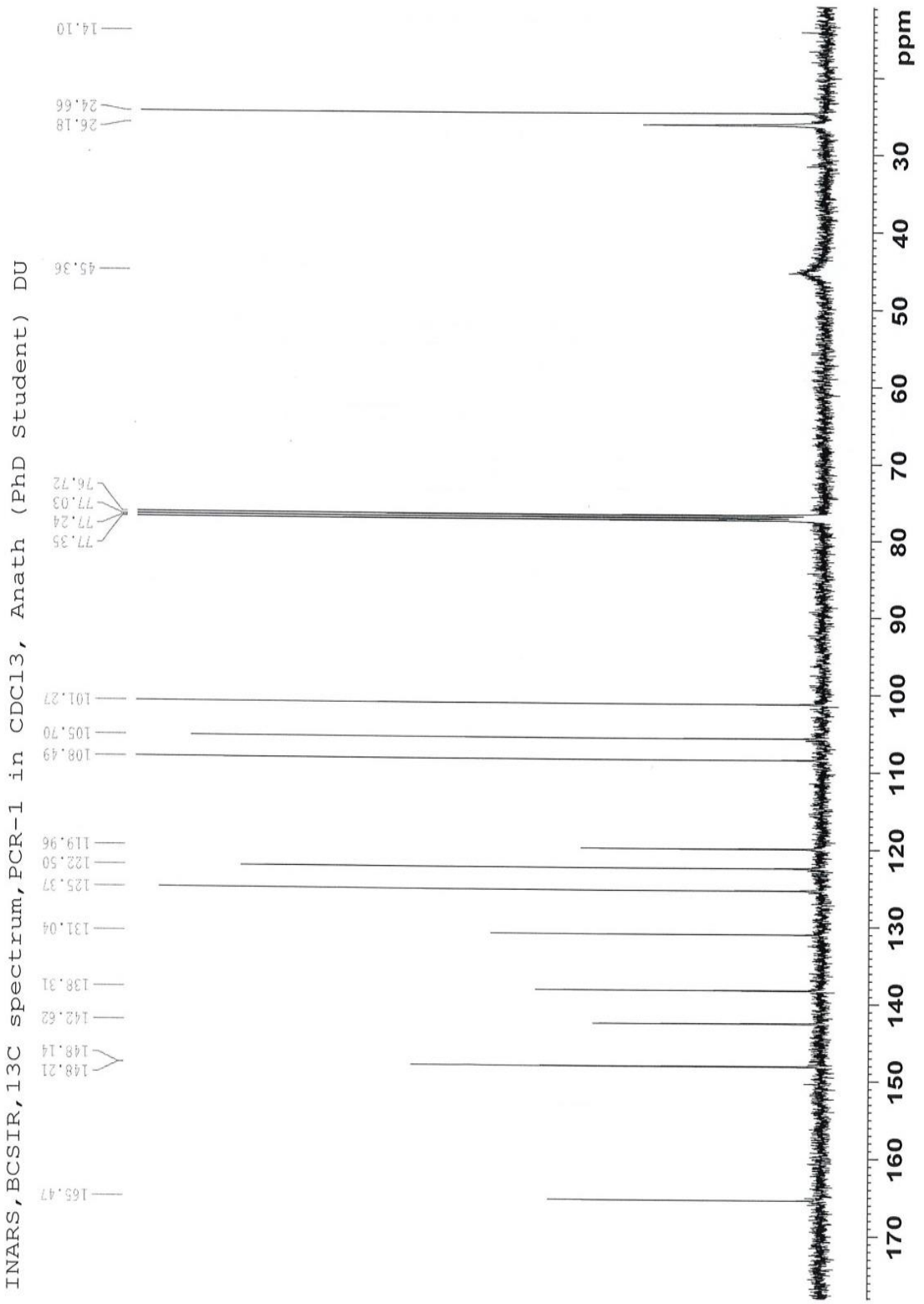


Figure 3.14: Expanded ^{13}C NMR spectrum of compound-2

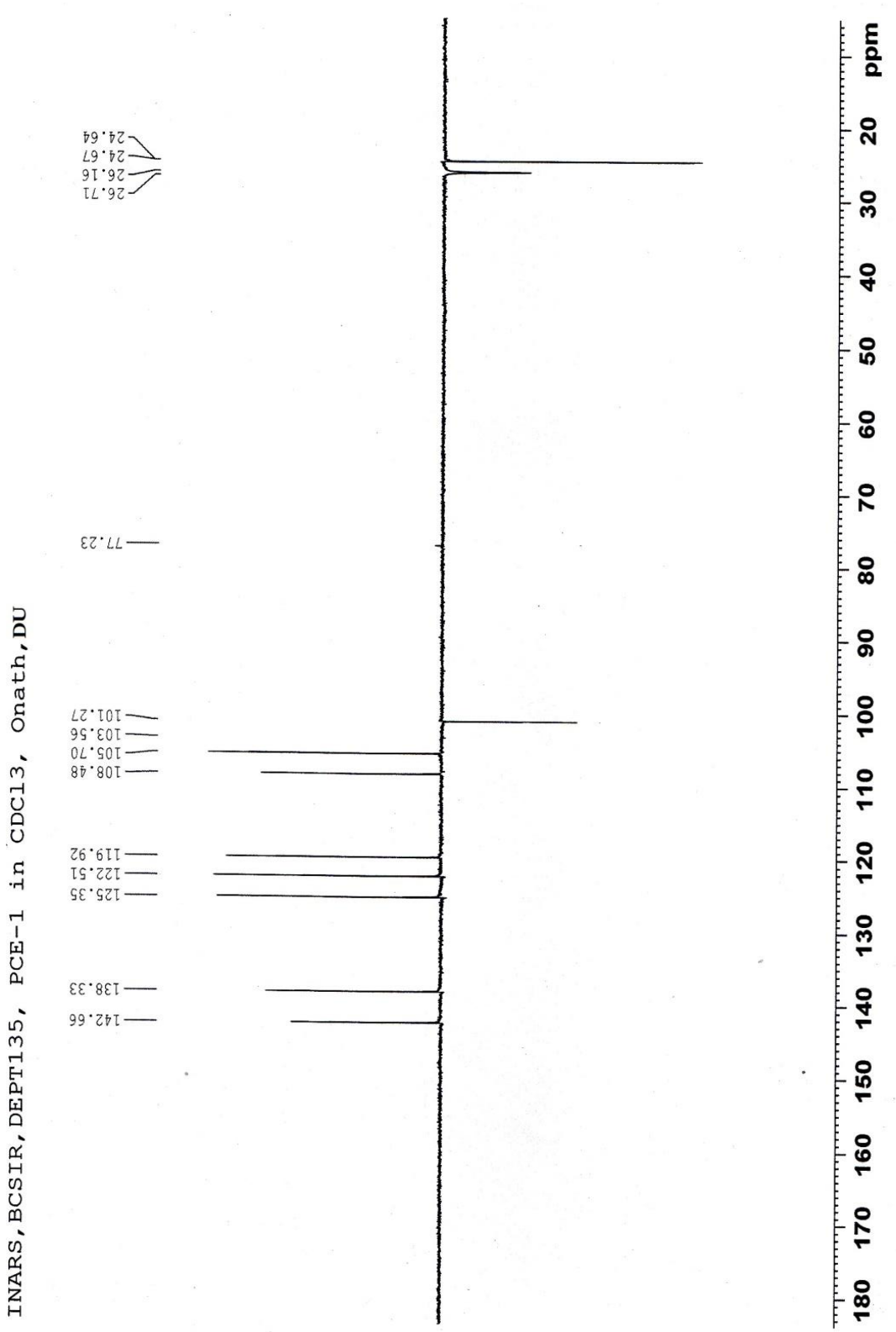


Figure 3.15: DEPT-135 spectrum of compound-2

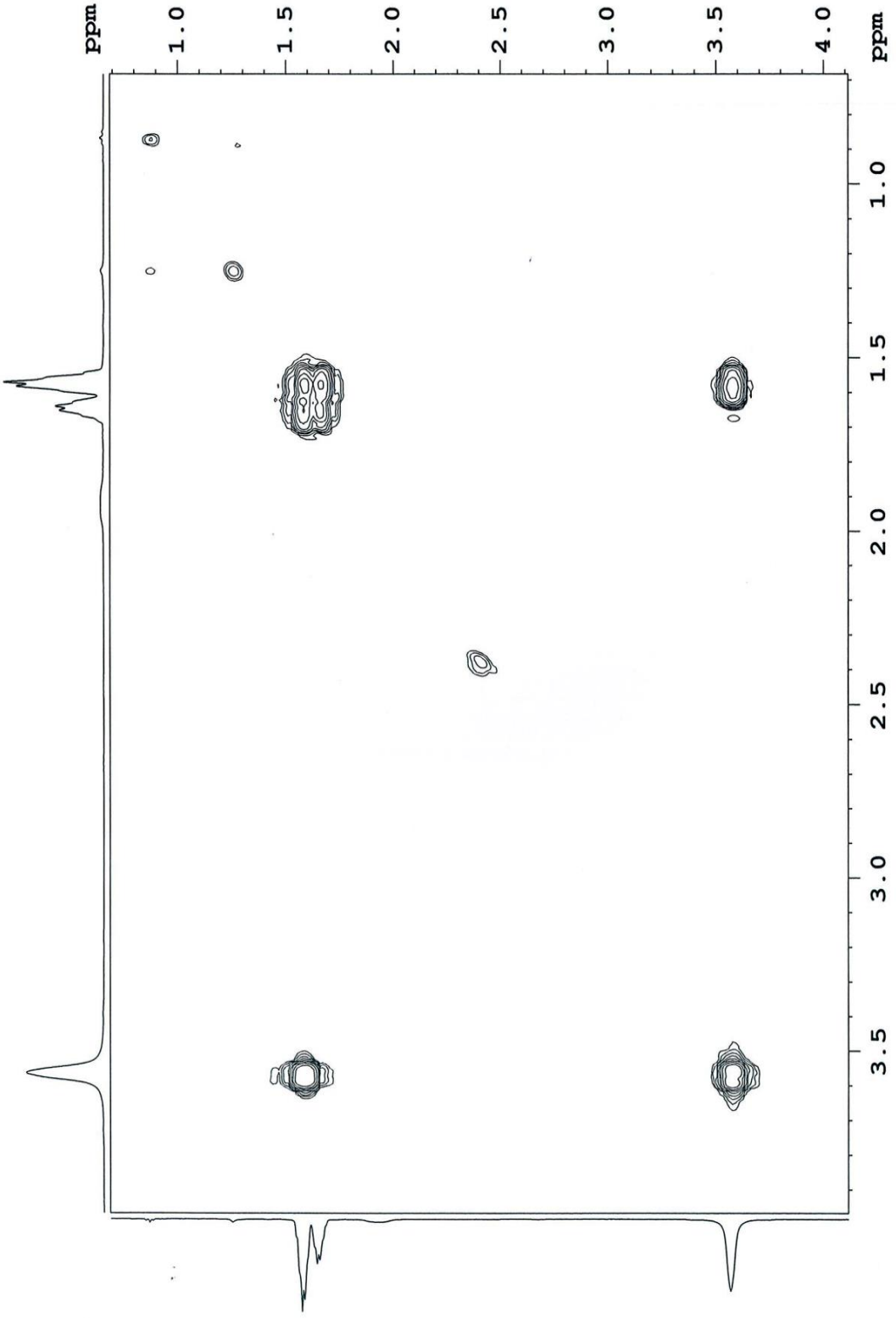


Figure 3.16: Cosy-45 spectrum of compound-2

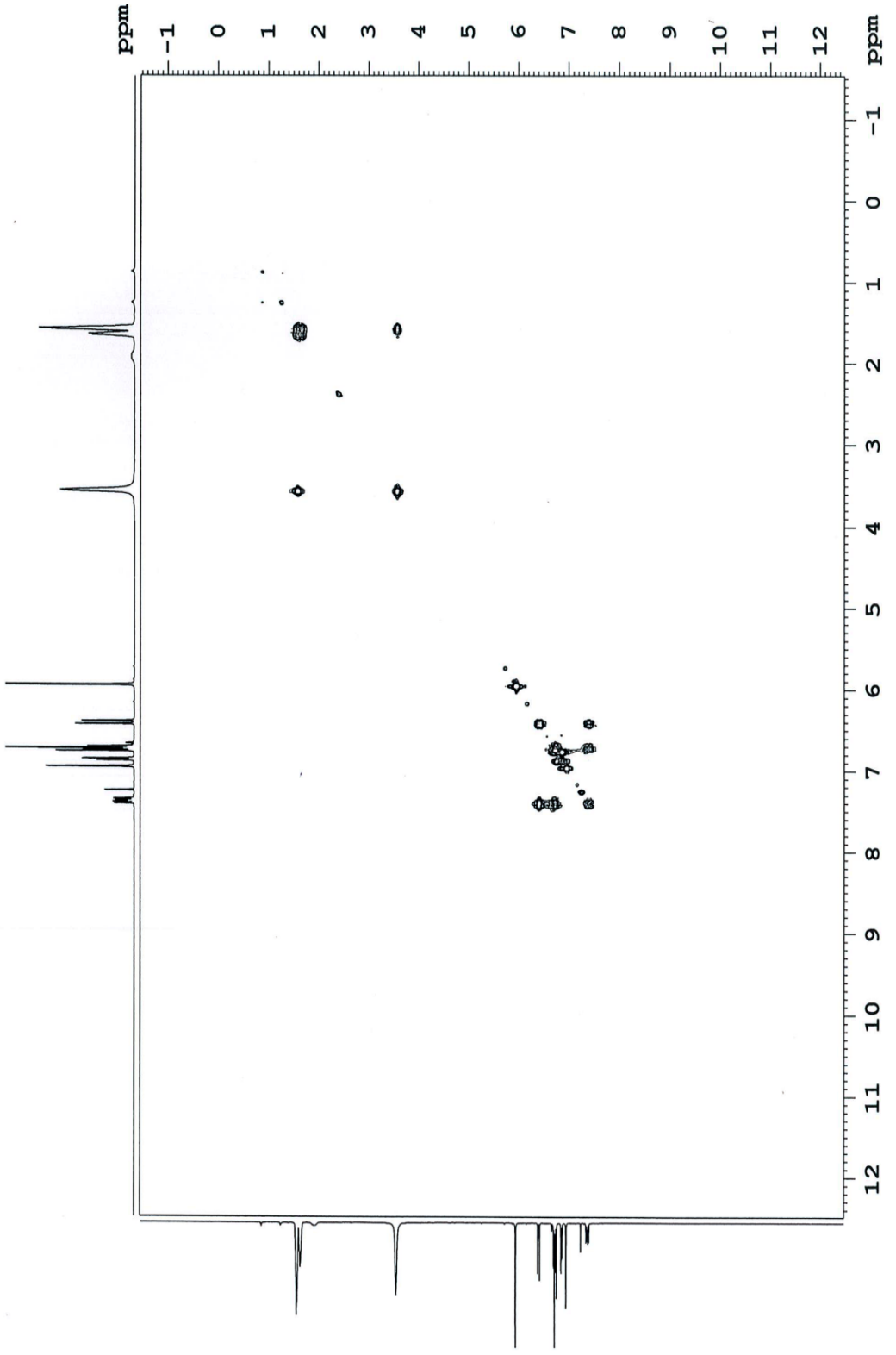


Figure 3.17: Cosy-45 spectrum of compound-2

The IR, ^{1}H-NMR and ^{13}C-NMR data of the **compound-2** compared with the published data **[Ref. 16] of Piperine,** the tentative structure **compound-2** was suggested as-

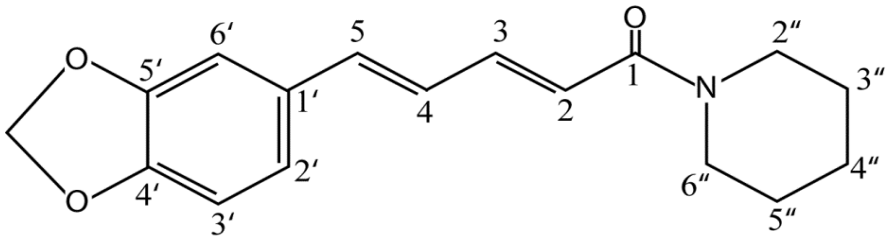


Figure 3.18: Structure of compound-2 (Piperine)

3.1.3 Characterization of compound-3

The compound-3 (~7.0 mg) was a white crystalline solid having R_f value: 0.36 (40% EtOAc in dichloromethane) and its melting point was found to be 142-143^{o}C. It was soluble in dichloromethane. On spraying with vanillin-sulfuric acid spray reagent, followed by heating at 105°C for several minutes, purple color appeared.

The IR spectrum (in KBr) of the compound-3 showed the absorption at 3178 cm^{-1} for O-H stretching, at 3032 cm^{-1} due to aromatic C-H stretching, at 1711 cm^{-1} which indicated the presence of carbonyl group and the absorption at 1490 cm^{-} for methyl group.

The ^{1}H-NMR spectrum (400 MHz, in $CDCl_3$) of the compound-3 showed the peaks at δ 0.806, 1.08 ppm due to methyl protons, A number of signals at δ 2.14-2.03 ppm (J = 11.2 Hz) and at δ 0.806 ppm due to methylene and methyl protons present in the compound, at δ 5.37 ppm for alcoholic proton flanked with tertiary carbon (C-OH), and the signals at δ 6.85 to 7.47 ppm due to aromatic ring protons.

The ^{13}C-NMR spectrum (100 MHz, in $CDCl_3$) of compound-3 showed the presence of carbonyl carbon at δ 178·5 ppm; at δ 130.27, 129.8, 165.31 and 147.96 for tertiary carbons; at δ 60.08, 53.7 for methylene and methine carbons; at δ 129.2, 128.9 for aromatic C-H carbons. The significant chemical shift at δ 14.1, 18.7 for methyl carbons present in this compound. These ^{1}H NMR and ^{13}C-NMR data are very close agreement with the published data **[Ref. 18]**

The ^{1}H NMR and ^{13}C NMR data of compound-3 were tabulated and compared with the published data of **5, 6-dihydroxy- 7, 8-dimethyl isoflavan-4-one [Ref. 18]** in **Table 3.3**

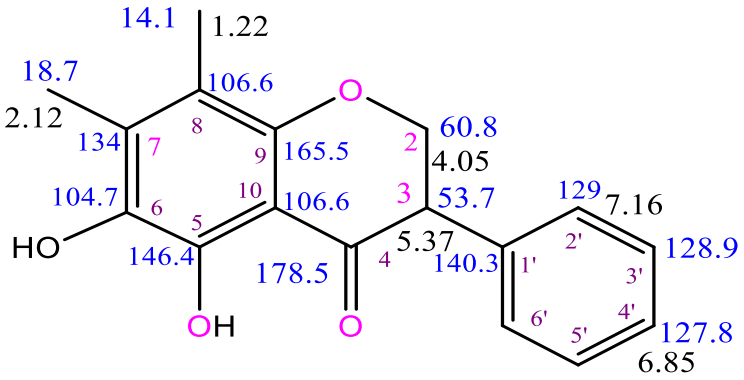


Figure 3.19: Structure of compound-3 showing ^{1}H-NMR and ^{13}C-NMR signals

Table 3.2: **The ^{1}H- NMR and ^{13}C-NMR data of compound-3 and 5, 6-dihydroxy- 7, 8-dimethyl isoflavan-4-one [Ref. 18]**

Position No.	Chemical shift δ in ppm*		Chemical shift δ in ppm of isoflavan **(**Kumar, R. A., Rao, K., Kumar, N. V., Nanduri, S. and Rajagopal, S. (2004)	
	δ_H (mult., J in Hz)	δ_C	δ_H (mult., J in Hz)	δ_C
2	4.05 (2H, m)	60.08	4.01 (2H, m)	60.6
3	5.37 (1H, s)	53.7	5.81 (1H, s)	51.7
4	-	178.5	-	178.2
5	-	146.4	-	145.0
6	-	140.7	-	104.1
7	-	134.0	-	134.8
8		106.6	-	107.2
9	-	165.5	-	165.4
10	-	106.6	-	105.3
1′	-	140.3	-	138.2
2′, 6′	7.16 (2H, m)	129.2	7.15 (2H, m)	130.5
3′, 5′	7.45 (2H, m)	128.9	7.35 (2H, m)	128.2
4′	6.85(1H, m)	127.8	6.72 (1H, m)	127.0
CH_3 at C-7	2.12 (3H, m)	18.7	2.11 (3H, m)	18.6
CH_3 at C-8	1.22 (3H, m)	14.1	2.22 (3H, s)	14.3

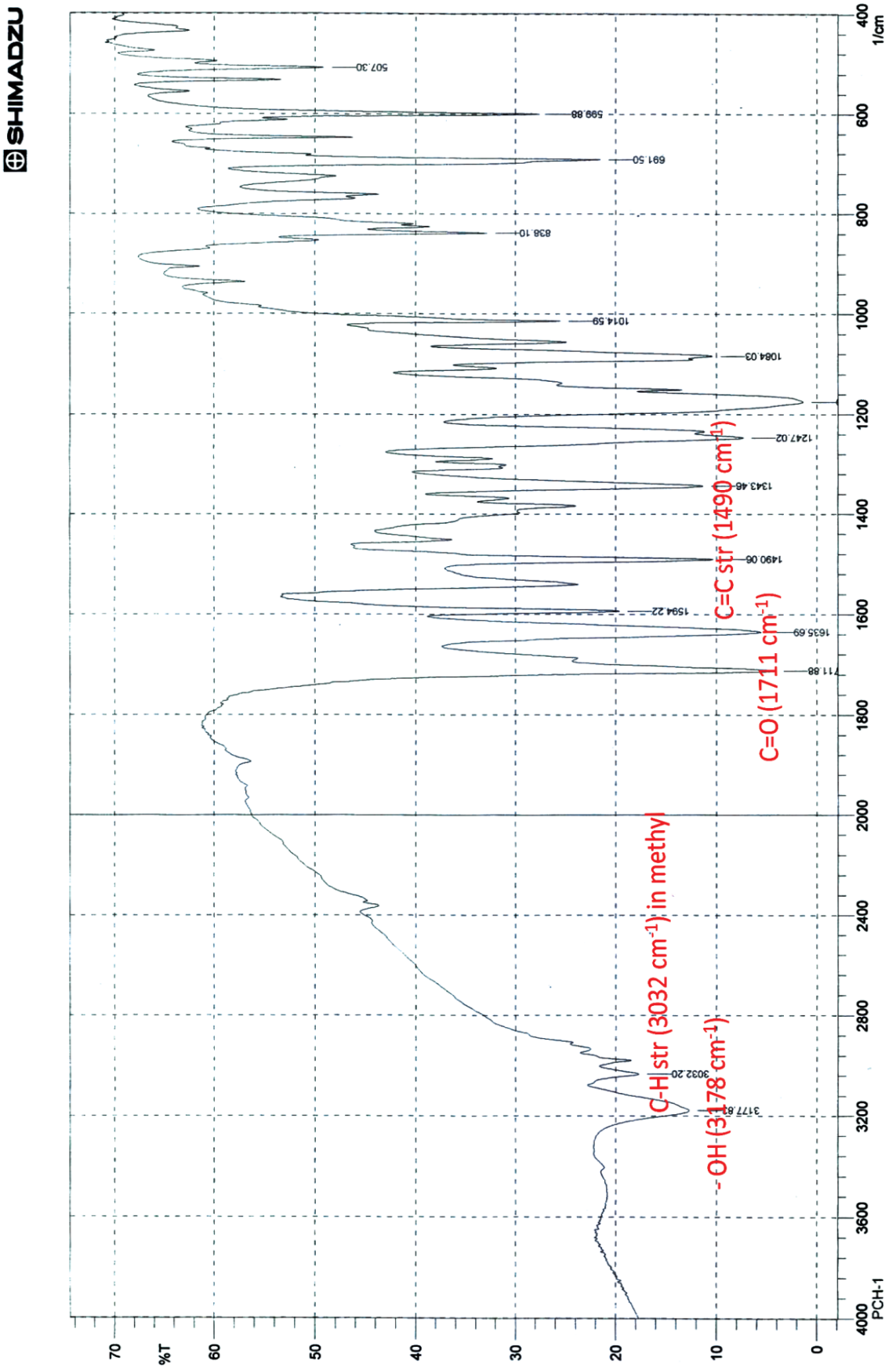


Figure 3.20: IR spectrum of compound-3

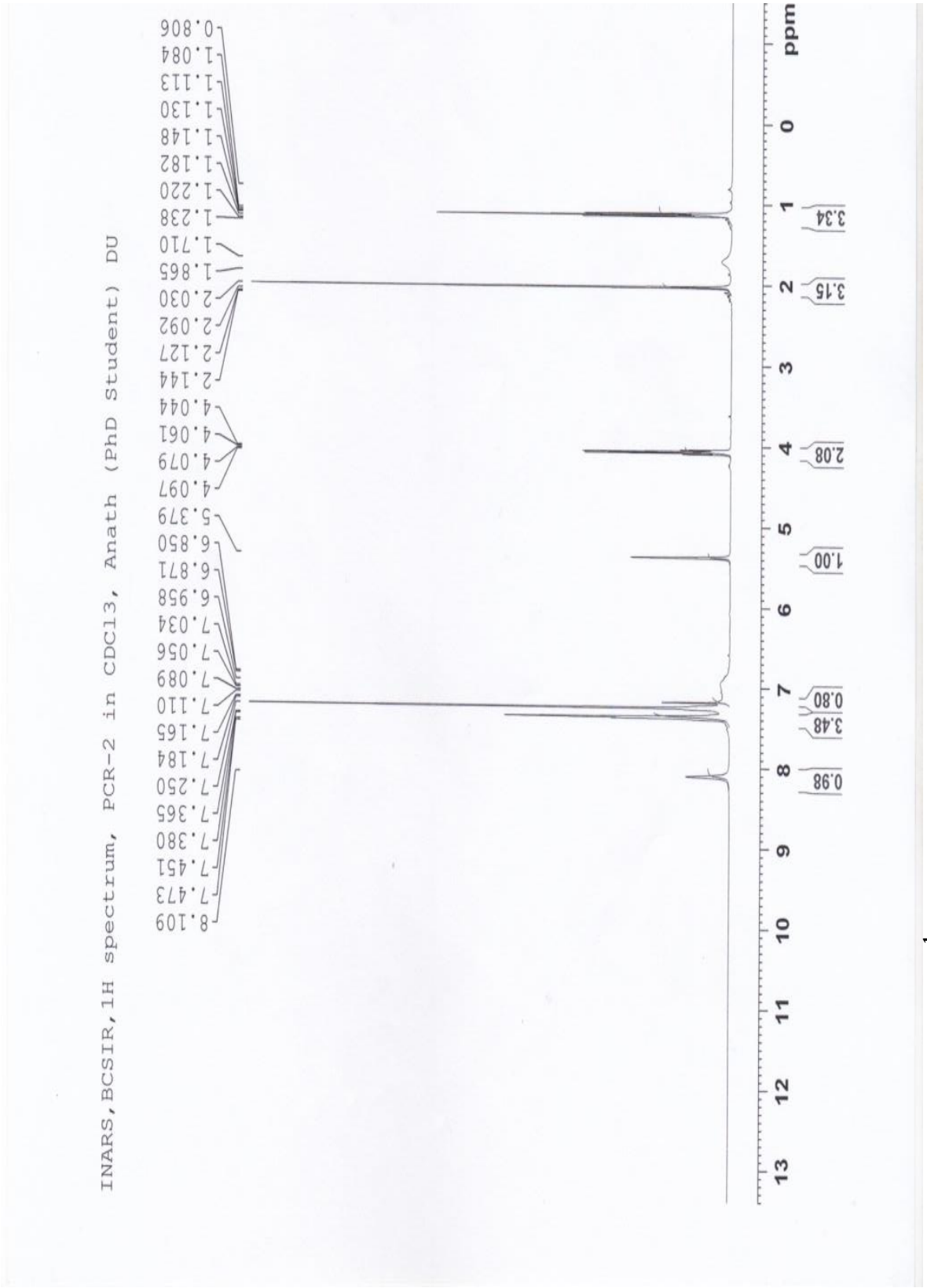


Figure 3.21: ^{1}H NMR spectrum of compund-3

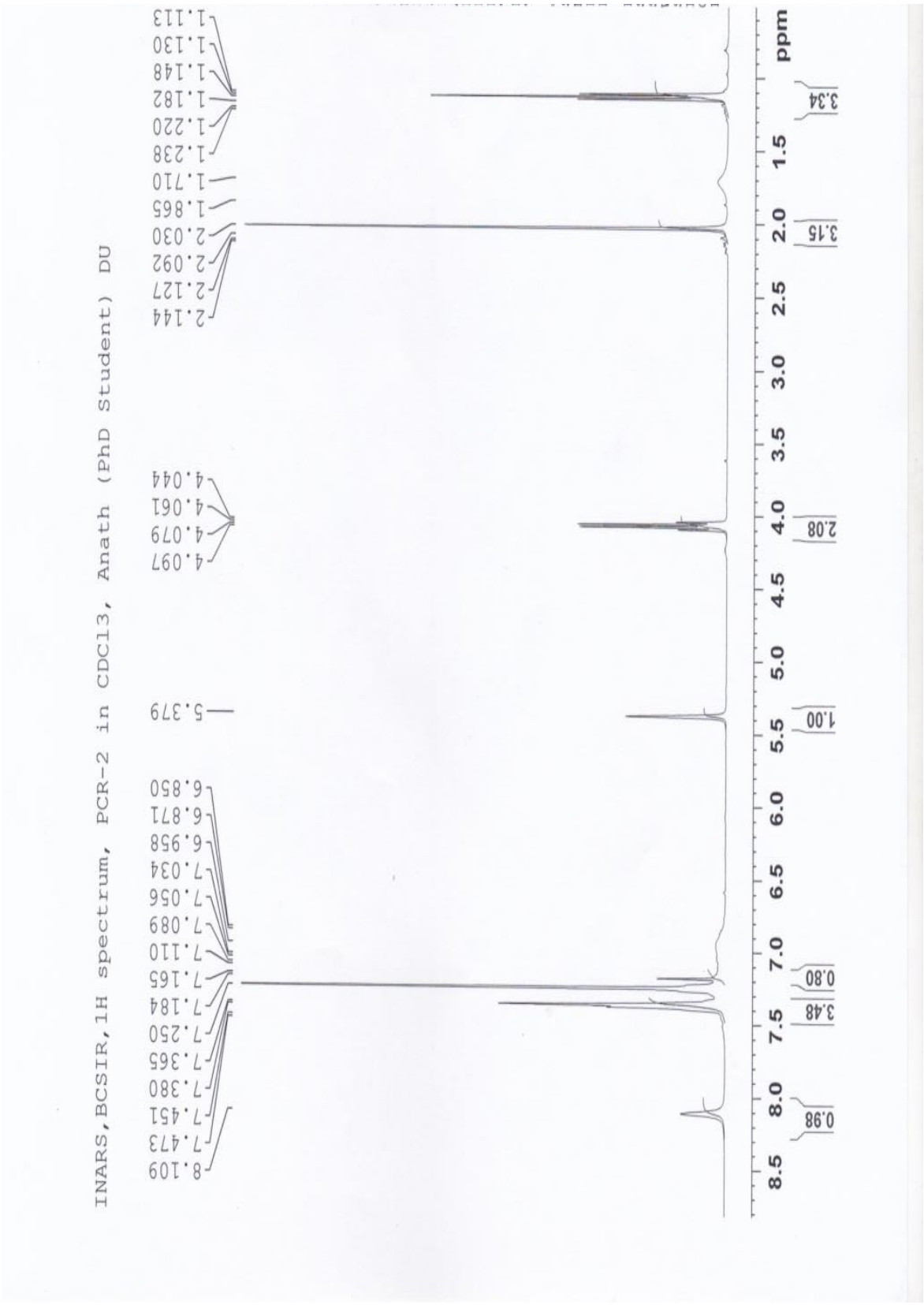


Figure 3.22: Expanded ^{1}H NMR spectrum of compund-3

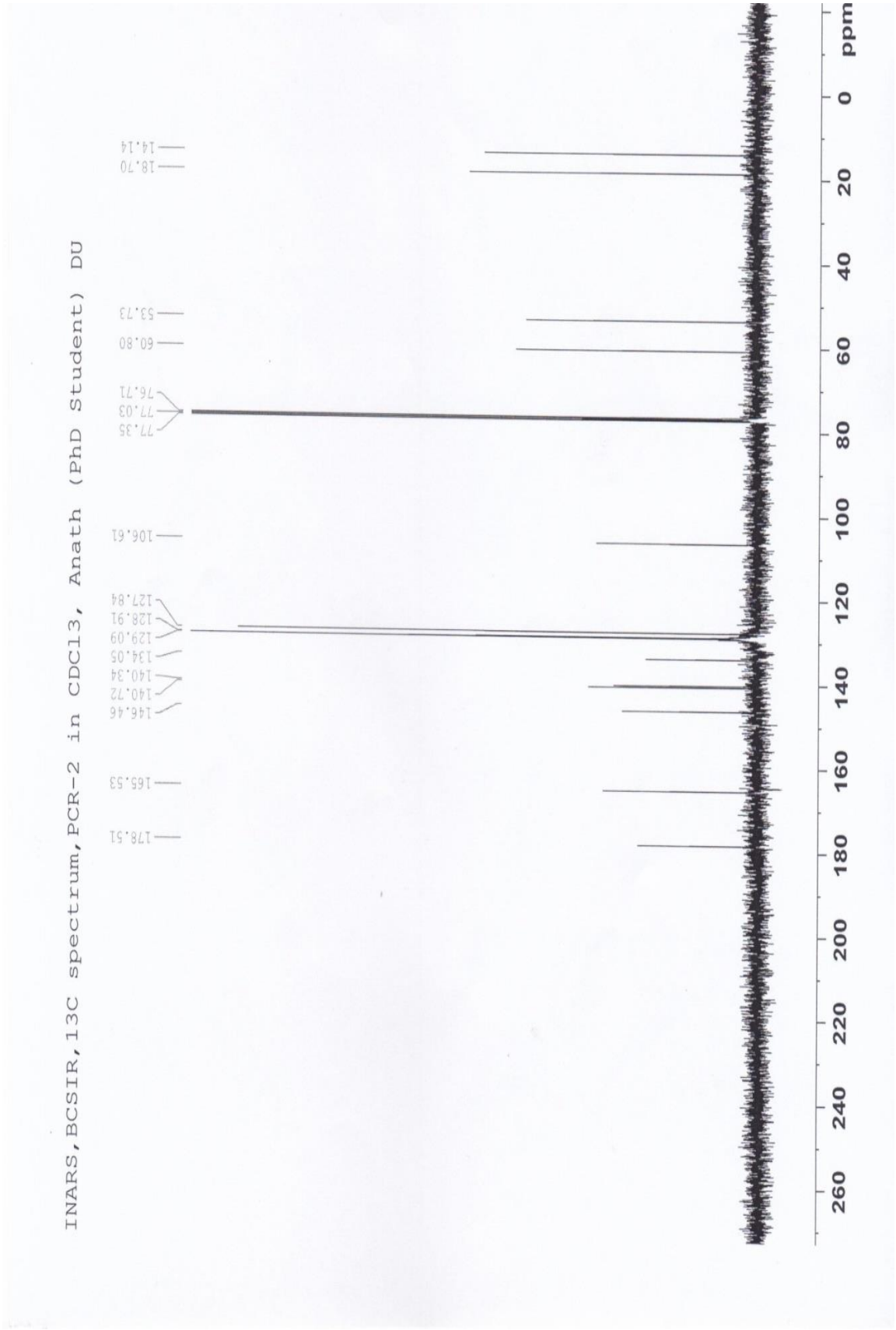


Figure 3.23: ^{13}C NMR spectrum of compund-3

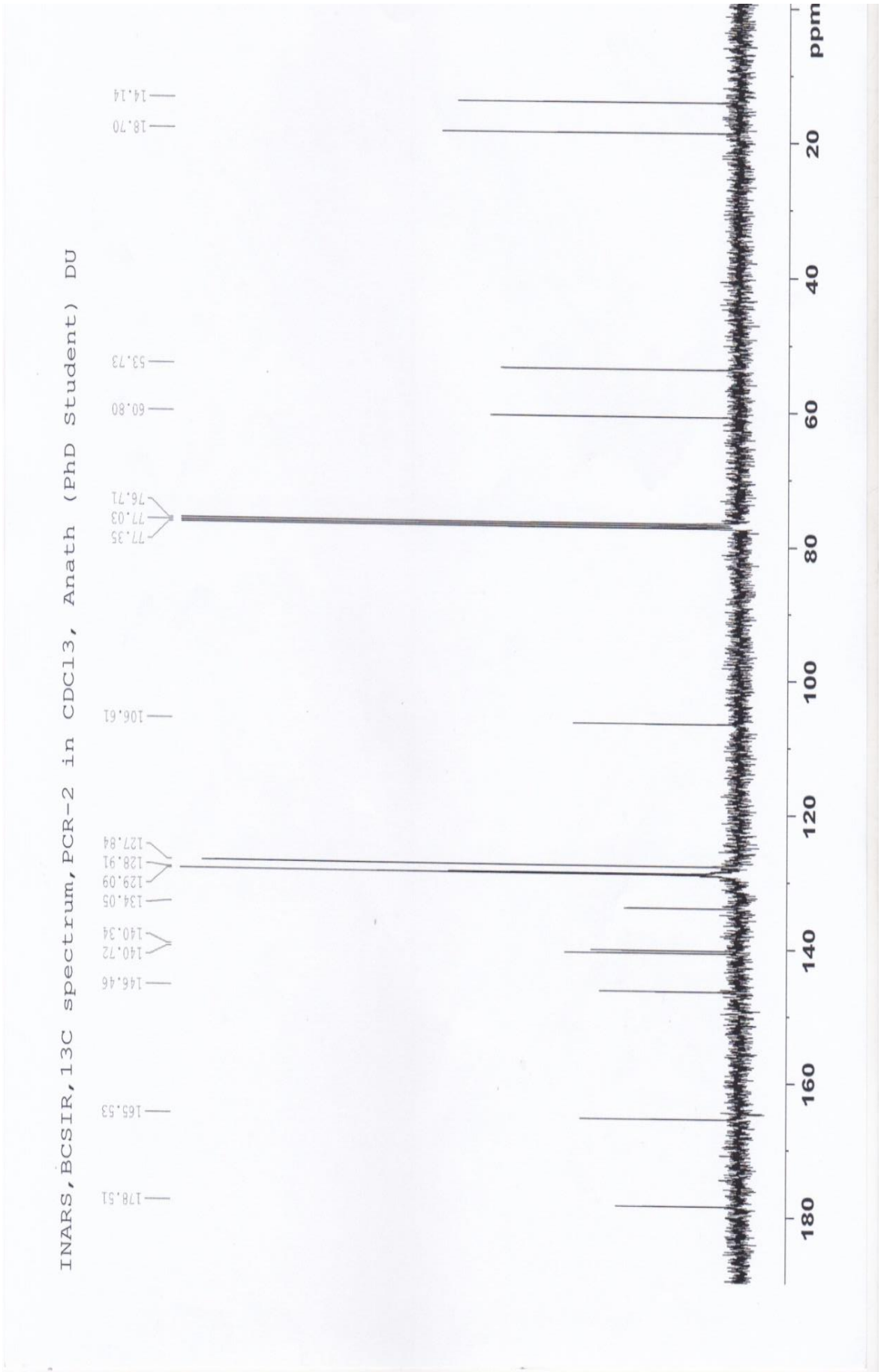


Figure 3.24: Expanded ^{13}C NMR spectrum of compund-3

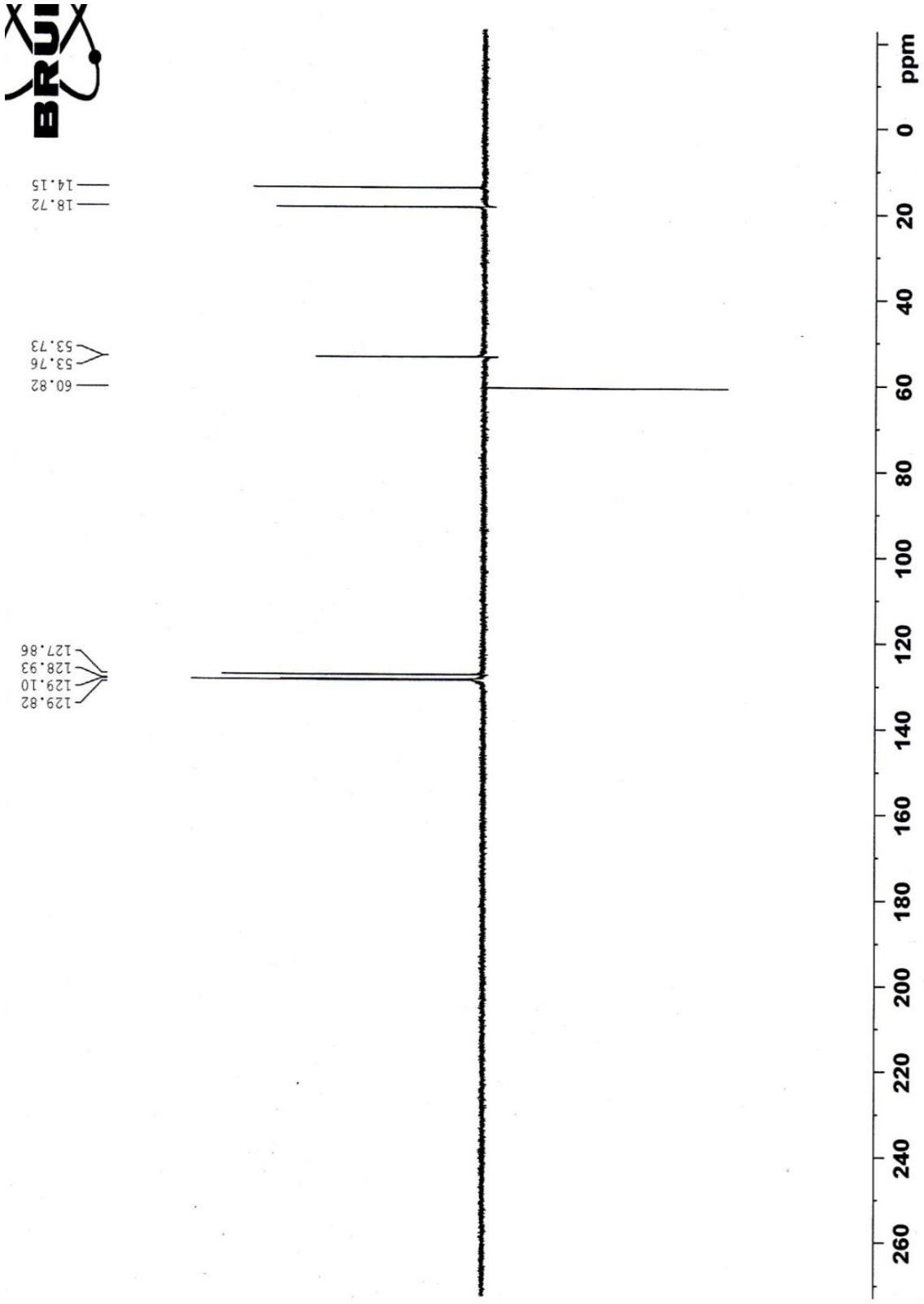


Figure 3.25: DEPT-135 spectrum of compund-3

The IR, ^{1}H-NMR and ^{13}C-NMR data of the **compound-3** compared with the published data **[Ref. 18] 5, 6-dihydroxy- 7, 8-dimethyl isoflavan-4-one.,** the tentative structure **compound-3** was suggested as-

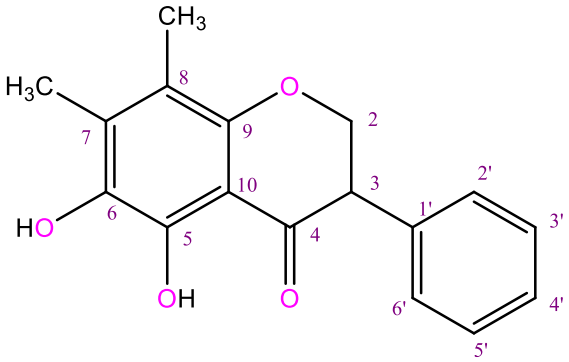


Figure 3.26: Structure of compound-3 (5, 6-dihydroxy-7, 8-dimethyl isoflavan-4-one)

3.1.4 Characterization of compound-4

The compound-4 (~7.5 mg) was a white crystalline solid having R_f value: 0.51 (5% EtOAc in dichloromethane). Its melting point, molecular formula and molecular weight was found to be 112-113^{o}C, $C_{16}H_{13}O_2Cl$ and (M^+ 273). It was soluble in dichloromethane. On spraying with vanillin-sulfuric acid spray reagent, followed by heating at 105°C for several minutes, purple color appeared.

The IR spectrum (in KBr) of the compound-4 indicates the absorption band at 2938 cm^{-1} for aromatic C-H stretching, at 1711 cm^{-1} due to for carbonyl group, at 1512 cm^{-1} for C-H bending due to methyl group and absorption at 1217 cm^{-1} for C-H bending due to methylene group.

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of the compound-4 showed the chemical shift at 6.93 and 7.95 ppm for aromatic protons, at δ 7.36 (6H, d, J= 15.6 Hz), 7.78 (1H, J=15.6 Hz) for methine protons and the significant chemical shift at δ 3.85 (s) indicates the compound contains methoxy proton.

The ^{13}C-NMR spectrum (100 MHz, $CDCl_3$) of the compound-4 showed the signals at δ 189.29 for carbonyl carbon, at δ 130.39, 114.50, 148.69, 129.87 and 128.91 for aromatic carbons, at δ 161.88, 127.45, 136.82 and 138.82 for tertiary carbons. The significant chemical shift at δ 55.48 for methoxy carbon and at δ 119.17, 145.29 for two unsaturated carbons.

The HSQC experiment presented that the protons at δ 7.36, 7.78, 7.60, 6.93, 7.95, 7.46 and 3.85 are directly attached to the carbons at δ 119.17, 145.29, 130.39, 114.50, 129.87, 128.91 and 55.48 respectively.

The long-range proton-carbon coupling results of the HMBC testing are shown in **Figure 3.27** is of the supporting information. The proton at δ 6.93 showed strong long-range coupling to carbons 4′ (δ 161.88), and 1′ (δ 127.45). The proton at δ 7.36 showed strong long-range coupling to carbons 1 (δ 189.28) and 1′ (δ 127.45), at δ 7.78 strong long-range coupling to carbons 1 (δ 189.28) and 2′ (δ 130.39) and the proton at δ 7.60 showed strong long-range coupling to carbons 3 (δ 145.29) and 4′ (δ 161.88). The penetrating cross-peaks amongst the proton at δ 7.95 and 7.46 to carbons at δ 4′′ (138.99) and 1′′(136.82). The other HMBC (H $\rightarrow$C) correlations were revealed in the **Figure 3.27** which supported the given structure of the compound-4.

From the ESI-MS spectrum of the compound-4 the peaks at m/z, 273 and 296 were due to $[M+H]^+$ and $[M+Na]^+$ respectively. The m/z values at 273 and 275 with an approximate intensity of 3:1 confirmed the presence of Cl atom in the parent compound. Thus, the molecular weight of compound-4 is 273 which were correctly matched with the calculated value of the molecular weight of the compound-4. Ref. [25]

The ^{1}H NMR, ^{13}C NMR, HSQC and HMBC data of compound-4 were tabulated in **Table 3.3**

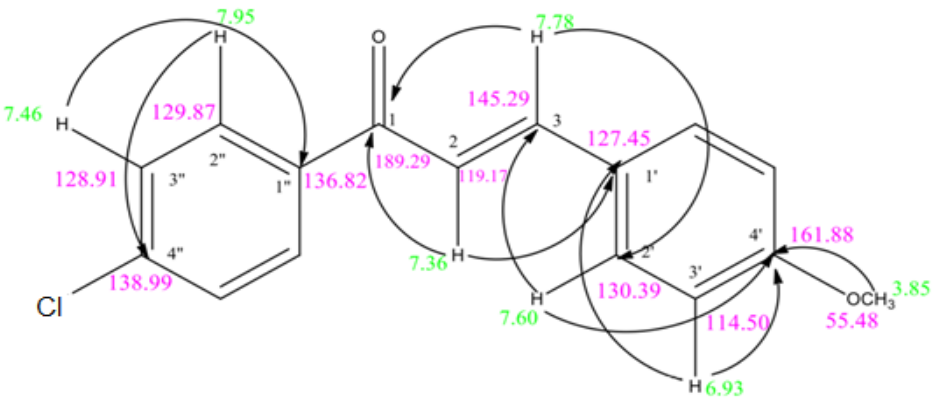


Figure 3.27: Structure of compound-4 showing ^{1}H NMR, ^{13}C NMR signals and HMBC (H$\longrightarrow$ C) correlations

Table 3.3: ^{1}H, ^{13}C-NMR, HMBC, HSQC data of compound-4 [Ref. 25]

Position No.	Chemical shift δ in ppm*		HSQC (^{1}H ⟶ ^{13}C)	HMBC (^{1}H ⟶ ^{13}C)	
	δ_H (mult, J in Hz)	δ_C	^{1}J	^{2}J	^{3}J
1	-	189.29	-	-	-
2	7.36, d, 15.6	119.17	C-2	C-1	C-1′
3	7.78, d, 15.6	145.29	C-3	-	C-1, C-2′
1′	-	127.45	-	-	-
2′	7.60, d, 8.8	130.39	C-2′	-	C-3, C-4′
3′	6.93, d, 8.8	114.50	C-3′	C-4′	C-1′
4′	-	161.88	-	-	-
1′′	-	136.82	-	-	-
2′′	7.95, d, 8.5	129.87	C-2′′	-	C-4′′, C-1
3′′	7.46, d, 8.8	128.91	C-3′′	-	C-1′′
4′′	-	138.82	-	-	-
4′-OMe	3.85, s	55.48	55.48	-	C-4′

*^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) in $CDCl_3$

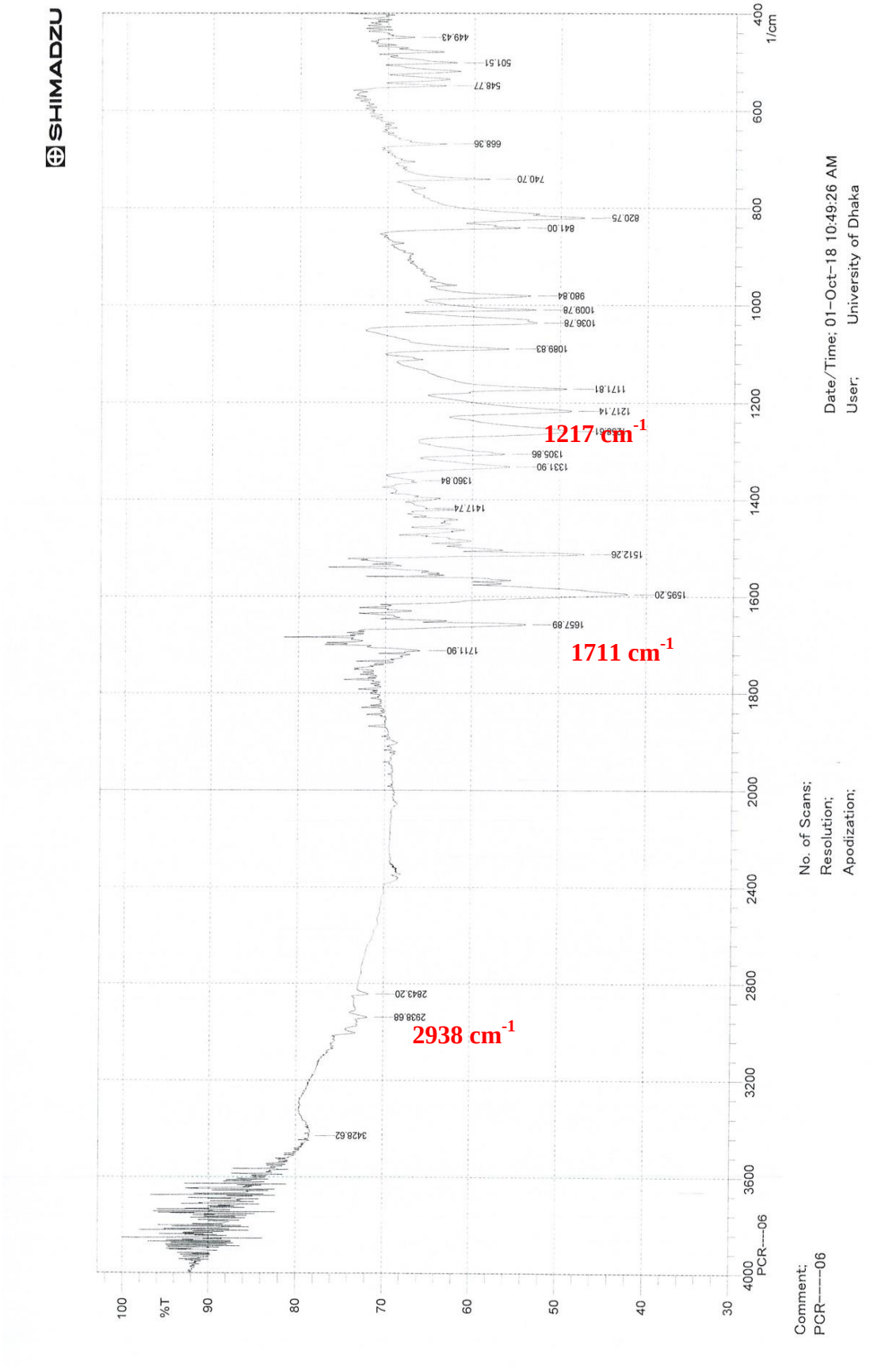


Figure 3.28: IR spectrum of compound-4

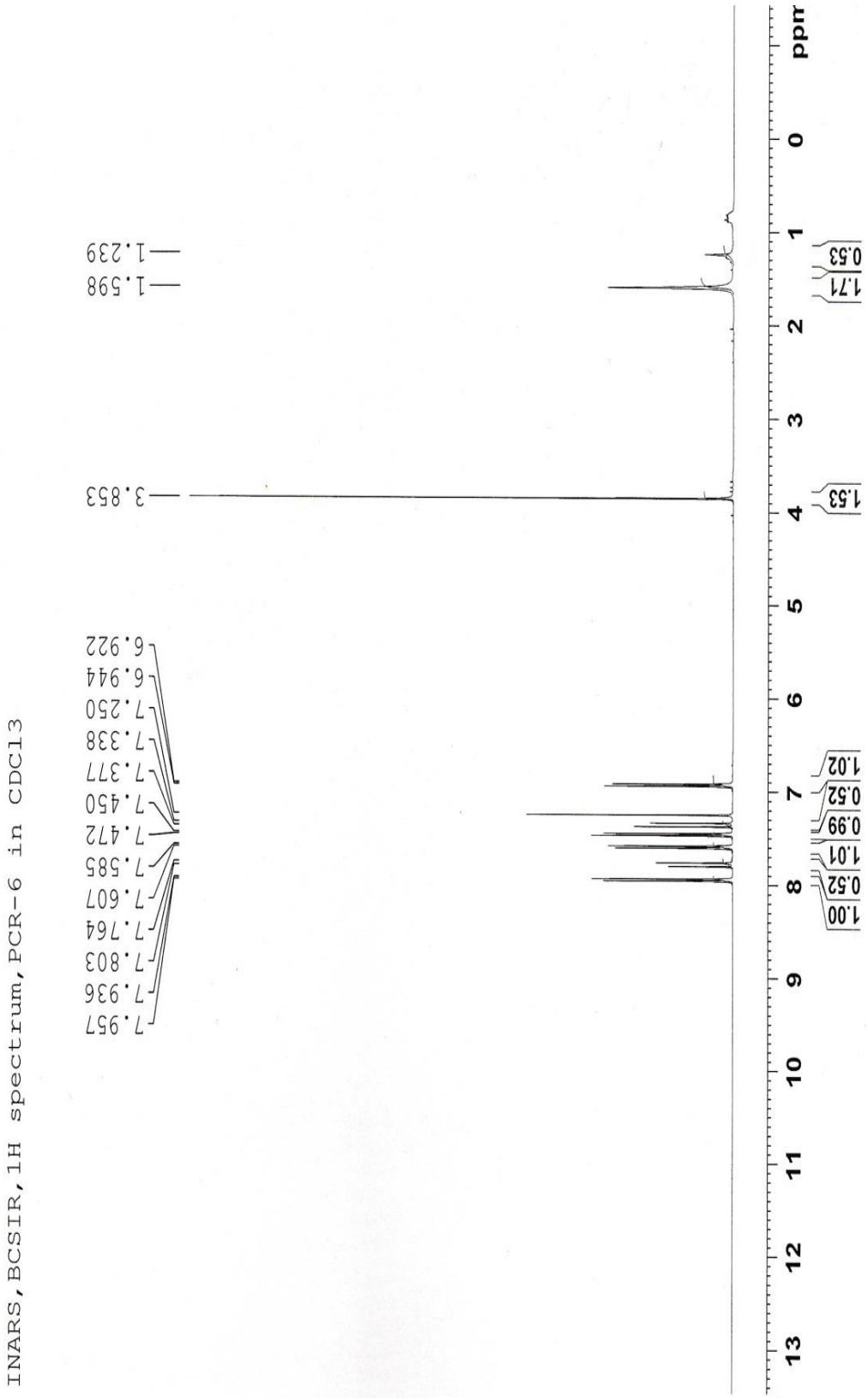


Figure 3.29: ^{1}H NMR spectrum of compund-4

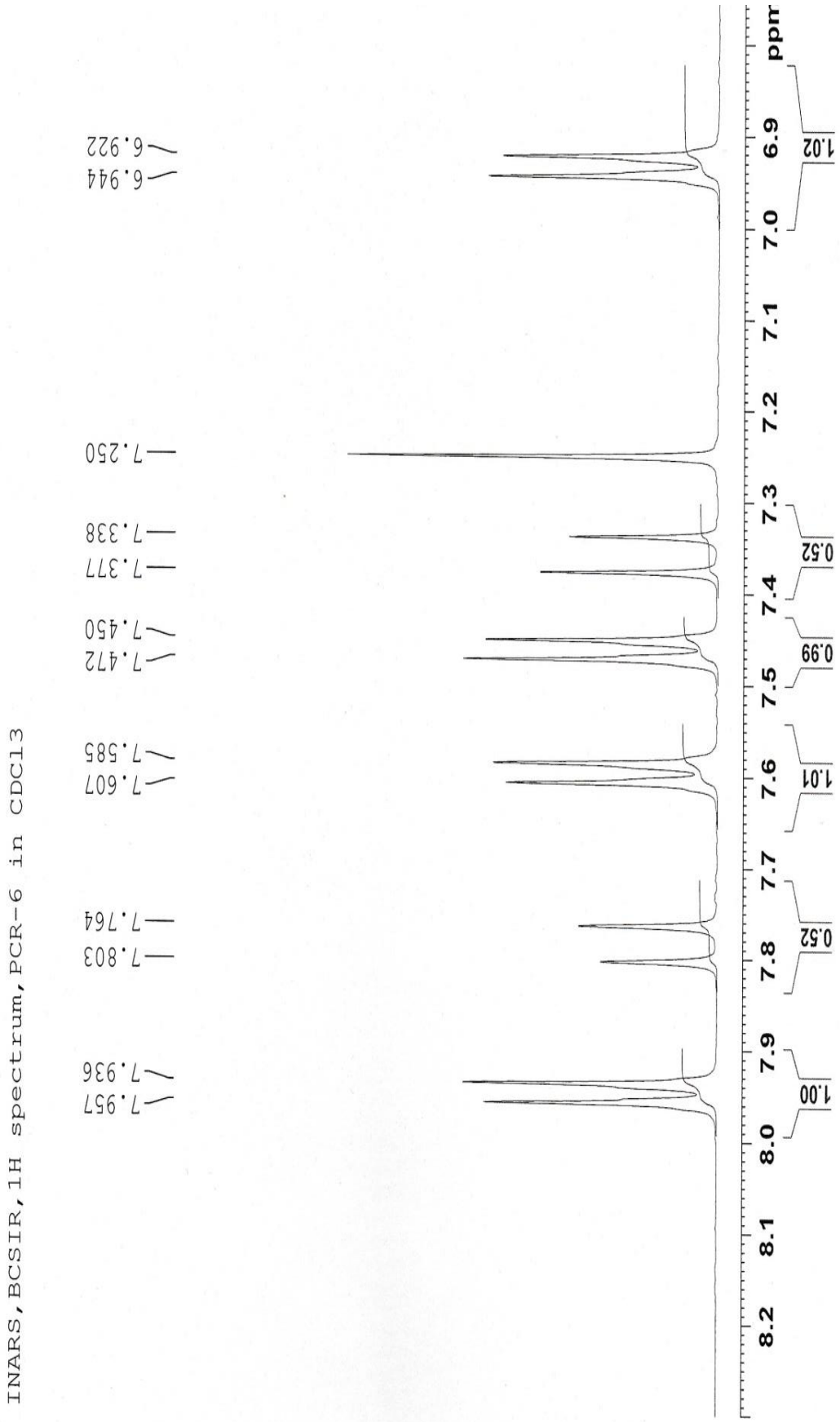


Figure 3.30: Expanded ^{1}H NMR spectrum of compound-4

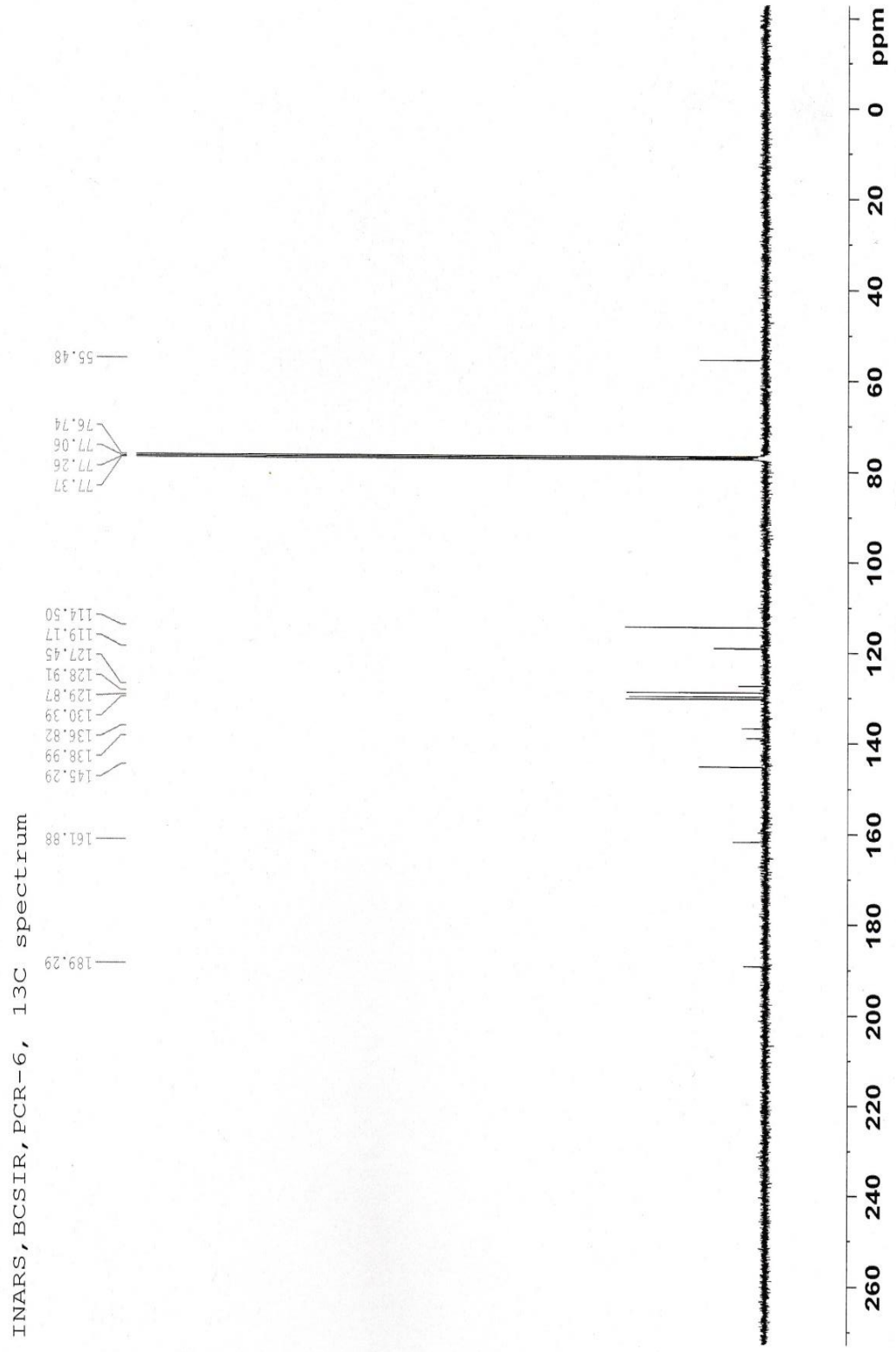


Figure 3.31: ^{13}C NMR spectrum of compund-4

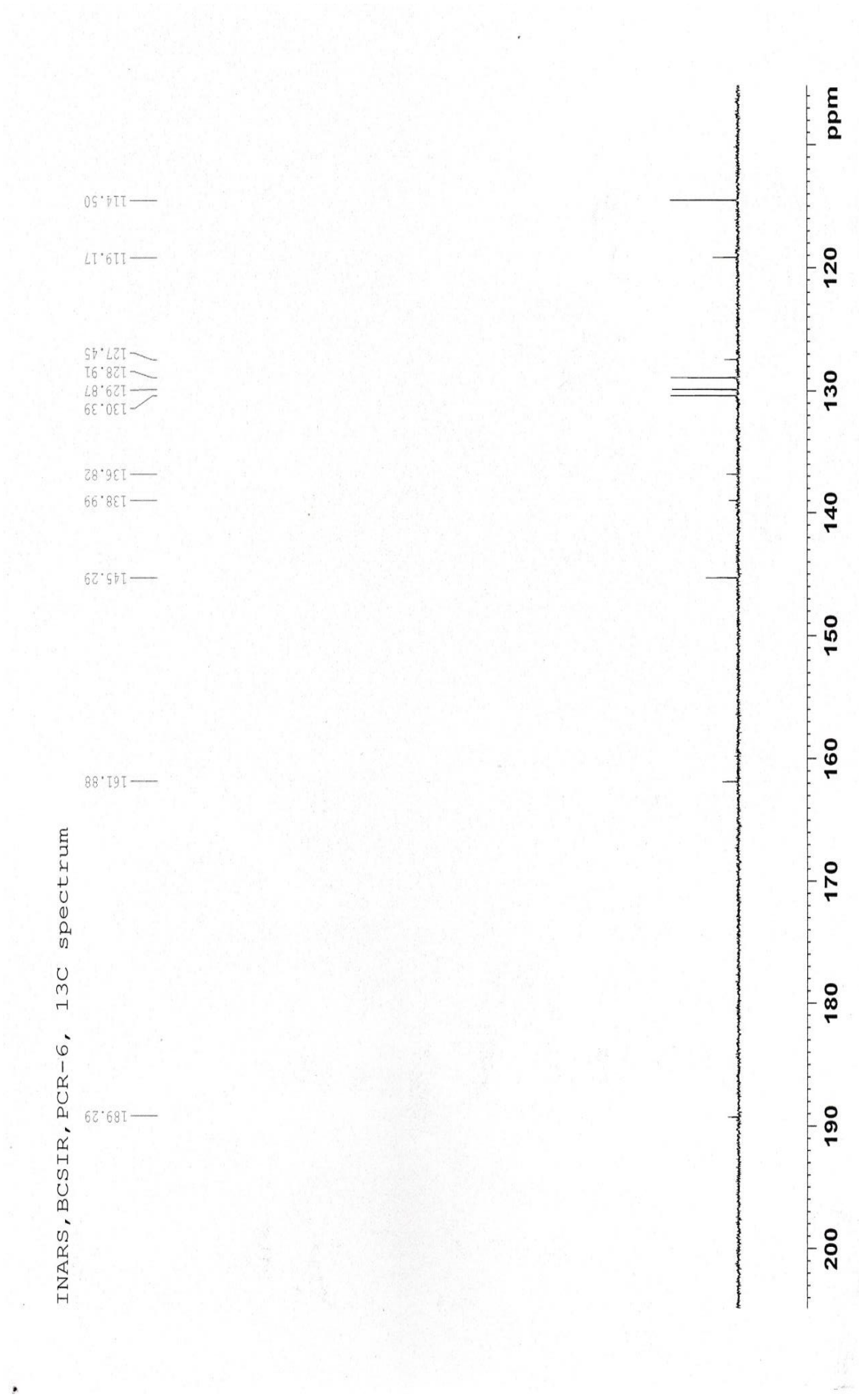


Figure 3.32: Expanded ^{13}C NMR spectrum of compund-4

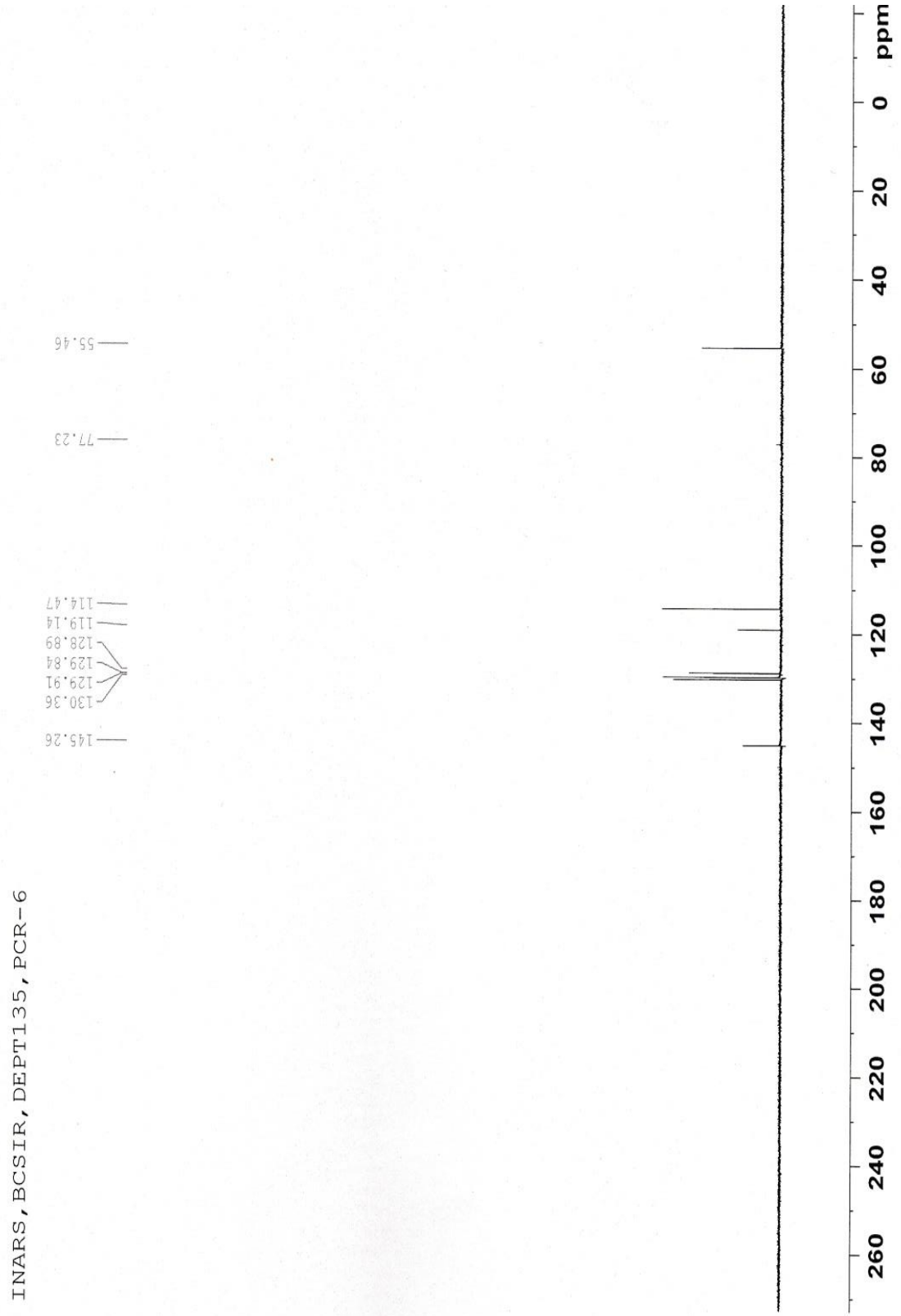


Figure 3.33: DEPT-135 spectrum of compund-4

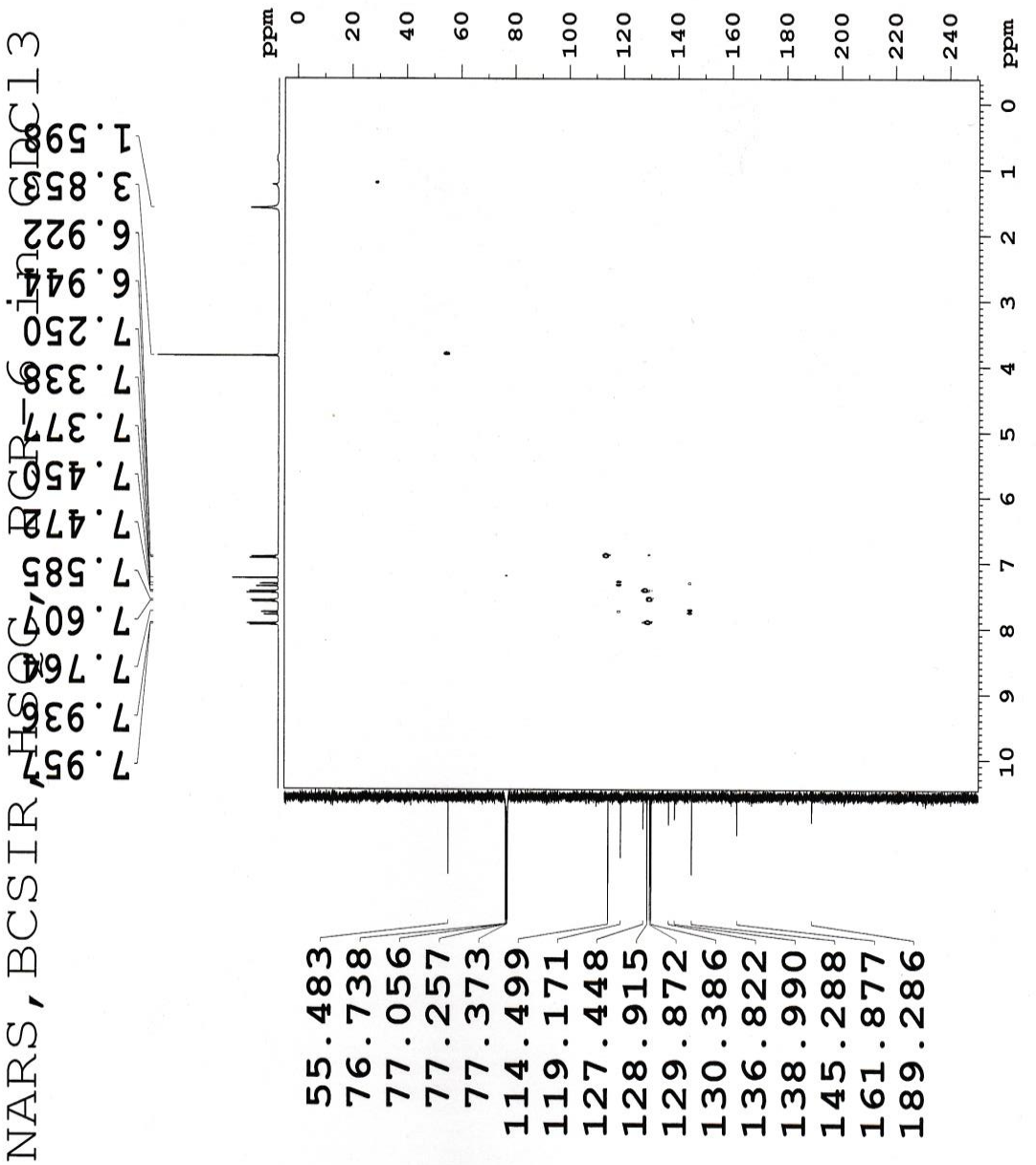


Figure 3.34: HSQC spectrum of compund-4

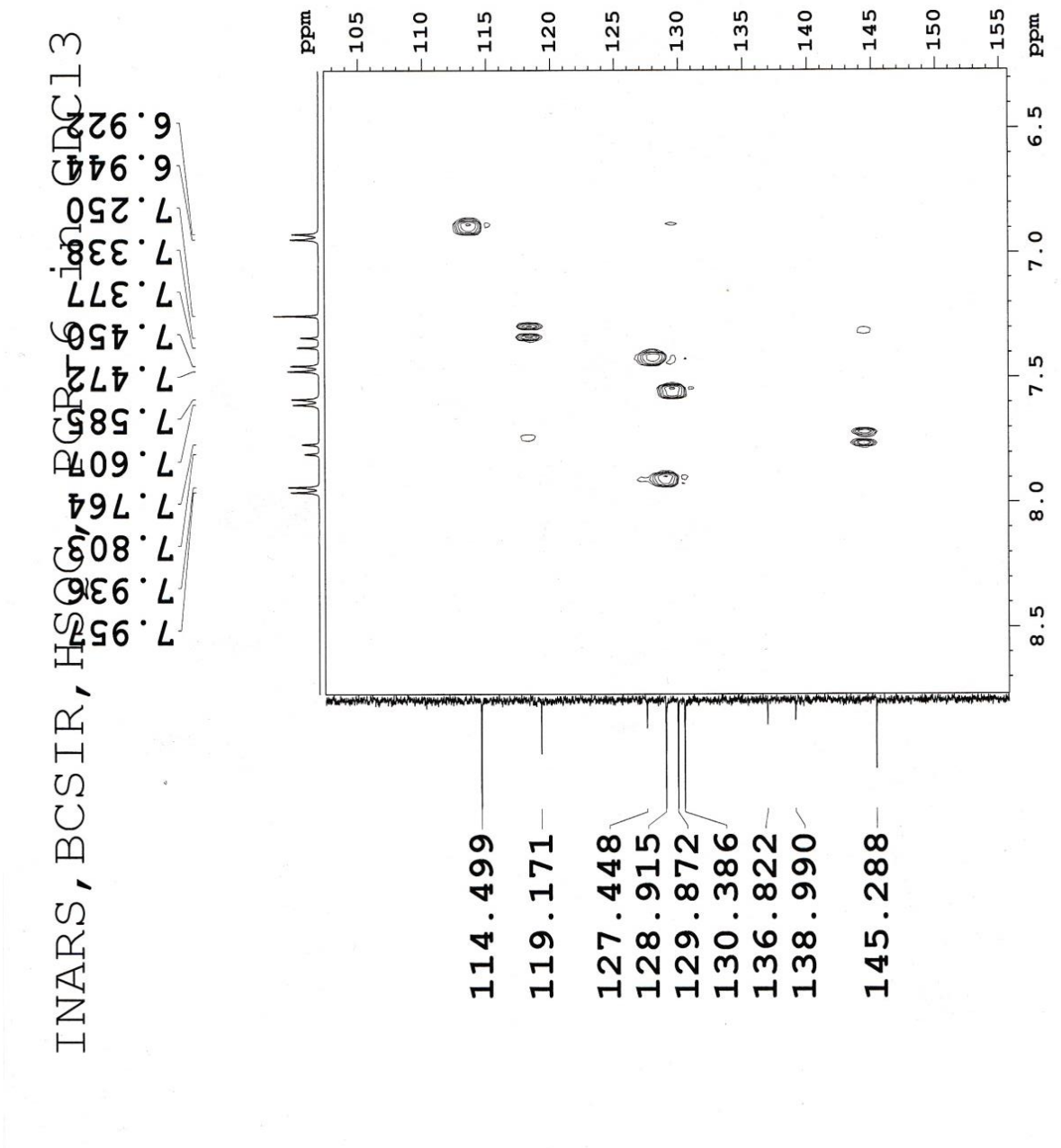


Figure 3.35: HSQC spectrum of compund-4

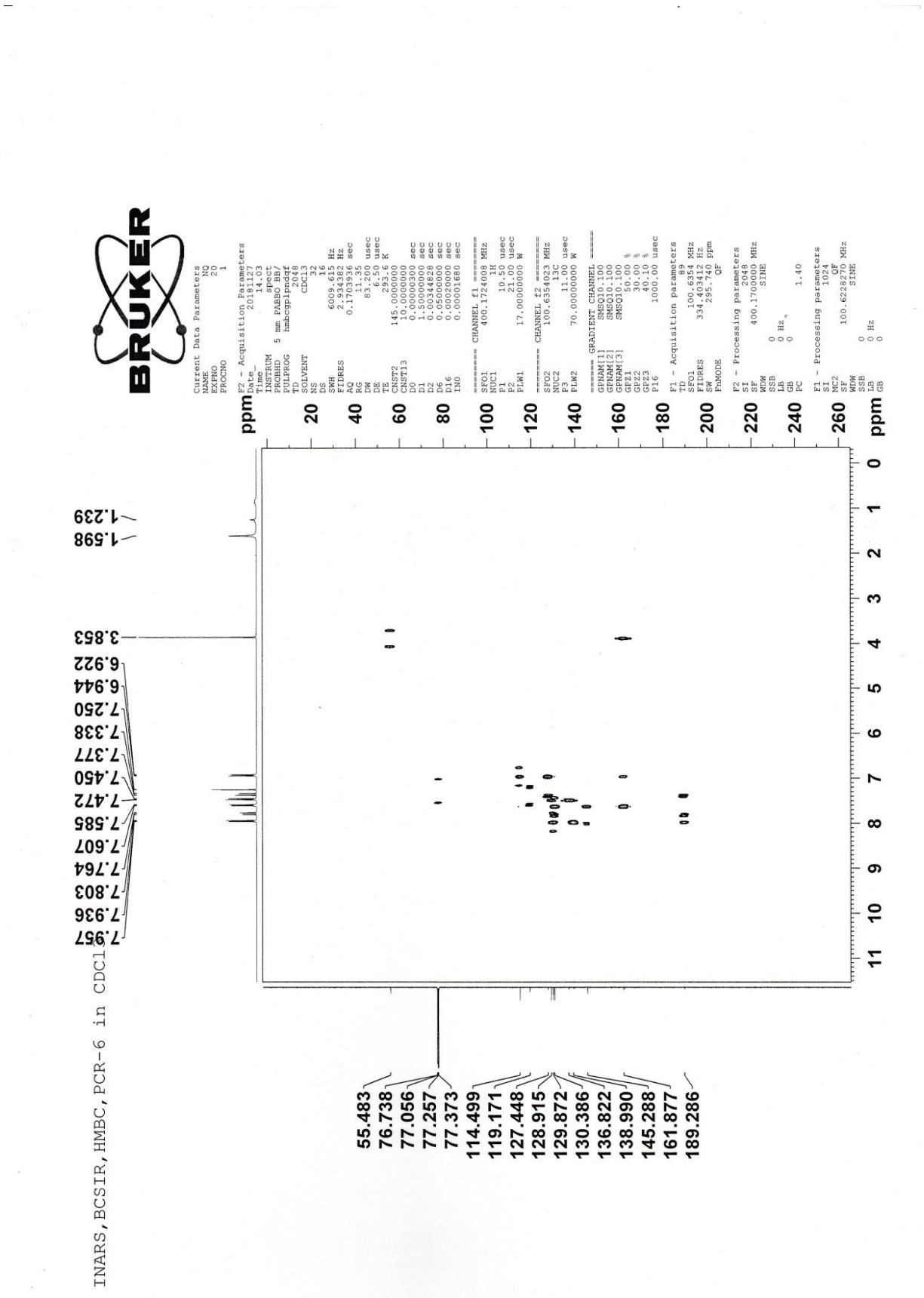


Figure 3.36: HMBC spectrum of compund-4

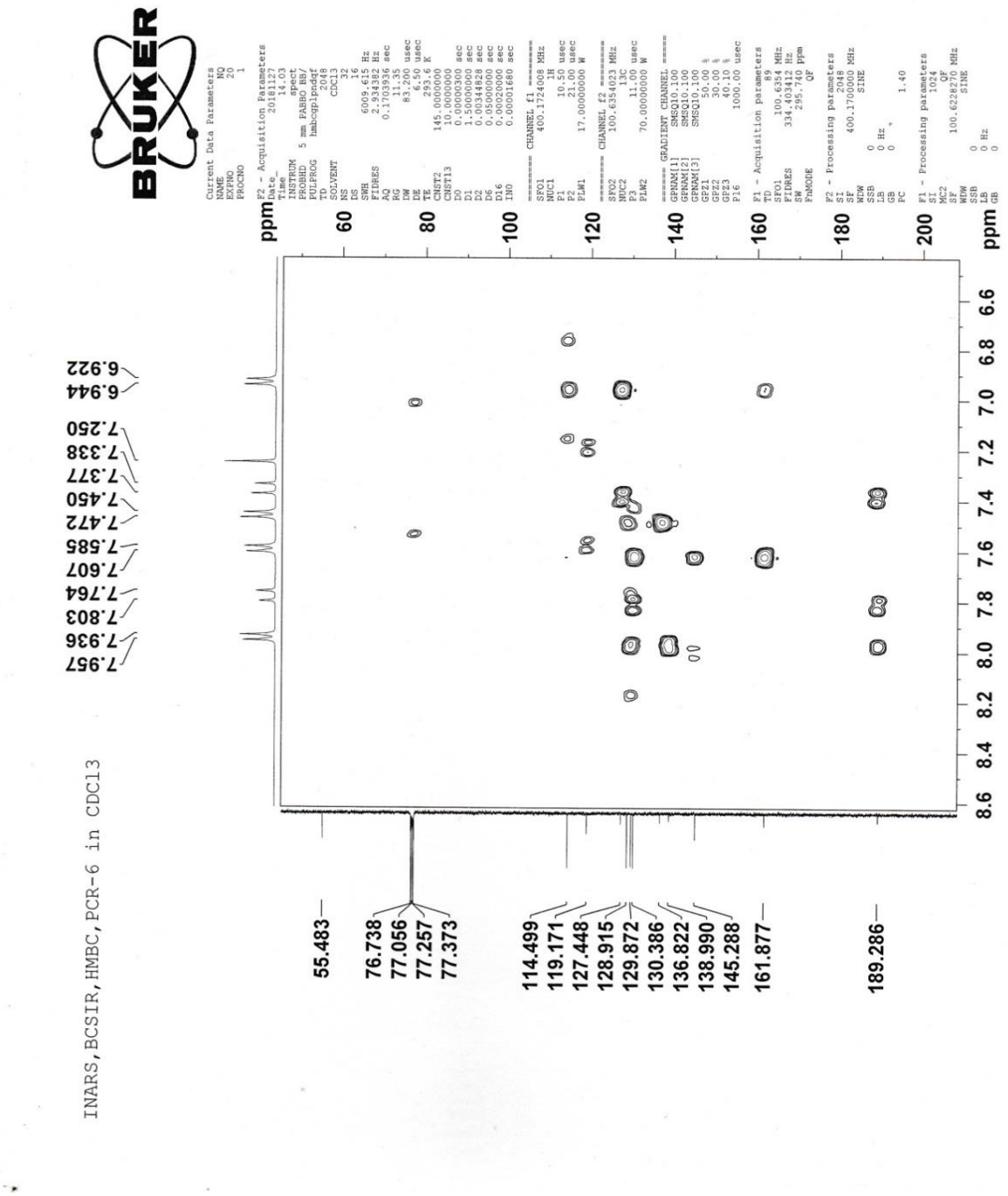
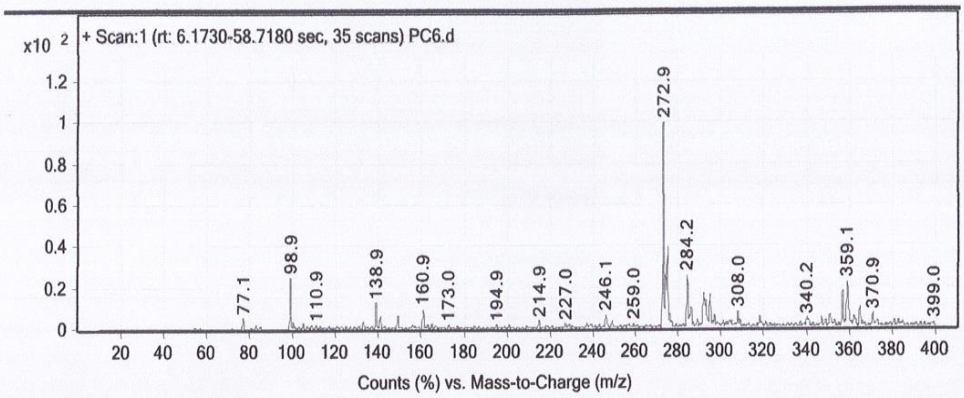


Figure 3.37: HMBC spectrum of compund-4

Qualitative Analysis Report



Peak List

m/z	z	Abund
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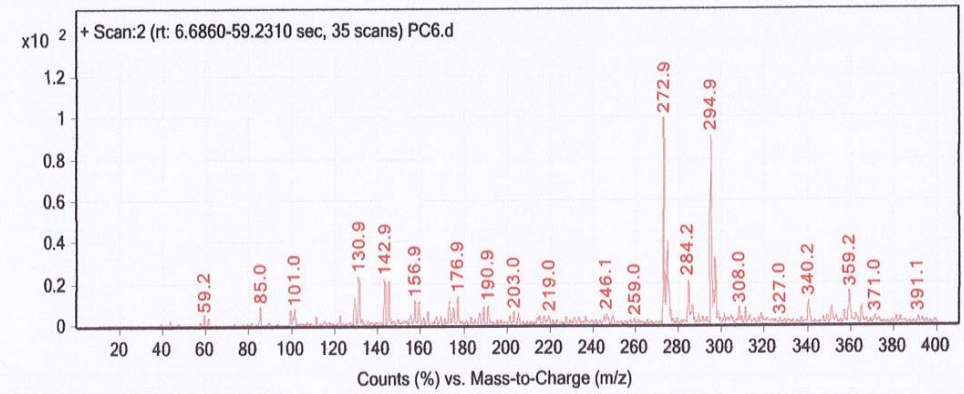
272.9	1	2905022
274	1	761949.63
275	1	1144336.38
284.2	1	740274.06
292		498502.16
292.8		388700.31
294.9	1	474455.59
356.9	1	520909.09
359.1		646836.81

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "+ TIC Scan"	70	0	ESI



Peak List

m/z	z	Abund
130.9	1	689603.13
142.9		658045.63
144.9		619424.94
272.9	1	2954648.5
274	1	743947.38
275	1	1184201.38
284.2	1	637471.69
294.9	1	2703735
295.9	1	475814.97

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Figure 3.38: ESI-MS spectrum of compund-4

According to the IR, ^{1}H-NMR and ^{13}C-NMR, HSQC, HMBC and ESI- mass spectrum data of the **compound-4** the tentative structure was suggested as-

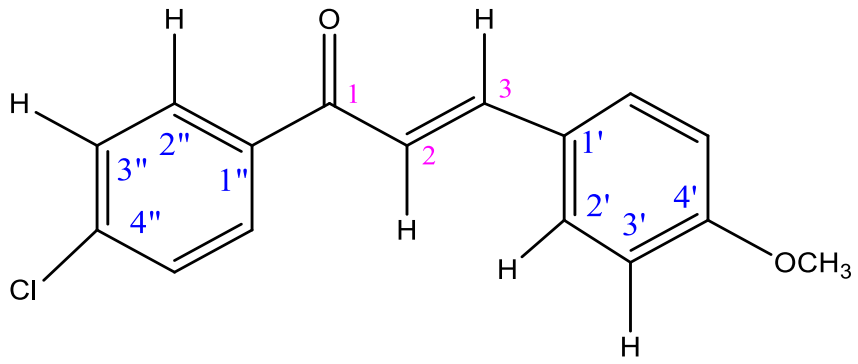


Fig 3.39: Compound-4 [1-(4"-chlorophenyl)-3-(4'-methoxyphenyl) prop-2-en-1-one]

3.5 Characterization of compound-5

The compound-5 (~6.5 mg) was a white crystalline solid having R_f value: 0.42 (10% EtOAc in dichloromethane). Its melting point, molecular formula and molecular weight was found to be 117-118^{o}C, $C_{16}H_{13}OCl$ and (M^{+} 257) respectively. It was soluble in dichloromethane. On spraying with vanillin-sulfuric acid spray reagent, followed by heating at 105°C for several minutes, purple color appeared.

The IR spectrum (in KBr) of the compound-5 showed the absorption at 2917 cm^{-1} for aromatic C-H stretching, at 1658 cm^{-1} due to C-O stretching for carbonyl group, at 1331 cm^{-1} for C-H bending due to methyl group and the absorption at 1222 cm^{-1} for C-H bending due to methylene group.

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of the compound-5 showed the signals at 7.23 and 7.95 for aromatic protons, at δ 7.44 (6H, d, J= 15.6 Hz), 7.79 (1H, J=15.6 Hz) for methine protons and the important chemical shift at δ 2.39 for methyl proton.

The ^{13}C-NMR spectrum (100 MHz in $CDCl_3$) of compound-5 showed the signals at δ 189.35 due to carbonyl carbon, at δ 129.80, 129.92, 128.60 and 128.95 for aromatic (C-H) carbons, at δ 141.41, 131.98, 136.68 and 139.12 for tertiary carbons and the signals at δ 21.61 for methyl carbon.

The HSQC experiment showed that the protons at δ 7.23, 7.54, 7.44, 7.79, 7.95, 7.47 and 2.39 are directly attached to the carbons at δ 128.95, 128.60, 120.51, 145.52, 129.92, 129.80 and 141.41 respectively.

The long-range proton-carbon link results of the HMBC research are shown in **Figure 3.40** is of the subsidiary evidence. The proton at δ 7.95 showed strong long-range coupling to carbons at C-1 (189.35), C- 3''(δ 129.80) and C- 4'' (δ139.15). The proton at δ 7.79 showed strong long-range coupling to carbons 1 (δ 189.35) and 2′ (δ 128.60). The spectrum showed the proton at δ 7.54 strong long-range coupling to carbons 3 (δ 145.52) and 4′ (δ 141.41). The proton at δ 7.23 showed strong long-range coupling to carbons at 4′-Me (δ 21.61) and 1′(δ 131.98). The other HMBC (H ⟶C) correlations were shown in the **Figure 3.40** which supported the given structure of the compound-5.

From the ESI-MS spectrum of the compound-5 the peaks at m/z, 257 was due to the presence of $[M+H]^+$. The m/z values at 257 and 259 with an approximate intensity of 3:1 confirmed the presence of Cl atom in the parent compound. Thus, the molecular weight of compound-5 is 257 which is correctly matched with the calculated value of the molecular weight of the compound-5

The ^{1}H NMR, ^{13}C NMR, HSQC and HMBC data of compound-4 were tabulated in **Table 3.5**

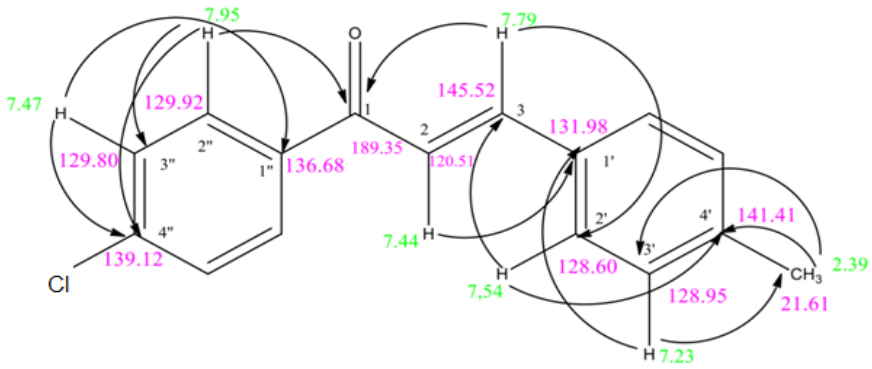


Figure 3.40: Structure of **compound-5** showing ^{1}H, NMR, ^{13}C NMR signals and HMBC (H⟶C) correlations

Table 3.4: The ^{1}H, ^{13}C-NMR, HMBC, HSQC data of compound-5 [Ref. 25]

Position No.	Chemical shift δ in ppm*		HSQC ($^{1}H \rightarrow ^{13}C$)	HMBC ($^{1}H \rightarrow ^{13}C$)	
	δ_H (mult, J in Hz)	δ_C	^{1}J	^{2}J	^{3}J
1	-	189.35	-	-	-
2	7.44, d, 15.6	120.51	C-2	-	C-1′
3	7.79, d, 15.6	145.52	C-3	-	C-2′
1′	-	131.98	-	-	-
2′	7.54, d, 8	128.60	C-2′	-	C-4′, C-3
3′	7.23, d, 8	128.95	C-3′	-	C-1′, 4′-Me
4′	-	141.41	-	-	-
1′′	-	136.68	-	-	-
2′′	7.95, d, 8.4	129.92	C-2′′	C-3′′	C-4′′, C-1
3′′	7.47, d, 8.4	129.80	C-3′′	C-4′′	C-1′′
4′′	-	139.12	-	-	-
4′-Me	2.39, s	21.61	21.61	C-4′	C-3′

*^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) in $CDCl_3$

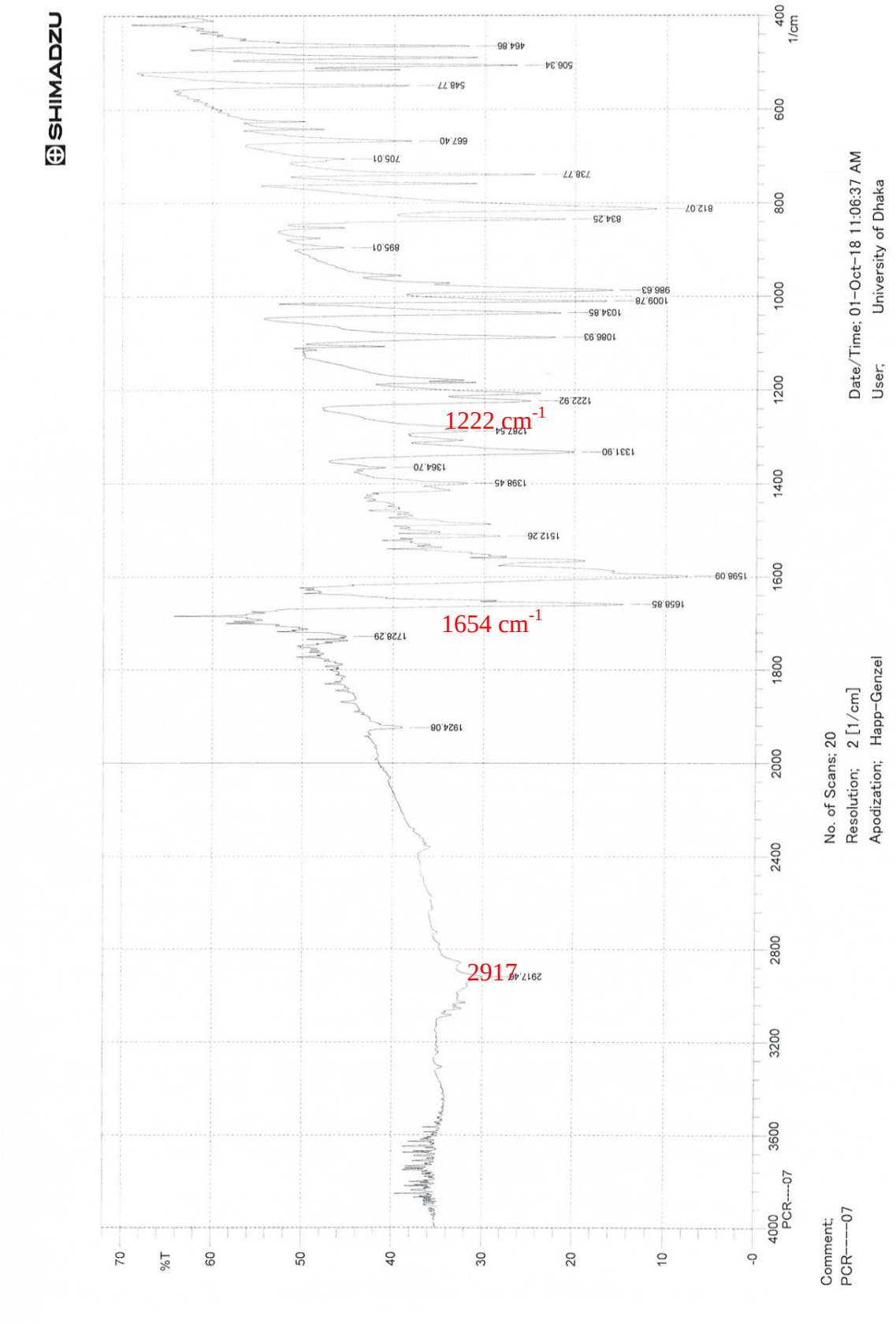


Figure 3.41: IR spectrum of compound-5

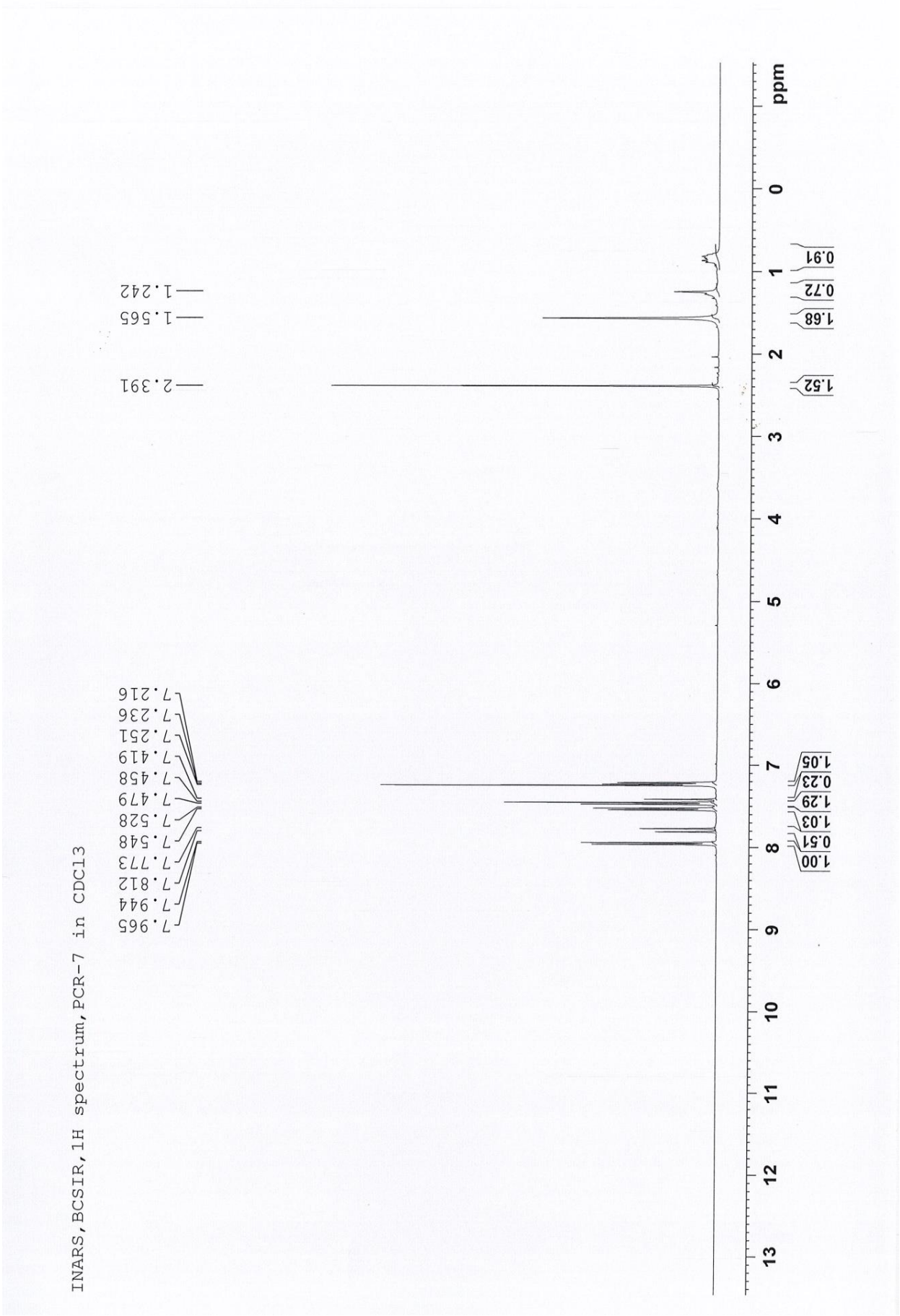


Figure 3.42: ^{1}H NMR spectrum of compund-5

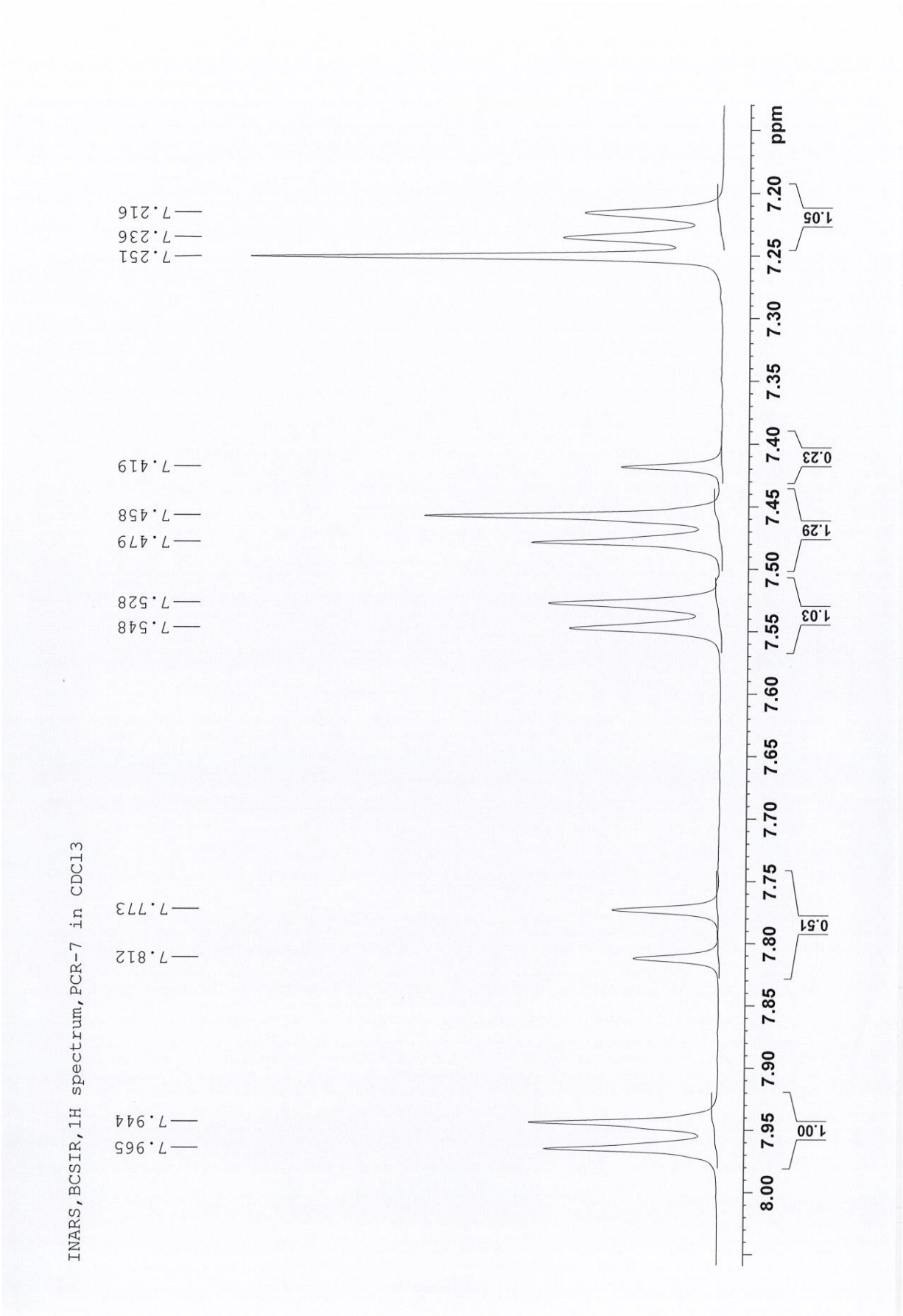


Figure 3.43: Expanded ^{1}H NMR spectrum of compound-5

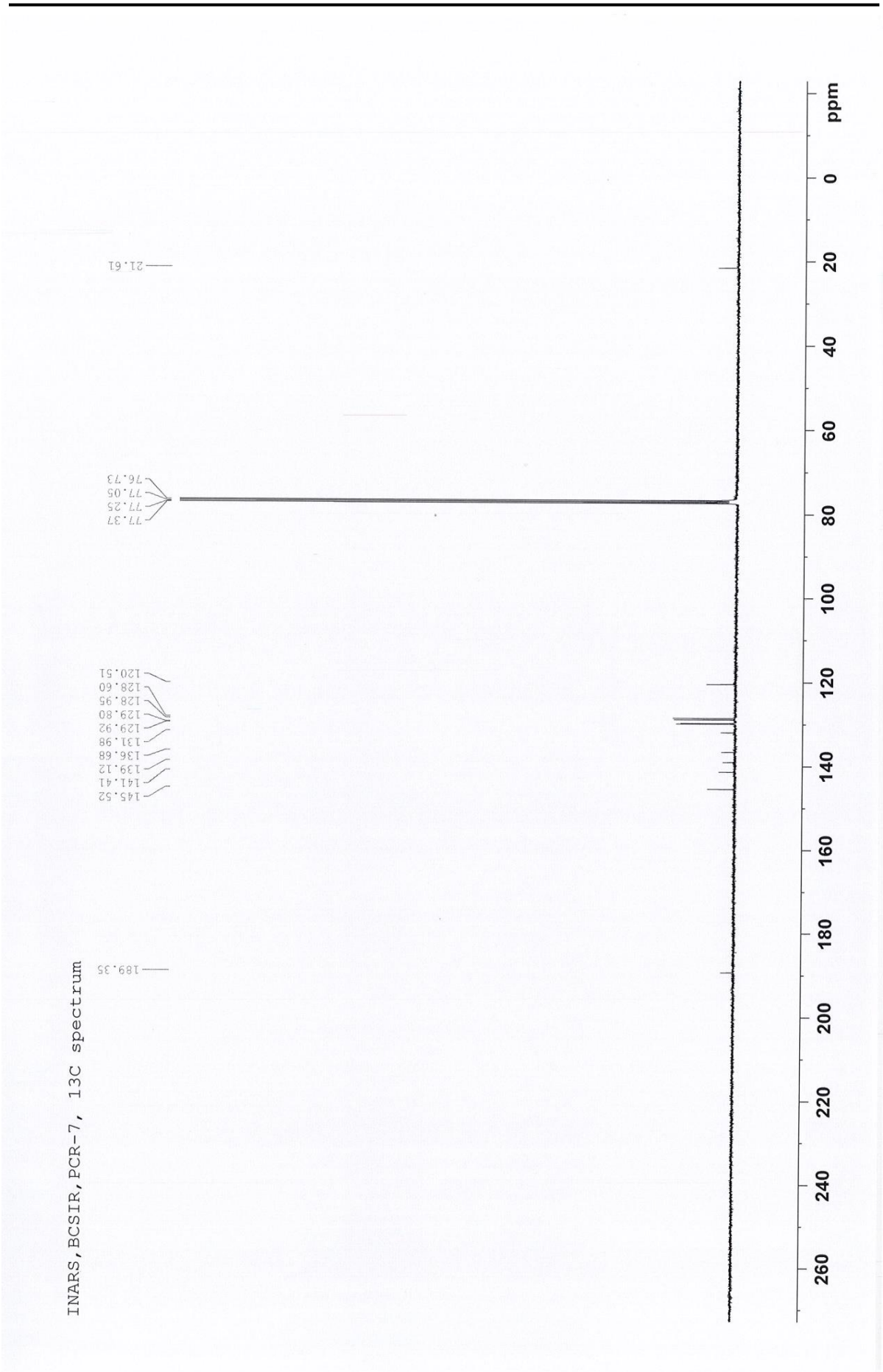


Figure 3.44: ^{13}C NMR spectrum of compund-5

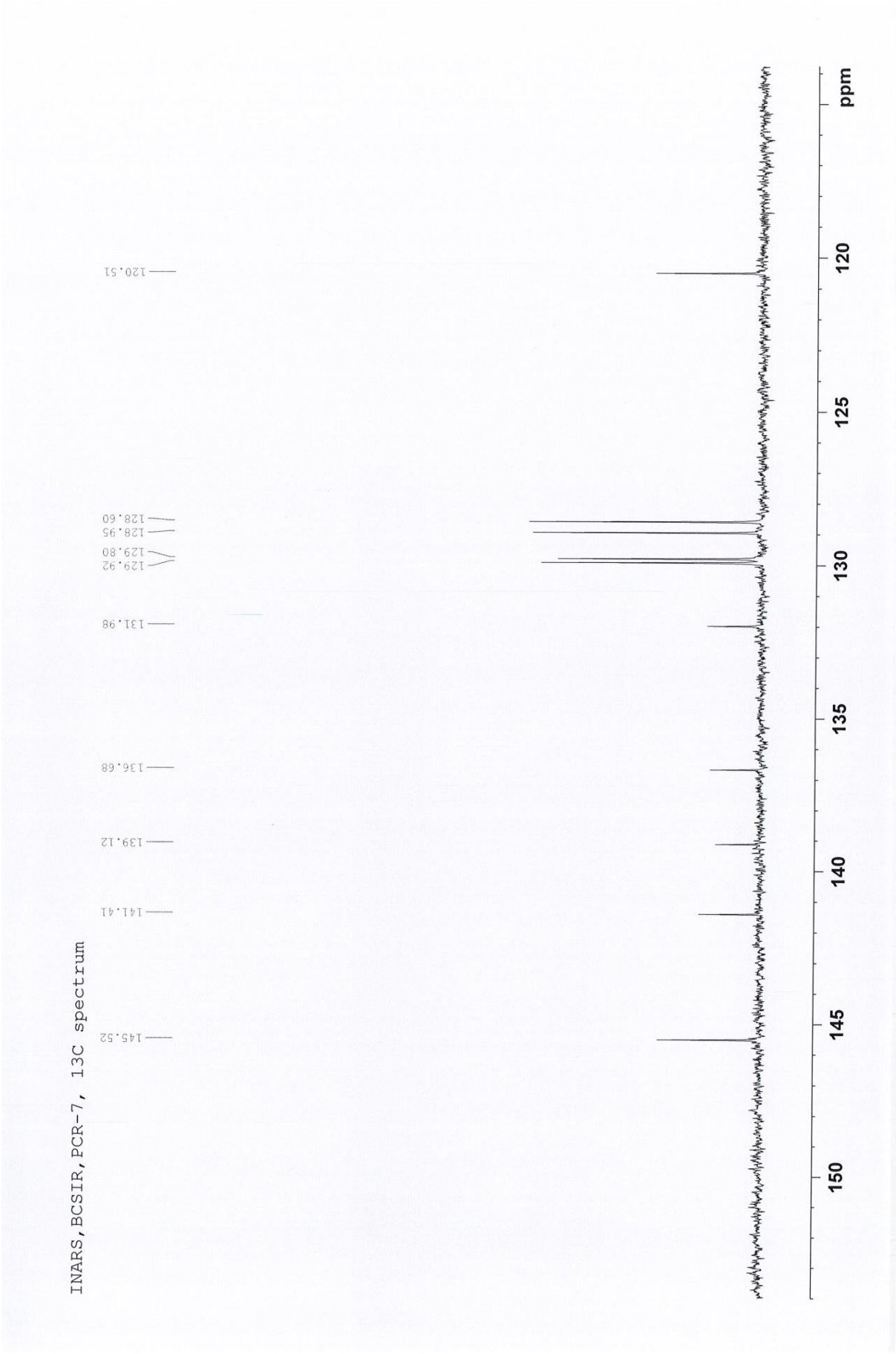


Figure 3.45: Expanded ^{13}C NMR spectrum of compund-5

Figure 3.46: DEPT-135 spectrum of compund-5

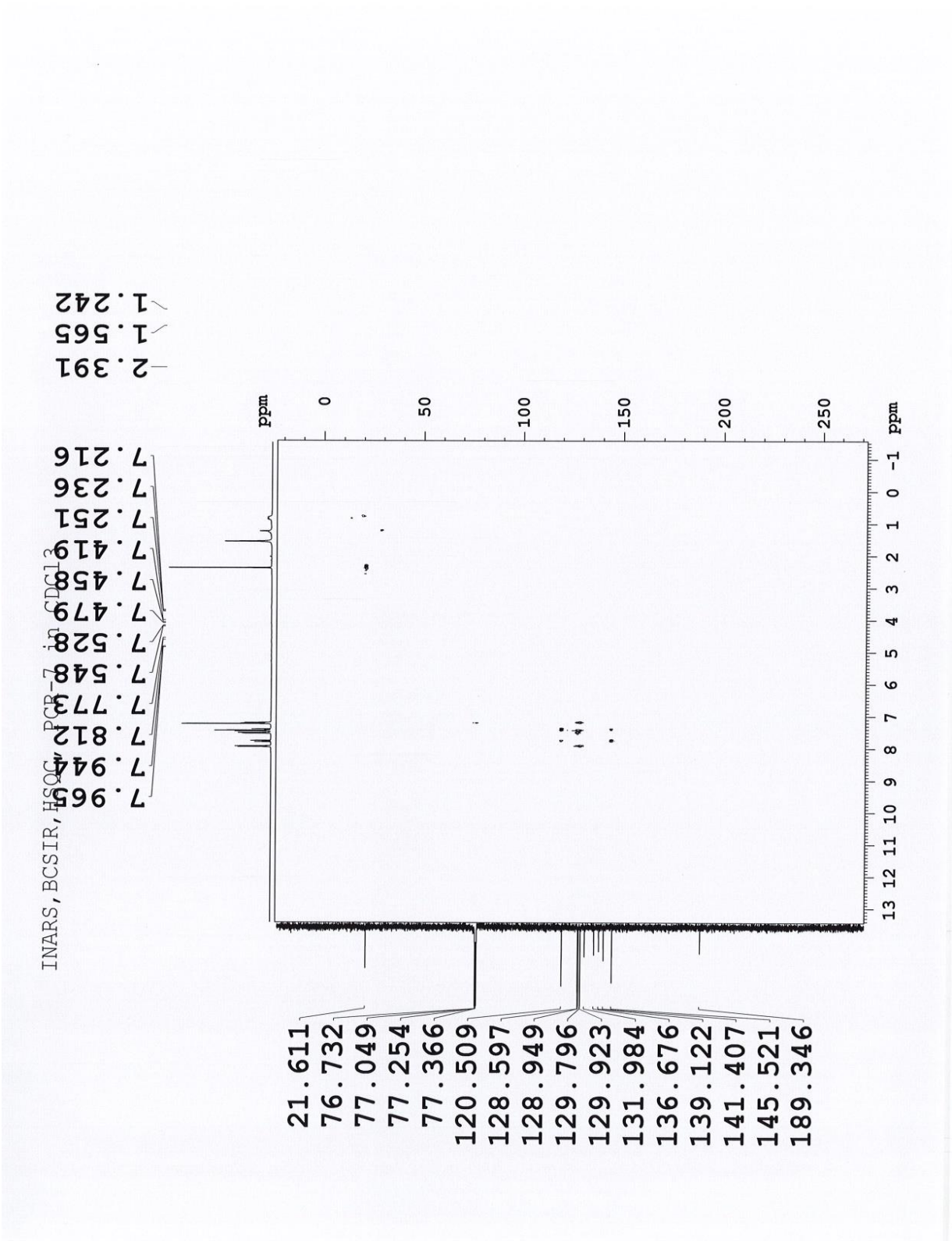


Figure 3.47: HSQC spectrum of compund-5

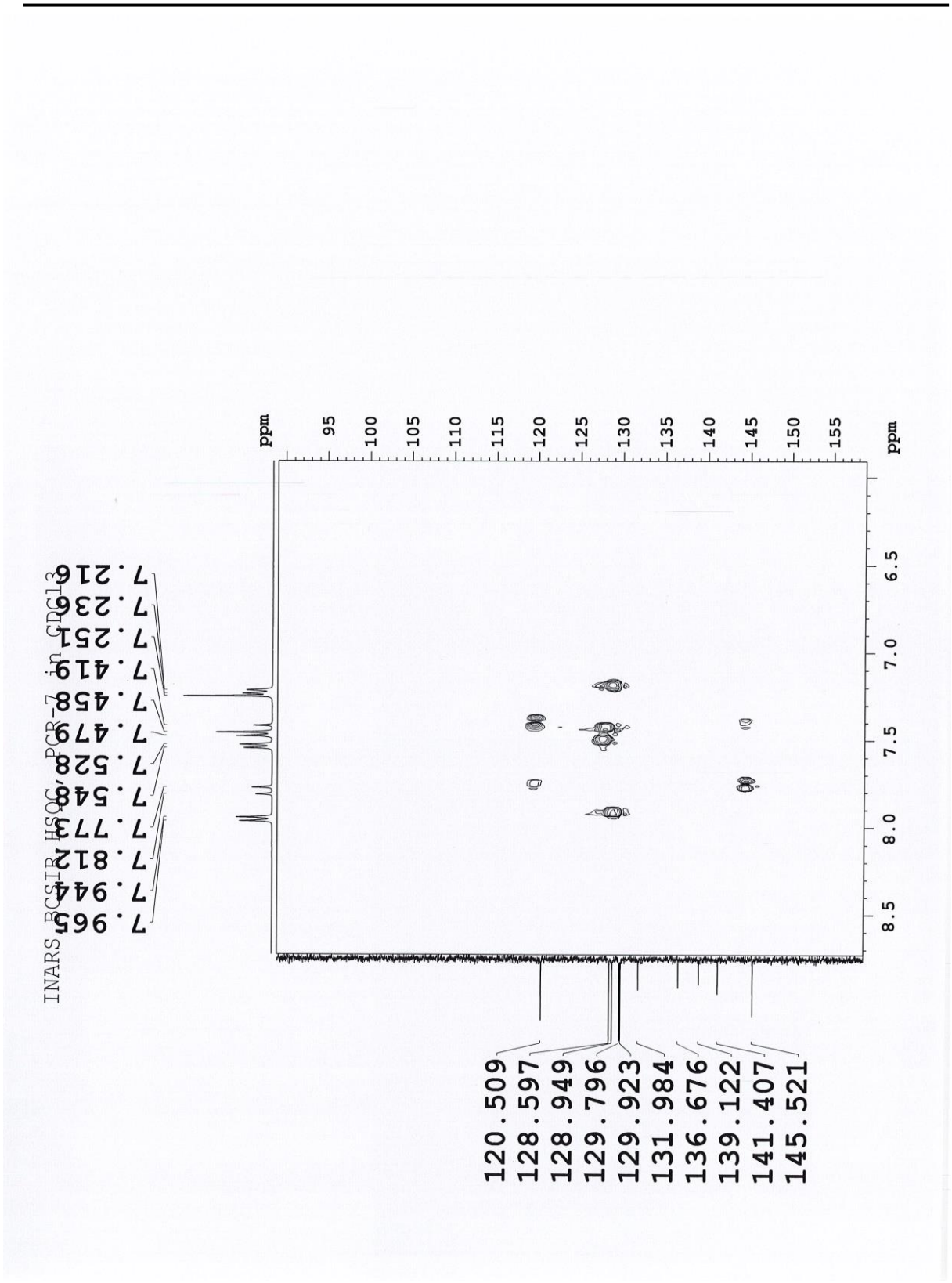


Figure 3.48: Expanded HSQC spectrum of compound-5

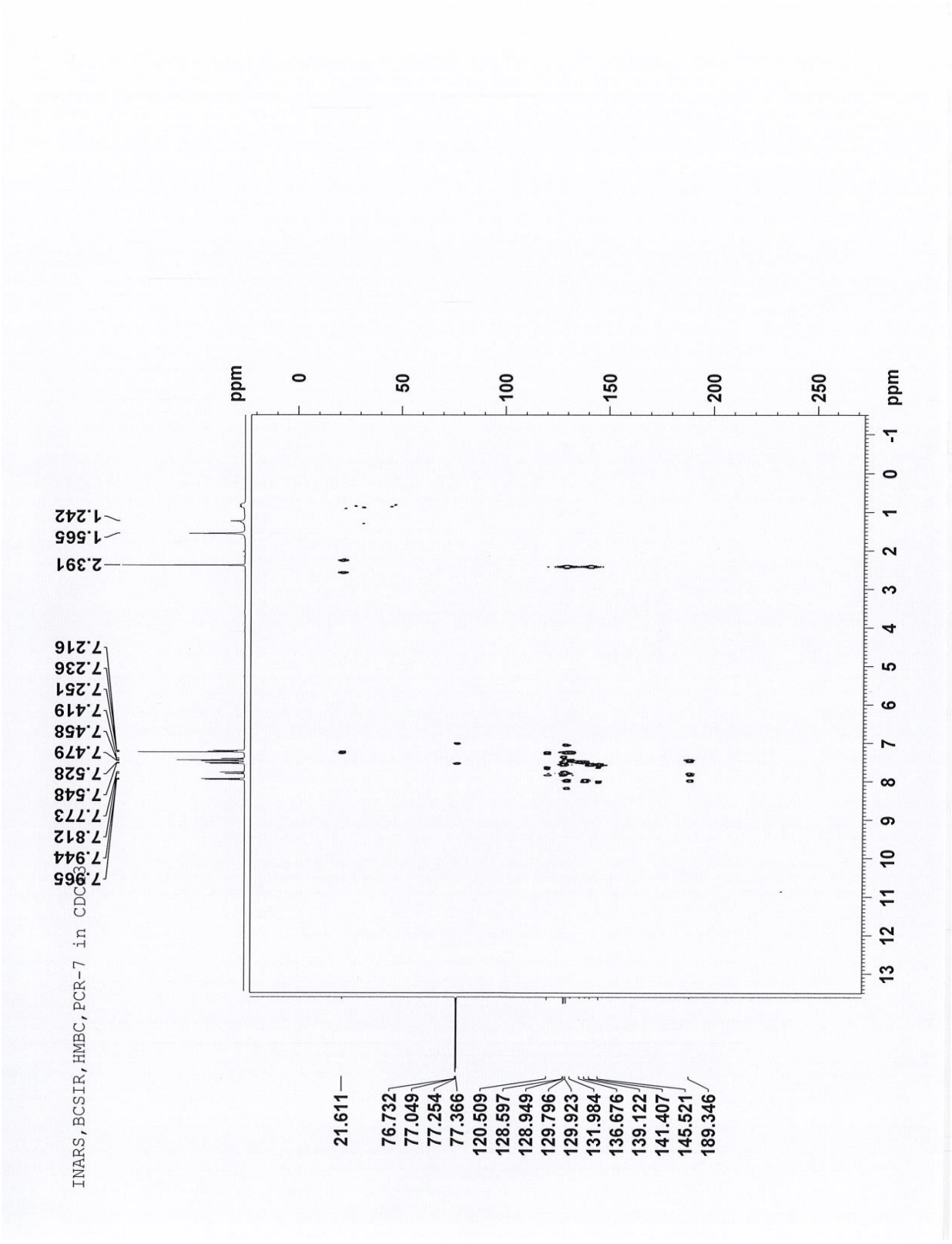


Figure 3.49: HMBC spectrum of compund-5

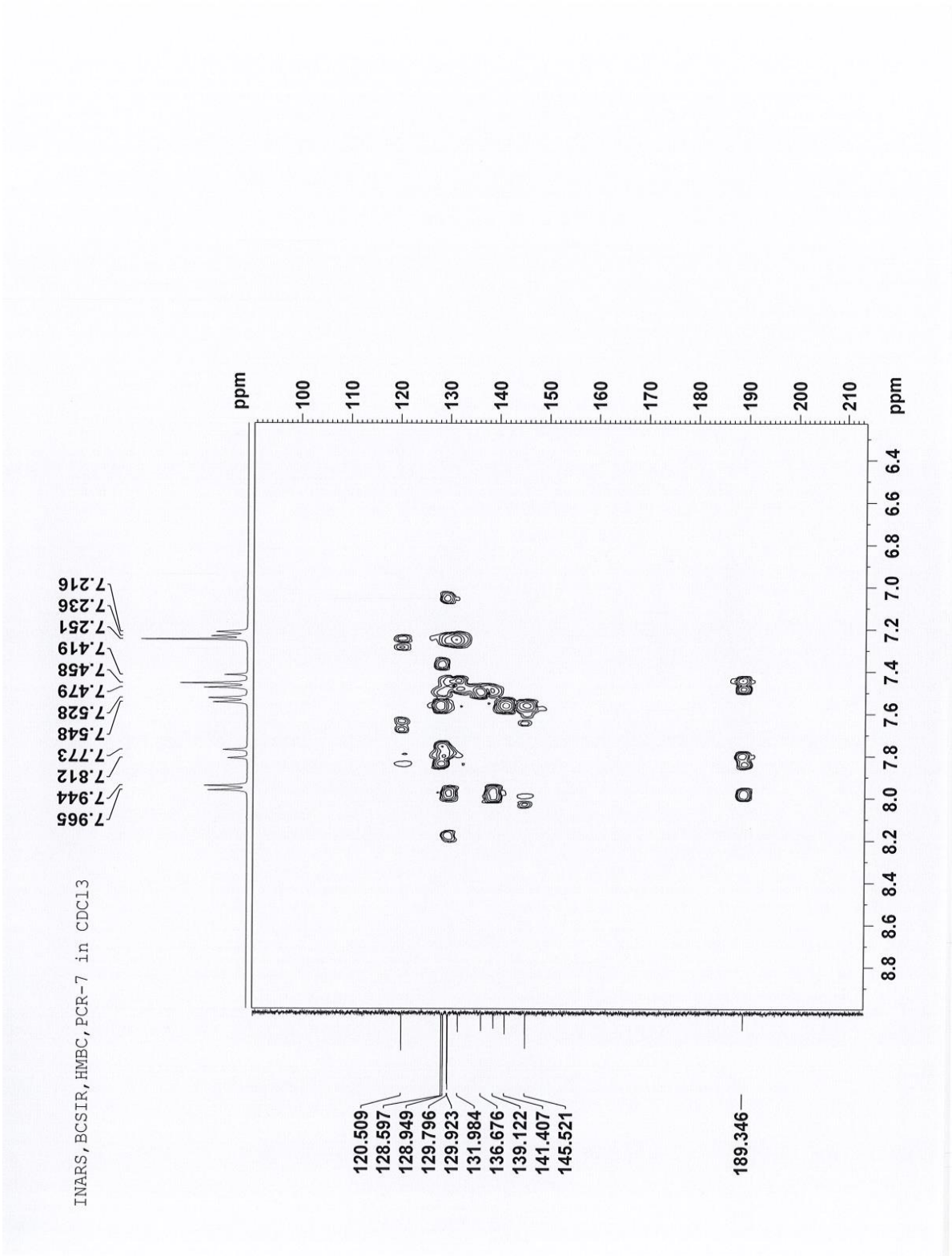
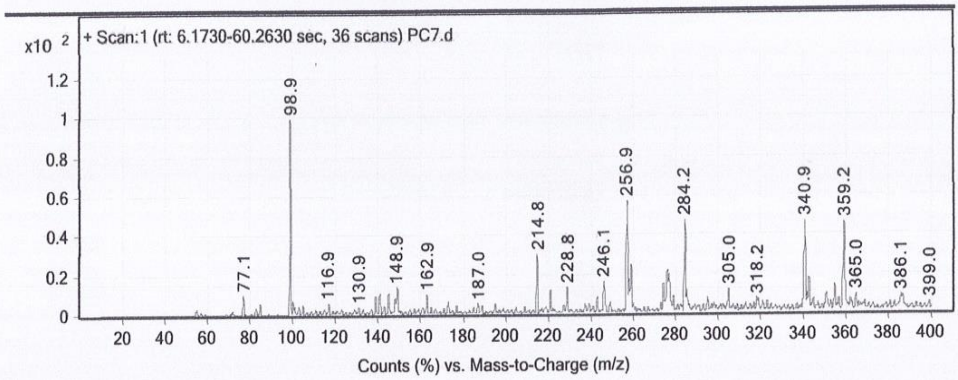


Figure 3.50: Expanded HMBC spectrum of compund-5

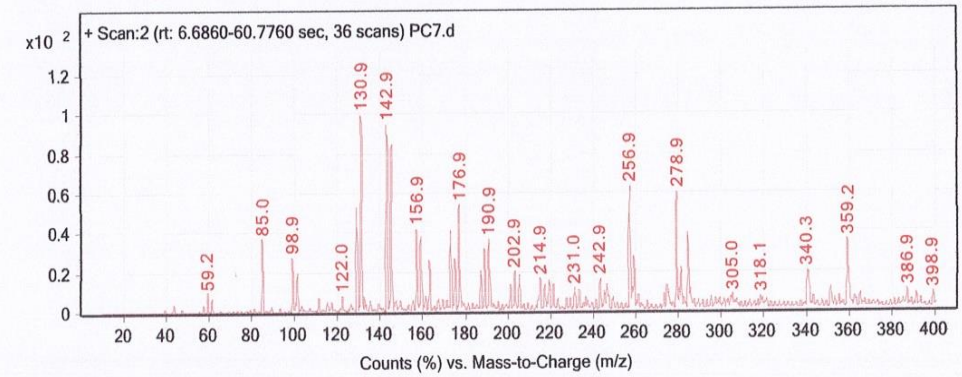
Qualitative Analysis Report



Peak List

m/z	z	Abund
98.9	1	1504730
214.8		458336.5
256.9	1	863287.25
258	1	268931.56
258.9	1	375474.38
276		330174.22
276.8		298910.19
284.2	1	712638.69
340.9	1	706317.56
359.2		694919.5

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "+ TIC Scan"	70	0	ESI



Peak List

m/z	z	Abund
128.9		813371.94
130.9	1	1539005
142.9	1	1460135.38
144.9	1	1303671.5
156.9		642997.94
173		632724.06
176.9		835270.38
256.9	1	971379.75
278.9	1	926018.63

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Figure 3.51: ESI-MS spectrum of compund-5

According to the IR, ^{1}H-NMR and ^{13}C-NMR, HSQC, HMBC and ESI-MS spectrum data of the **compound-5,** the tentative structure was suggested as-

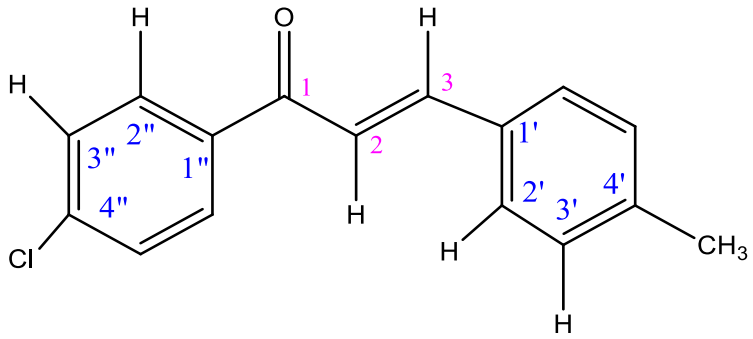


Figure 3.52: Compound-5 [1-(4"-chlorophenyl)-3-(p-tolyl)prop-2-en-1-one]

ANTIOXIDANT ACTIVITY

4.1 Introduction

The free radical scavenging activities (antioxidant capacity) of the plant extracts on the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were estimated by the method of Brand-Williams *et al.*, 1995.

2.0 ml of a methanol solution of the extract at different concentration were mixed with 3.0 ml of a DPPH methanol solution (20µg/ml). The antioxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by the plant extract as compared to that of tert-butyl-1-hydroxytoluene (BHT).

4.2. Materials & methods

DPPH was used to evaluate the free radical scavenging activity (antioxidant potential) of various compounds and medicinal plants (Choi *et al.*, 2000; Desmarchelier *et al.*, 1997)

4.2.1. Assay of free radical scavenging activity

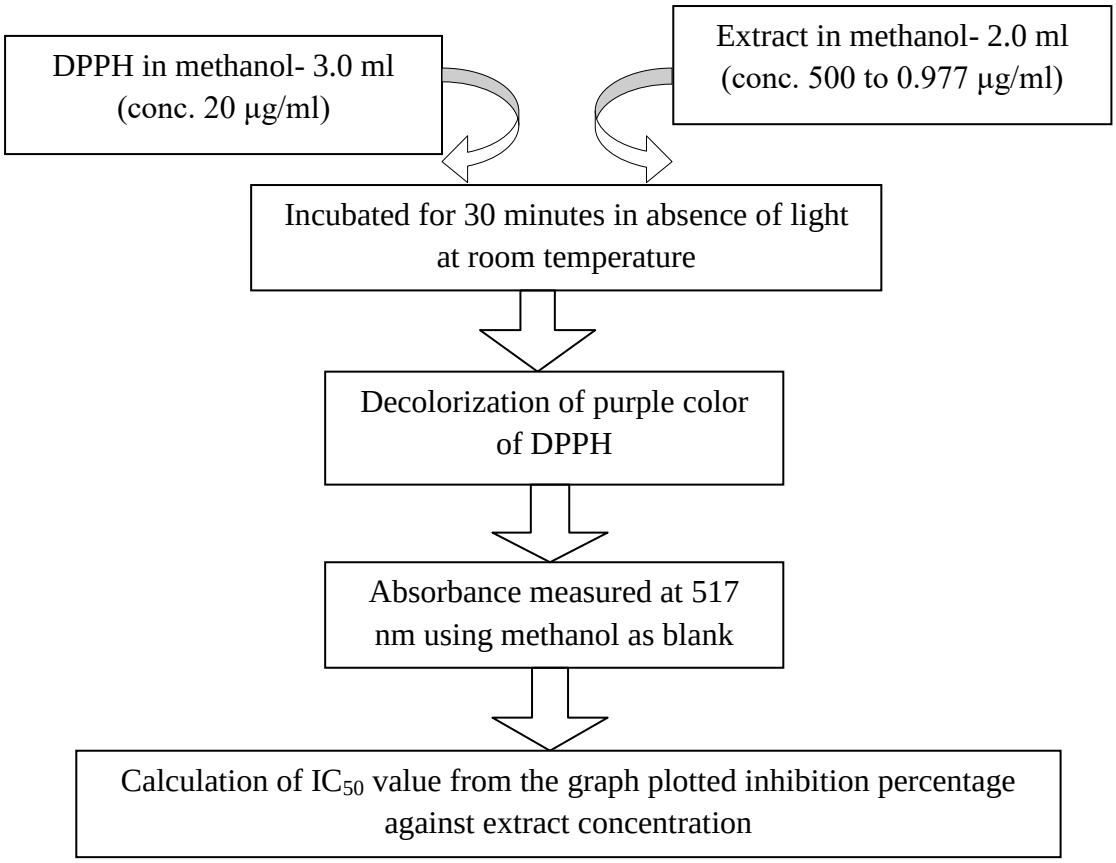


Figure 4.1: Schematic representation of the method of assaying free radical scavenging activity

4.3 Calculation

Calculated the inhibition of free radical DPPH in percent as follows:

$$\%\boldsymbol{Inhibition} = \frac{\boldsymbol{Absorbance\ of\ the\ control - Absorbance\ of\ the\ sample}}{\boldsymbol{Absorbance\ of\ the\ control}}$$

Then the percentages reserves were plotted against respective concentrations. Calculated the IC_{50} values as the concentration of each sample required to give 50% DPPH radical scavenging movement from the graph (linear regression curve) by Excel 2010 office software .

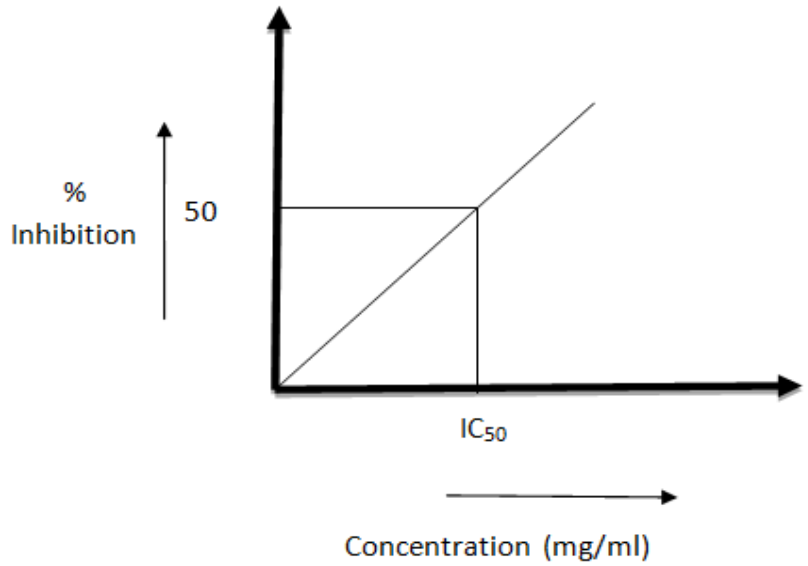


Figure 4.2: Plotting Concentration vs. % Inhibition graph for evaluate IC_{50}.

4.4 Results and discussion

4.4.1. Free radical scavenging activity (DPPH)

In this examination, HEX presented the highest free radical scavenging activity with an IC_{50} value 61.86 µg/mL for the roots of ***Piper chaba***. At the similar period the DCM, EAC and ME also demonstrated antioxidant potential having IC_{50} value 37.05, 15.43, and 25.78 µg/mL respectively.

Table 4.1: IC_{50} values of the roots of ***Piper chaba*** standard and partition extract.

Plant part	Sample code	Test Sample	IC_{50} (µg/mL)
Roots of *Piper chaba*	**HEX**	n-hexane soluble partitionate	61.86
	DCM	Dichloromethane soluble partitionate	37.05
	EAC	Ethyl acetate soluble partitionate	15.43
	ME	Methanol extract	25.78
Standard	**BHT**	Tert-butyl-1-hydroxytoluene	51.76

Table 4.2: IC_{50} value of tert-butyl-1-hydroxytoluene (BHT)

Test tube No.	Abs. of Control	Conc. of sample (µg/mL)	Abs. of sample	% inhibition	IC_{50} (µg/mL)
1	0.258	500	0.012	95.93	51.76
2		250	0.049	82.16	
3		125	0.104	60.98	
4		62.5	0.148	44.63	
5		31.25	0.216	19.37	
6		15.625	0.225	15.88	
7		7.813	0.246	8.28	
8		3.906	0.253	5.68	
9		1.953	0.262	2.87	
10		0.977	0.273	0.75	

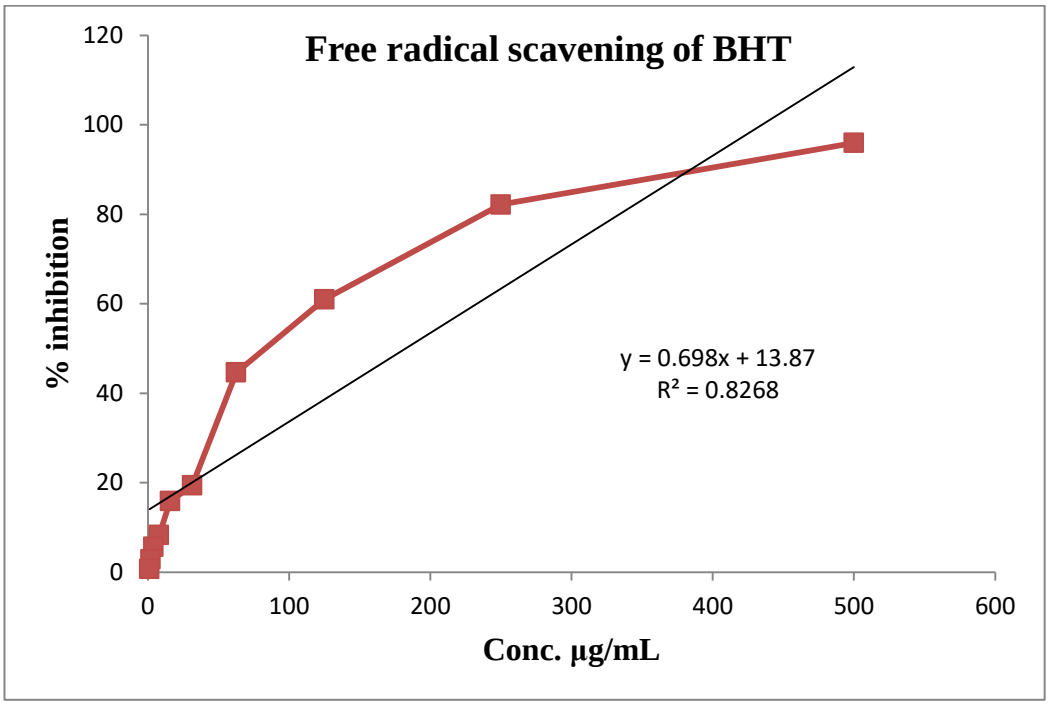


Figure 4.3: IC_{50} value of tert-butyl-1-hydroxytoluene (BHT)

Table 4.3: IC_{50} value of n-Hexane soluble partitionate (HEX) of roots of ***Piper chaba***

Test tube No.	Abs. of Control	Conc. of sample (µg/ml)	Abs. of sample	% inhibition	IC_{50} (µg/mL)
1	0.258	500	0.031	87.10	61.86
2		250	0.027	88.59	
3		125	0.087	65.92	
4		62.5	0.262	38.77	
5		31.25	0.207	22.62	
6		15.625	0.253	5.18	
7		7.813	0.257	5.20	
8		3.906	0.252	4.04	
9		1.953	0.242	7.21	
10		0.977	0.261	0.72	

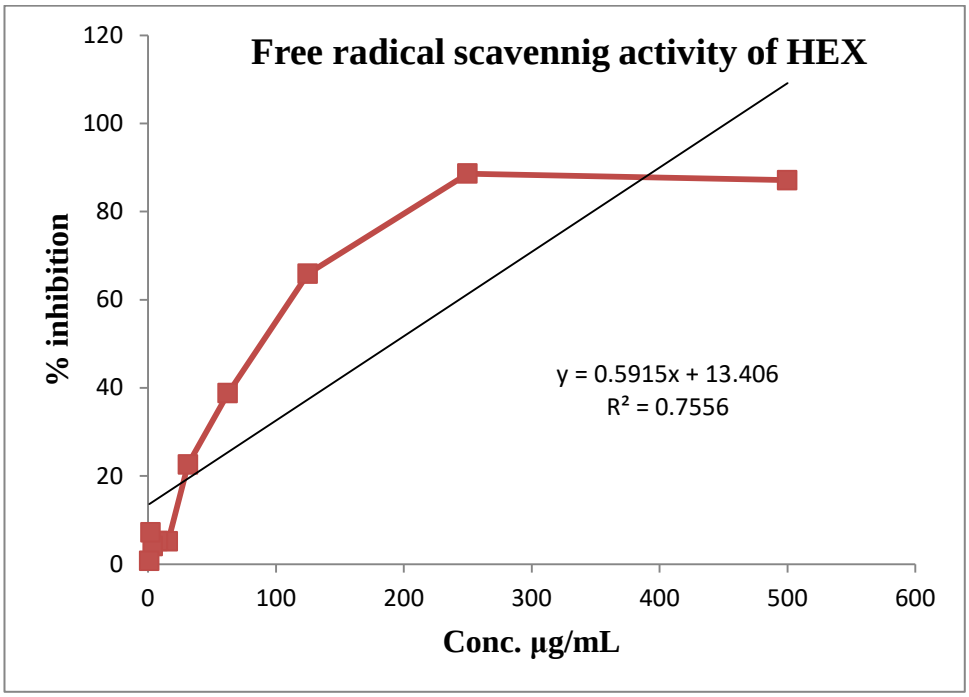


Figure 4.4: IC_{50} value of n-hexane soluble partitionate of roots of ***Piper chaba***

Table 4.4: IC_{50} value of Dichloromethane soluble partitionate (DCM) of roots of ***Piper chaba***

Test tube No.	Abs. of Control	Conc. of sample (µg/ml)	Abs. of sample	% inhibition	IC_{50} (µg/ml)
1	0.258	**500**	0.081	68.52	37.05
2		**250**	0.031	86.61	
3		**125**	0.017	93.30	
4		**62.5**	0.024	90.31	
5		**31.25**	0.091	65.04	
6		**15.625**	0.152	42.34	
7		7.813	0.201	24.52	
8		3.906	0.231	12.02	
9		1.953	0.242	8.53	
10		0.977	0.240	6.02	

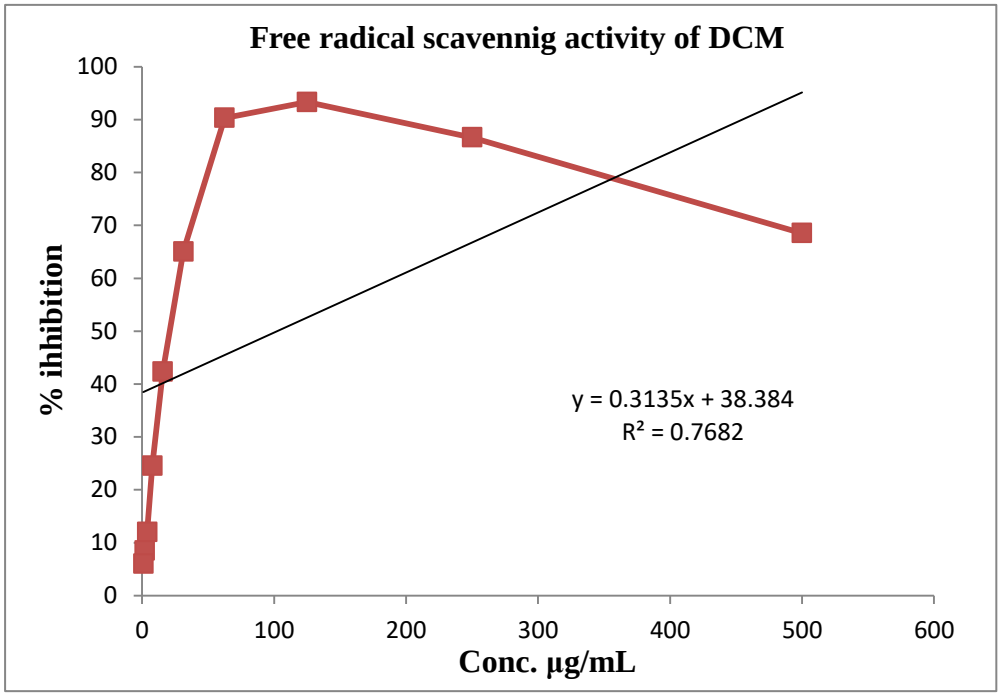


Figure 4.5: IC_{50} value of DCM soluble partitionate of roots of ***Piper chaba***

Table 4.5: IC_{50} value of ethyl acetate soluble partitionate (EAC) of roots of ***Piper chaba***

Test tube No.	Abs. of Control	Conc. of sample (µg/ml)	Abs. of sample	% inhibition	IC_{50} (µg/ml)
1	0.258	500	0.017	94.06	15.43
2		250	0.012	95.16	
3		125	0.007	96.64	
4		62.5	0.002	98.63	
5		31.25	0.006	96.40	
6		15.625	0.071	71.32	
7		7.813	0.134	48.60	
8		3.906	0.189	29.32	
9		1.953	0.231	13.74	
10		0.977	0.242	9.27	

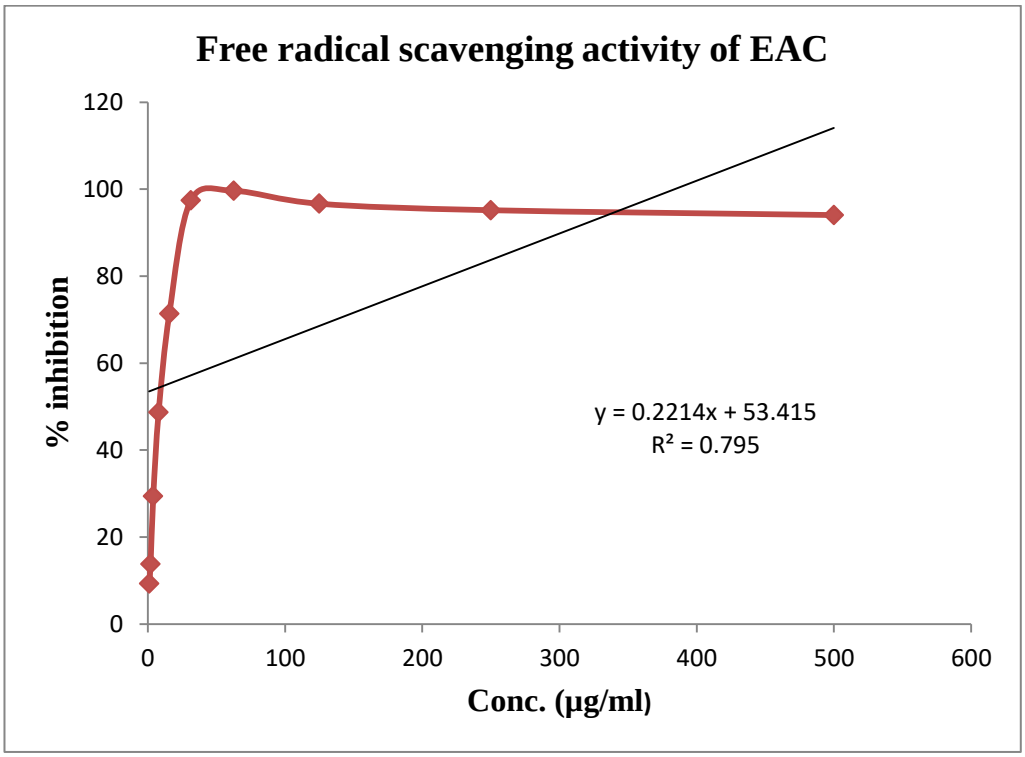


Figure 4.6: IC_{50} value of EtOAc soluble partitionate of roots of ***Piper chaba***

Table 4.6: IC_{50} value of methanolic extract (ME) of roots of ***Piper chaba***

Test tube No.	Abs. of Control	Conc. of sample (µg/ml)	Abs. of sample	% inhibition	IC_{50} (µg/ml)
1	0.258	500	0.010	95.81	25.78
2		250	0.012	95.12	
3		125	0.010	96.27	
4		62.5	0.001	99.24	
5		31.25	0.025	89.92	
6		15.625	0.120	54.25	
7		7.813	0.140	44.14	
8		3.906	0.226	15.62	
9		1.953	0.247	7.82	
10		0.977	0.244	7.71	

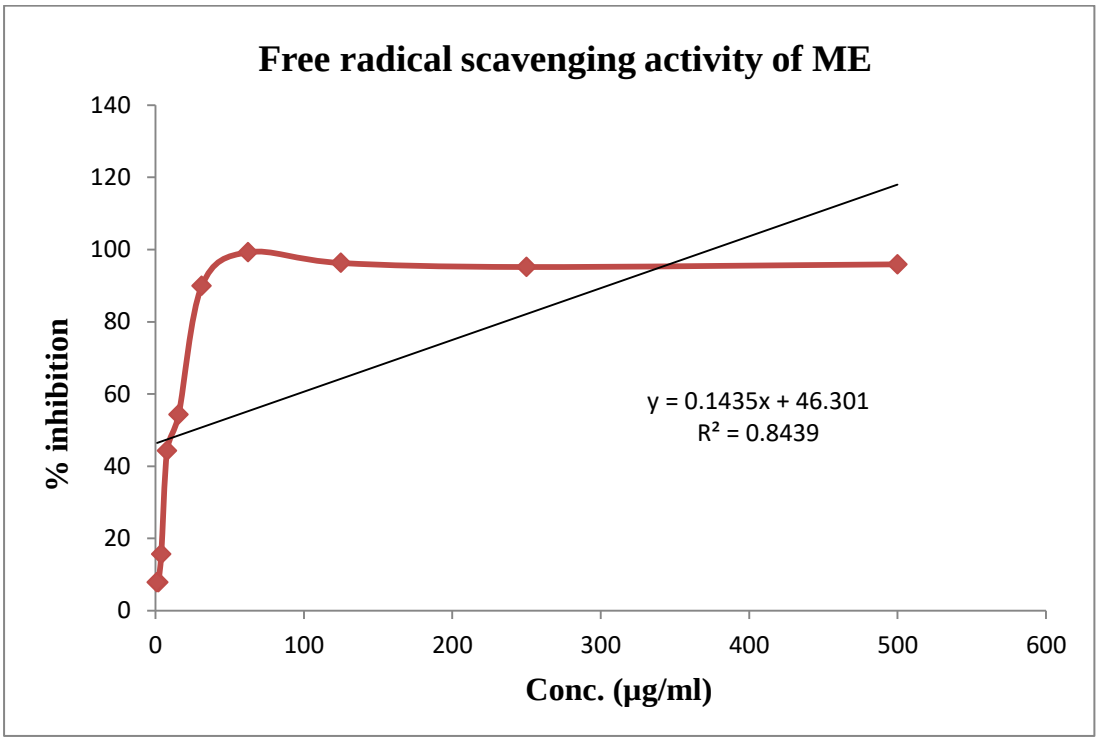


Figure 4.7: IC_{50} value of methanolic extract of roots of ***Piper chaba***

ANTIMICROBIAL SCREENING

5.1. Principle of disc diffusion method

In this classical method, on nutrient agar medium uniformly seeded with the test microorganisms dried and sterilized filter paper discs (6 mm diameter) containing the test samples of known amounts are placed. Antibiotics diffuse from a confined source through the nutrient agar gel and create a concentration ascent. Standard antibiotic (ciprofloxacin) discs and blank discs are used as positive and negative control.

To allow maximum diffusion of the test materials to surrounding media these plates are kept at low temperature (4°C) for 16 to 24 hours (Barry, 1976). For optimum growth of the organisms the plates are then inverted and incubated at 37°C for 24 hours.

The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The diameter of zone of inhibition expressed in millimeter is then measured to determine antimicrobial activity of the test agent (Barry, 1976; Bayer *et al.*, 1966).

In the present study the crude extracts as well as fractions were tested for antimicrobial activity by disc diffusion method. The experiment is carried out more than once and the mean of the readings is required (Bayer *et al.*, 1966)

5.2 Experimental procedure

The experiment was done by following the method illustrated by Bayer *et al.*, 1966

5.2.1 Reagents and apparatus

Apparatus : Filter paper discs petri plate, inoculating ring, sterile cotton, sterile forceps, spirit burner, micropipette screw cap test tubes, nose mask , Hand gloves, Laminar air flow hood, Autoclave, Incubator**,** Refrigerator

Reagents: Nutrient agar medium, chloroform and ethanol

5.2.2 Test materials

Partitioned crude n-hexane, ethyl acetate extract, methanol, ethanol extract and five pure compounds. 300 μg per disc the amount of test sample was taken.

5.2.3 Test Organisms

Table 5.1: List of Test Bacteria and Fungi

Gram-positive Bacteria	Gram-negative Bacteria	Fungi
Sarcina Lutea	*Escherichia coli*	*Sacharomyces cerevacae*
Bacillus megaterium	*Pseudomonas Aureus*	*Aspergillus niger*
Staphylococcus aureus	*Shigella boydii*	*Candida albicans*
Bacillus cereus	*Salmonella typhi*	
Bacillus subtilis	*Salmonella paratyphi*	
	Shigella dysenteriae	
	Vibrio mimicus	

Table 5.2: Antimicrobial activity of crude n-hexane, ethyl acetate, methanol and ethanol extracts of *Piper chaba.*

Bacteria and fungi	n-hexane extract	EtOAc extract	methanol extract	ethanol extract	kanamycin
	300 (µg/disc)				30(µg/disc)
Gram-positive bacteria					
Bacillus cereus	---	6.5	6.5	6.5	32
Bacillus megaterium	---	6.5	--	6.5	32
Bacillus subtilis	14	13	---	---	33
Staphylococcus aureus	13	11	14	13	33
Sarcina lutea	12	12	---	---	33
Gram-negative bacteria					
Escherichia coli	12	11	12	10	33
Salmonella paratyphi	10	10	---	---	33
Salmonella typhi	12	11	---	---	33
Shigella boydii	---	9	---	---	33
Shigella dysenteriae	12	11	---	---	33
Vibrio mimicus	13	9	---	---	32
Pseudomonas aeruginosa	6.5	6.5	7	7	33
Fungi					
Candida albicans	13	10	---	---	32
Aspergillus niger	10	18	18	11	32
Sacharomyces cerevacae	13	9	---	---	32

"----------" Indicates 'No activity'

Table 5.3: Antimicrobial activity of the five compounds (com-1, com-2, com-3, com-4 and com-5) of EtOAc extract of the roots of *Piper chaba.*

Bacteria and fungi	Comp-1	Comp-2	Comp-3	Comp-4	Comp-5	Kanamycin
	30(µg/ disc)					
Bacillus cereus	---	7	---	9	---	32
Bacillus megaterium	---	13	---	---	---	32
Bacillus subtilis	6	---	---	---	---	32
Staphylococcus aureus	11	12	8	5	---	33
Sarcina lutea	....	---	---	---	---	33
Escherichia coli	12	---	---	---	---	33
Salmonella paratyphi	10	---	---	---	---	33
Salmonella typhi	10	---	---	---	---	33
Shigella boydii	9	---	---	---	---	33
Shigella dysenteriae	11	---	---	---	---	33
Vibrio mimicus	9	---	---	---	---	32
Vibrio parahemolyticus	10	---	---	---	---	33
Candida albicans	10	---	---	---	---	32
Aspergillus niger	10	14	---	---	---	32
Sacharomyces cerevacae	9	---	---	---	---	32

"---" Indicates 'No activity'

5.3 Results and discussion of *in vitro* Antimicrobial screening of the roots of *Piper chaba* H.

Separated crude methanol, ethanol, n-hexane and ethyl acetate extracts and five pure compounds were tested (**comp-1, comp-2, comp-3, comp-4 and com-5**) for antimicrobial activity against a number of both Gram-positive and Gram-negative bacteria and fungi. Standard disc of kanamycin (30µg/disc) was used for comparison purpose.

The crude n-hexane extract displayed slight antimicrobial action in contradiction of most of the test organisms accept *Bacillus cereus, Bacillus megaterium* micro-organisms **(Table: 5.2)** and the ethyl acetate extracts exhibited antimicrobial activity against all of the test micro-organisms and fungi **(Table: 5.2)**. The methanol extract exhibited mild antimicrobial activity against the Gram-positive micro-organisms *Bacillus cereus, Staphylococcus aureus,* Gram-negative *Escherichia coli, Pseudomonas aeruginosa* and fungi *Aspergillus niger.* The ethanol extract exhibited mild antimicrobial activity against Gram-positive micro-organisms *Bacillus cereus, Bacillus megaterium, Staphylococcus aureus and* Gram-negative bacteria, *Pseudomonas aeruginosa and Escherichia coli.*

The only one compound **(compound-1)** showed the similar activity as n-hexane crude extract (9-12 mm inhibition zone) against all the micro-organisms and fungi except *Bacillus cereus, Bacillus megaterium*. The rest of the pure **compound-2** (**Piperine), compound-3 and compound-4** showed resistance against the three *Bacillus cereus, Bacillus megaterium* and *Staphylococcus aureus* micro-organisms and the fungi *Aspergillus niger.* The one pure compound (**compound-5**) of ethyl acetate extract exhibited no antimicrobial activity against most of the test organisms and fungi **(Table: 5.3)**

INTRODUCTION

1.0 Description of *Bombax ceiba* L

Bombax ceiba is a lofty, deciduous tree up to 45 m tall and 7 m or more in girth with horizontally spreading branches and young stems covered with stout, hard prickles. The bark is pale ash to silver grey in color. Flowers are large in diameter, red in color and numerous with copious nectar. The fruits are brown capsule-like upto 15 mm long, filled with numerous black seeds which are irregular ovoid in shape, smooth and oily with dense silky hair. The fruit pulp is sweet and edible. Shemul trees have compound leaves which are palmate in appearance. It is digitate, large, spreading, glabrous which has common petiole and the size of leaf is 15-30 cm long. The size of the leaflets varies from 10 cm to 20 cm. New leaves usually do not appear until flowering is over. The young stem and branches are covered with sharp, straight, stout prickles up to 1.2 cm long with woody conical bases.

1.1. Taxonomy

Kingdom : Plantae – Plants

Subkingdom : Tracheobionta – Vuscular plants

Superdivision : Spermatophyta – Seed plants

Division : Magnoliophyta– Flowering plants.

Class : Magnoliopsida – Dicotyledons

Subclass : Dilleniidae

Order : Malvales

Family : Bombacaceae –Kapok –tree family

Genus : *Bombax* L.

Species : *Bombax ceiba* L.

Scientific name : *Bombax ceiba* L.

Synonym : *Bombax malabaricum DC. Salmalia malabaricum DC.*

Common names : Silk cotton tree, kapok tree.

Local name : Shemul

1.3 General botanical data

Plant structure: Trees, 10-15m tall, crown often dome-shaped. Branches verticillate. Bark yellow-brown. Foliage buds 2-5 mm long, occasionally longer, the primary scales often spreading.

Bark: Bark of *Bombax ceiba* looks pale ashy to silver grey, 1.8 -2.5 cm thick, smooth up to middle age, becoming rough with irregular vertical cracks on older trees.

Leaves: *Bombax ceiba* tree has the compound leaves which is palmate in appearance. It is exactly appears as the palms appear in man. It is digitate, large, spreading, glabrous which has common petiole, and the size of leaf is 15-30 cm long. One leaf is composed of several leaflets. Five leaflets are common in one leaf but sometimes up to the seven leaflets could be found. The size of leaflets varies from 10 to 20 cm. generally the leaflets found in the centre are longer as in the fingers in palm. The leaflets are lanceolate, acuminate, more or less coriaceous and entirely glabrous.

Flowers: The bright red flowers, which appear in January to March, are large and conspicuous on the leafless trees. It presents a strikingly remarkable sight in winter and spring when the usually bare branches are covered with large, fleshy, red flowers. Birds are attracted to them, and are probably responsible for their pollination. These flowers form a scarlet carpet on the ground for few weeks (2-3 weeks) after dropping. The flowers of *Bombax ceiba* are very showy, attractive and visible from long distances also. Because of its beautiful and attractive flowers, people like to plant it as the ornamental plant in the botanical garden, garden or as the avenue species. Flowers are numerous, large, 10-12.5 cm across. It clustered towards the ends of branches at the time of flowering. It has the thick, fleshy and cup shaped Sepals. It bears generally 5 petals in one flower which are 7.5-15 cm long oblong, recurved above, and fleshy, of bright crimson (rarely yellow or orange) color.

Capsule: The pods are about 12-21 cm in length, oblong-oval in shape, locucidally 5 valved; valves woody, downy outside, lined with silky hairs within.

Seeds: Within the capsule it has many seeds which are ovoid, smooth, 5-8 mm long in size. These seeds are oily and surrounded by a thick mass of long silky hairs or floss, hence easily blown about by wind (Rajendra, K.C. 2007).

Roots: The morphological studies of roots *of Bombax ceiba* show the shape of the root is more or less cylindrical, slightly tapering. Color of the peeled root is yellowish brown to

dark brown while a peeled root is pale yellow with a rough surface. All the organoleptic features studied have been summarized in **Table.1.2**

Table 1.1: Organoleptic features of *Bombax ceiba* roots

Features	Observations
Shape	Cylindrical
Width	2-6 cm
Length	18-51 cm
Color	Peeled-pale yellow, unpeeled-yellowish brown to dark brown
Odor	Faint and characteristic
Taste	Characteristic, free from bitterness

1.4 Photographs of *Bombax ceiba* L.

(A) Leaves of *Bombax ceiba*

(B) Flowers of *Bombax ceiba*

(C) Fruits of *B. ceiba*

(D) Root of *B. ceiba*

Figure 1.1: General outlook of *Bombax ceiba* (A) Leaves of *B. ceiba* (B) Flowers of *B. ceiba* (C) Fruits of *B. ceiba* (D) Root of *B. ceiba*

Root: Sesquiterpene lactone isolated from the roots of a plant species identified as *Salmalia malbaricum* (syn. *Bombax ceiba*) was previously identified as hemigossylic acid lactone-7- methyl ether. 2D NMR experiments have shown that this was a new compound, isohemigossylic acid lactone-2-methyl ether (Puckhaber LS *et.al,* 2001). A detailed exploration of phytochemical properties along with the TLC ratios of various extracts of B.

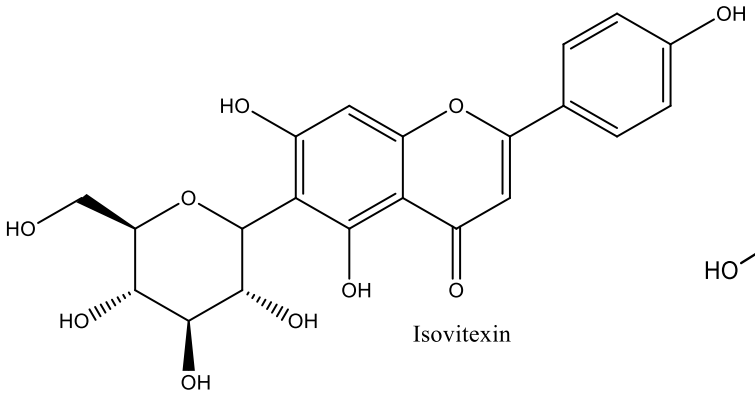
ceiba was also conducted which showed that the alcoholic and water extracts indicate the presence of alkaloids, flavonoids, glycosides, coumarins, proteins and amino acids.

Stem bark: It is reported that it contains lupeol, β-sitosterol, shamimicin ceiba naphthaquinone, simailn-A and simalin-B, mangiferin, epicatechin-7-O-β -xylopyranoside, epicatechin-3-O-β-xylopyranoside, shamiminol, stigmasta-3,5-diene, lupenone and opuntiol.

Flowers: Three new phenolic compounds and twenty known compounds were isolated from the flowers of the *Bombax ceiba*.Their chemical structures were elucidated by spectroscopic analyses (IR, ESI-MS, HR-ESI-MS, 1D- and 2D-NMR) (Yu-Bo Zhang *et.al*, 2015).

1.5 General description and chemical investigation of *Bombax ceiba* L.

Sesquiterpene lactone isolated from the roots of a plant species identified as *Salmalia malbaricum* (syn. *Bombax ceiba*) was previously identified as hemigossylic acid lactone-7-methyl ether. 2D NMR experiments have shown that this was a new compound, isohemigossylic acid lactone-2-methyl ether (Puckhaber LS *et.al*, 2001). A detailed exploration of phytochemical properties along with the TLC ratios of various extracts of B. ceiba was also conducted which showed that the alcoholic and water extracts indicate the presence of alkaloids, flavonoids, glycosides, coumarins, proteins and amino acids. It is reported that it contains lupeol, β-sitosterol, shamimicin ceiba naphthaquinone, simailn-A and simalin-B, mangiferin, epicatechin-7-O- β -xylopyranoside, epicatechin-3-O- β -xylopyranoside, shamiminol, stigmasta-3,5-diene, lupenone and opuntiol.



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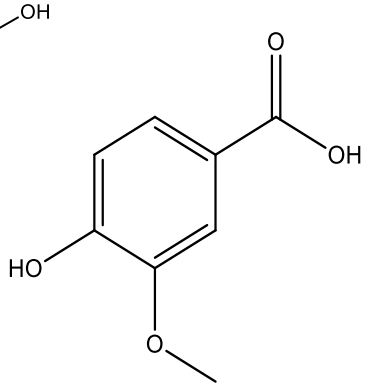


Vanillic acid

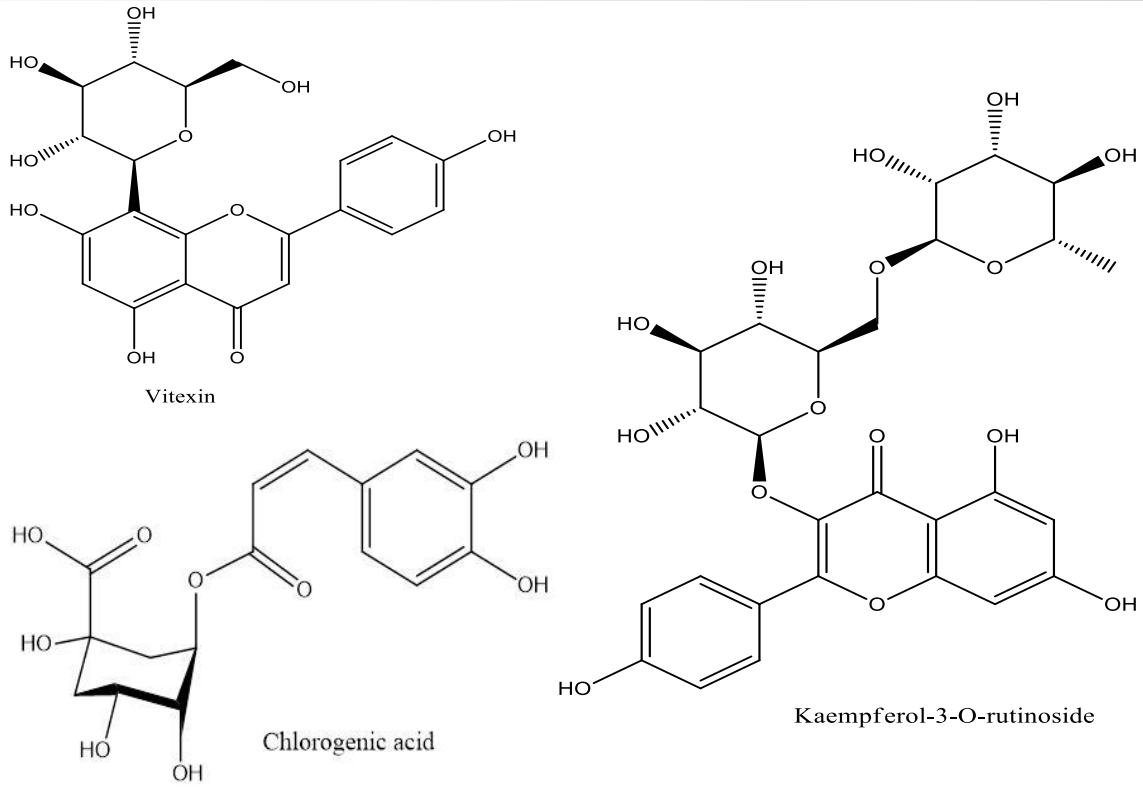


Figure 1.2: Phytoconstituents of *Bombax ceiba*

1.6 Medicinal importance of *Bombax ceiba* L.

Nocturnal Emission, Semen Problems: A detailed phytochemical analysis has been carried out on the roots of plant *Bombax ceiba* L. Tender roots of Bombax ceiba took, cleaned and dried in shade. Then dried to make powders. This powder cures seminal disorder and nocturnal emission.

Anti-inflammatory Activity: The aqueous extract of *Bombax ceiba* showed a certain beneficial effects, suggesting this plant has a protective role on inflammatory bowel disease cases.

Anti-diarrhoeal: Diarrhea is one of the top ten causes of death worldwide and is a leading cause of death in children in developing countries; especially under five years of age. The bark juice was mixed with the bark juice of *Magnifera indica* and *P. guajava* and drunk to cure dysentery and intestinal spasm. The resin was also taken orally to treat worms and diarrhoea; root juice was consumed to treat abdominal pain and gonorrhea.

Muscular Injury: B. ceiba barks and roots were used to treat muscular injury.

Blood Purification: Leaves of Bombax ceiba taken to grind with water and filtered. Drinking this works as blood purifier.

Leucorrhoea: Taking *Bombax ceiba* root powder twice a day with water used as a treatment.

Over Bleeding in menstruation: *Bombax ceiba* root powder (100g), mulethi (50g) , swarn geru (25g) mixed, taking this powder twice a day with water or milk reduces this problem.

Acne, Skin Blemish and Pigmentation: Thorny part of stem of shemul tree taken to make paste with water. Applying on affected area also lightens scar marks due to boils, freckles, acne vulgarize and burns.

Cold and Cough: Shemul root powder mixed with black pepper and dry ginger powder. Taking in small amount to cure cold and cough.

Anthelmintics: *Bombax ceiba* leaves exerted anthelmintic effects, a property that some plants and medicines have to help the body expel helminthes or parasitic worms.

Wounds: Paste of its bark can applied on wound.

Weakness: From its flower green base part taken, cleaned and dried in shade. Then grinded to make powder. Mixing one spoon powder, honey (2 table spoon), desi ghee (1 table spoon) in milk and drink to recover weakness.

Improve Breast Milk: The bark of semal root cleaned, dried and grinded to make powder. Taking twice to improve breast milk.

Leprosy: The seeds and roots of B. ceiba were used in the treatment of leprosy.

Anti-infertility Activity: *Bombax ceiba* L. possessed anti-fertility and pregnancy-terminating effects and stopped spermatogenesis in male rats. None of the female mice that daily consumed *Bombax ceiba* L mixed food became pregnant when mated with the male of potential fertility who did not receive the treatment.[73] The observations suggested an anti-spermatogenic or anti-androgenic abilities as well as ovulation preventive effect of the plant. Hence, using of the herb during pregnancy should be avoided. *Bombax ceiba* L. proved to affect spermatogenesis in rats by preventing cytokinesis of the dividing spermatogenic cell lines with appearances of sertoli cell damage and a spermatotoxic effect.

Miscellaneous Uses: Root bark extract was given as a tonic in case of sexual debility and also as nervine tonic. Root powder mixed with sugar candy and milk was taken to avoid impotency. The roots powdered with those of Chlorophytum, Capparis sepiaria and fruits of Pedalium murex were taken with water as a tonic for 7–8 days to calm body heat (Chaudhary, H.P. *et al.* 2012 ; Rameshwar, V. *et al.* 2014).

EXPERIMENTAL

2.1 Collection of the plant and identification of species

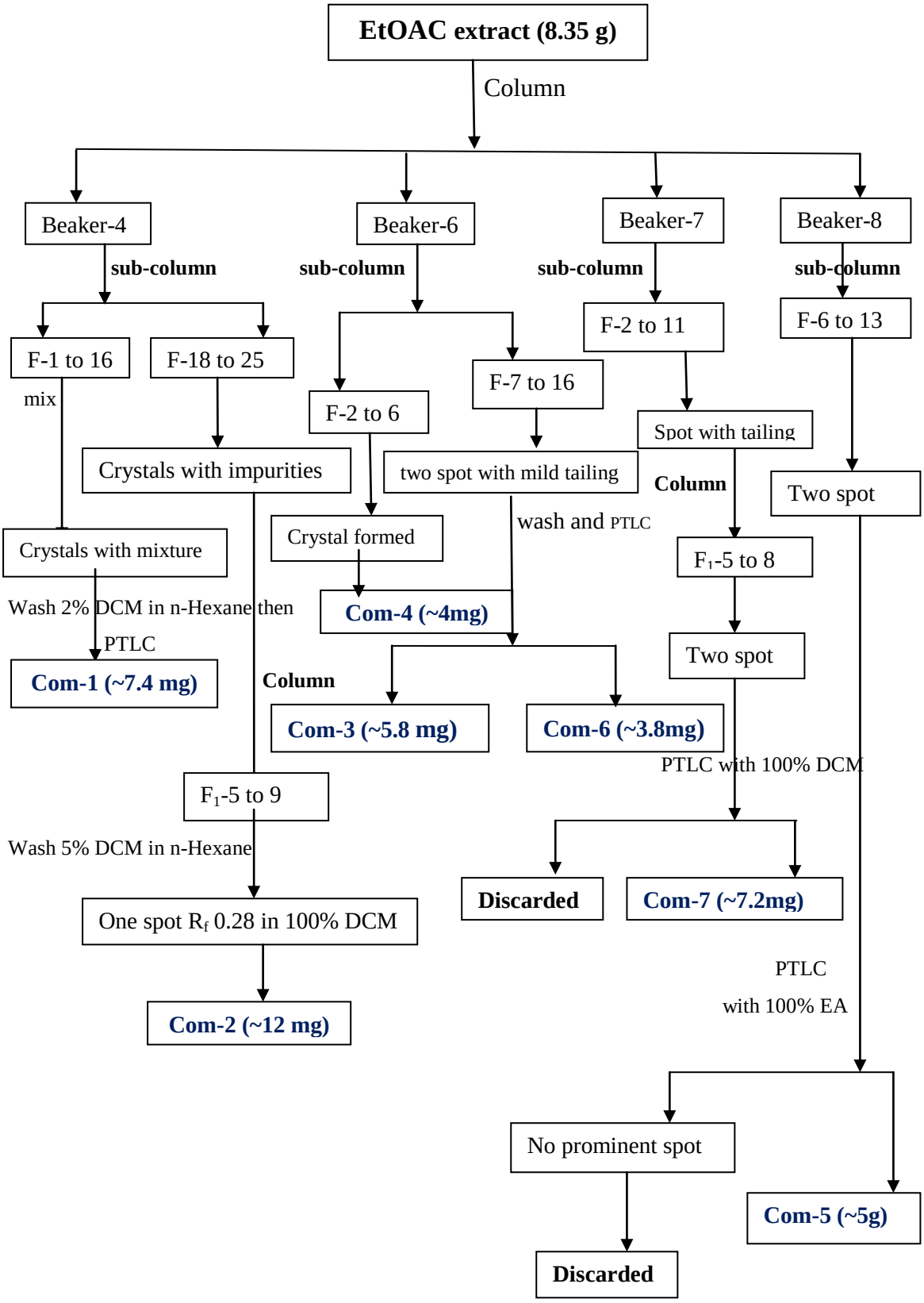
The roots of the plant of *Bombax ceiba* L. was collected from Boraigram Upazila in the district of Nator, Bangladesh. The taxonomy of the plant was confirmed consulting with the National Herbarium's Botanist. A voucher specimen of this plant was deposited at Bangladesh National Herbarium.

2.2 Phytochemical screening (Edeoga H.O. *et.al*. 2005) of the roots of *Bombax ceiba* L.

This experimental works are same and was discussed in previous section Part-A **page 24 to 25.**

2.3 Extraction, partition and Fractionation of the compounds from the roots of *Bombax ceiba* L.

This experimental works are same and was discussed in previous section Part-A **page 23 to 35.**



Scheme of isolating different compounds from the ethyl acetate extract of the roots of *Bombax ceiba* L.

2.4 Isolation and purification of the compound-1, 2, 3, 4, 5, 6 and 7 by column chromatography

This experimental works are same and was discussed in previous section Part-A **page 38 to 45**

2.5. Characterization of the isolated compounds

2.5.1. Physical properties of the compound-1

Physical state : A white crystalline solid

Solubility : Soluble in dichloromethane

Melting point : 150-152 ^{o}C

R_f value : R_F value 0.69 (100 % Dichloromethane)

Amount : ~4.5 mg.

^{1}H-NMR Spectroscopic Analysis

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound-1 showed peaks at δ 13.9 (1H, s), 11.0 (1H, s), 7.69 (1H, s), 6.94 (1H, s), 6.28 (1H, s), 3.78 (3H, s), 3.66 (1H, sept, J = 6.8 Hz), 2.46 (3H, s) and 1.38 (6H, d, J = 6.8 Hz) ppm.

^{13}C-NMR Spectroscopic Analysis

The ^{13}C-NMR spectrum (100 MHz, $CDCl_3$) of compound-1 showed main chemical shift at δ 197.14, 165.98, 157.41, 148.69, 140.70, 125.16, 123.52, 122.38, 121.731, 113.43, 110.24, 59.60, 29.40, 23.11 and 16.25 ppm.

2.5.2. Physical properties of the compound-2

Physical state : A white crystalline solid

Solubility : Soluble in DCM

Melting point : 210-211 ^{o}C

R_f value : R_F value 0.27 (in 100% Dichloromethane)

Amount : ~7.5 mg.

^{1}H-NMR Spectroscopic Analysis

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound-2 showed peaks at δ 7.53 (1H, s), 7.02 (1H, s), 4.34 (3H, s), 3.67 (1H, sept. J= 6.8 Hz), 2.43 (3H, s), 1.41(6H, d, J=6.8 Hz) ppm.

^{13}C-NMR Spectroscopic analysis

The ^{13}C-NMR spectrum (100 MHz, $CDCl_3$) of compound-2 showed main chemical shift at δ 167.7, 156.97, 156.57, 140.25, 132.00, 131.48, 130.09, 119.71, 118.19, 113.63, 98.85, 59.83, 29.69, 23.43 and 18.23 ppm.

2.5.3. Physical properties of the compound-3

Physical state : A white crystalline solid

Solubility : Soluble in DCM + few drops of CD_3OD

R_f value : R_F value 0.57 (DCM: EtOAc =5:1)

Amount : ~3.5 mg

^{1}H-NMR Spectroscopic Analysis

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$+ few drops of CD_3OD) of compound-3 (**Figure 4.13)** showed peaks at δ 7.45 (1H, s), 6.87 (1H, s), 3.60 (1H, sept, J=6.8 Hz), 2.40 (3H, s), 1.34 (6H, d, J=6.8 Hz) ppm.

^{13}C-NMR Spectroscopic analysis

The ^{13}C-NMR spectrum (100 MHz, $CDCl_3$+ few drops of CD_3OD) of compound-3 (**Figure 4.14)** showed main chemical shift at δ 167.7, 156.97, 156.57, 140.25, 132.00, 131.48, 130.09, 119.71, 118.19, 113.63, 98.85, 59.83, 29.69, 23.43 and 18.23 ppm.

2.5.4. Physical properties of the compound-4

Physical state : A white crystalline solid

Solubility : Soluble in EtOAc, DMSO and MeOH

Melting point : 199-201^{o}C

R_f value : R_F value 0.57 (in 100% EtOAc)

Amount : ~4.5 mg.

^{1}H-NMR Spectroscopic Analysis

The ^{1}H-NMR spectrum (400 MHz, DMSO-d_6) of compound-4 showed peaks at δ 9.44 (2H, brs.), 7.34 (1H, d, J= 2.0 Hz), 7.30 (1H, dd, J= 8.4, 2.0 Hz) and 6.78 (1H, d, J= 8.4 Hz) ppm.

^{13}C-NMR Spectroscopic analysis

The ^{13}C-NMR spectrum (100 MHz, DMSO-d_6) of compound-4 showed main chemical shift at δ 167.79, 150.45, 145.34, 122.34, 122.19, 117.03 and 115.61 ppm.

2.5.5. Physical properties of the compound-5

Physical state : A white crystalline solid

Solubility : Soluble in EtOAc, DMSO and MeOH

Melting point : 210-212^{o}C

R_f value : R_F value 0.43 (in 100% EtOAc)

Amount : ~3.8 mg.

^{1}H-NMR Spectroscopic Analysis

The ^{1}H-NMR spectrum (400 MHz in $CDCl_3$) of compound-5 showed peaks at δ 2.15, 2.45, 3.28, 3.45 due to (1H, m, H-5) and (1H, m, H-4) and aromatic proton 6.94, 6.97, 7.12 and 7.16 ppm.

^{13}C-NMR Spectroscopic analysis

The ^{13}C-NMR spectrum (100 MHz in $CDCl_3$) of compound-5 showed main chemical shift at δ 29.08, 47.3, 48.3, 71.03, 119.3, 122.8, 124.1, 125.8, 127.9, 142.04 and 163.06 ppm.

2.5.6 Physical properties of the compound-6

Physical state : A white crystalline solid

Solubility : Soluble in DCM, EtOAc, DMSO and MeOH

Melting point : 203-204^{o}C

R_f value : R_F value 0.34 (in 5% EtOAc in DCM)

Amount : ~4.9 mg.

^{1}H-NMR Spectroscopic Analysis

The ^{1}H-NMR (400 MHz, in $CDCl_3$+CD_3OD) spectrum of compound-6 displayed the aromatic proton signals at 6.214 (1H, d, J=9.6Hz) and 6.818(s), 6. 865 (s) and 7.574 (1H, d, J=9.6). The other signals from BC-9 at δ 1.217, 2.211(q), 3.910(s).

^{13}C-NMR Spectroscopic analysis

In the ^{13}C-NMR (100 MHz, in $CDCl_3$+CD_3OD) spectrum of compound-6 displayed 10 different peaks were observed. Presence of O-methoxy (-OCH_3) observed at δ 56.40, carbonyl group (C=O) observed at δ 161.80 ppm. Five non-oxygenated aromatic carbons at δ 113.00, 143.53, 107.73, 103.26, 111.40. Three oxygenated aromatic carbon at δ 144.43, 150.22 and 150.09 ppm.

2.5.7 Physical properties of the compound-7

Physical state	: A white crystalline solid
Solubility	: Soluble in DCM, EtOAc, DMSO and MeOH
Melting point	: 120-121^{o}C
R_f value	: R_F value 0.56 (in 100% DCM)
Amount	: ~5.4 mg.

^{1}H-NMR Spectroscopic Analysis

The ^{1}H-NMR (400 MHz, in $CDCl_3$+CD_3OD) spectrum of compound-7 displayed the methylene proton signals 0.65 – 1.54 (m) and 1.67 (3H, s), 1.91(1H, m), 2.37 (s), 3.2 (1H, d), and 4.56 (2H, s), (4.71 1H, s).

^{13}C-NMR Spectroscopic analysis

In the ^{13}C-NMR (100 MHz, in $CDCl_3$+CD_3OD) spectrum of compound-7 different peaks were observed. The compound showed main chemical shift at δ 38.7, 28.0, 79.0, 38.8, 55.3, 18.3, 34.4, 40.8, 50.4, 37.1, 20.9, 25.1, 38.0, 42.8, 27.45, 35.6, 43.0, 48.3, 48.0, 150.9, 29.8, 40.0, 29.7, 15.3, 16.1, 15.9, 14.5, 18.0, 109.3 and 19.3.

RESULTS AND DISCUSSION

3.0 Preliminary investigation of the plant material

This procedure are same and was discussed in previous section Part-A **page-40**

3.1. Characterization of compound-1

The compound-1 (~ 4.5 mg) was a colorless crystalline solid having R_f value: 0.69 (in 100% DCM) and its melting point was found to be 150-152^{o}C. It was soluble in dichloromethane. On spraying with vanillin-sulfuric acid spray reagent, followed by heating at 105°C for several minutes, purple color appeared.

The ^{1}H NMR (400 MHz, in $CDCl_3$) spectrum of compound-1 displayed the aromatic proton signals at 6.86 and 7.69. The other signals from compound-1 at δ 1.37 (6H, d, J= 6.8 Hz), 3.67 (1H, sept, J= 6.8Hz), 3.78 (3H, s), 6.28 (1H, s), 11.01 (1H, s) and 13.90 (1H, s) were assigned to isopropyl methyl, isopropyl methine, methoxyl, C-2 hydroxyl, aldehyde and C-7 hydroxyl protons respectively. These assignments are in close agreement with the structure of isohemigossypol-1-methyl ether **Ref. [17]** (A.V.B. Sankaram *et al.* 1981).

The ^{13}C NMR (100 MHz, in $CDCl_3$) spectrum of compound-1 showed the presence of carbonyl group at δ 197.15; ten types of aromatic carbons at δ 165.98, 157.41, 148.69, 140.70, 125.16, 123.52, 122.38, 121.73, 113.43 and 110.24; one saturated oxygenated carbon at δ 59.60; and three saturated carbons at δ 29.68, 23.11 and 16.25.

The HSQC experiment showed that the protons at δ 11.01, 7.69, 6.94, 3.78, 3.67, 2.45 and 1.37 are directly attached to the carbons at δ 197.15, 122.38, 113.43, 59.60, 29.68, 16.25 and 23.11, respectively.

The long-range proton-carbon coupling results of the HMBC experiment are shown in **Figure 3.1** is of the supporting information. The isopropyl methine proton (H-12) showed strong coupling to carbons 5 (δ 157.41), 6 (δ 113.43), and 13 and 14 (δ 23.11). The intense cross-peaks between the proton at δ 13.87 and carbons at δ 110.24, 165.98 and 113.43 which established that one hydroxyl group is attached to C-7. The other HMBC (H$\longrightarrow$C) correlations were shown in the **Figure 3.1** which supported the given structure of the compound compound-1 as isohemigossypol-1-methyl ether.

Table 3.1: ^{1}H NMR, ^{13}C NMR data, HSQC and long-range HMBC for compound-1

Position No.	Chemical shift δ in ppm*		HSQC ($^1H \longrightarrow ^{13}C$)	HMBC ($^1H \longrightarrow ^{13}C$)	
	δ_H (mult, J in Hz)	δ_C	^1J	^2J	^3J
1	-	140.70	-	-	-
2	-	148.69	-	-	-
3	-	123.52	-	-	-
4	7.69, s	122.38	C-4	-	C-2, C-5, C-9,C-15
5	-	157.41	-	-	-
6	6.94, s	113.43	C-6	C-7	C-8,C-10, C-12
7	-	165.98	-	-	-
8	-	110.24	-	-	-
9	-	125.16	-	-	-
10	-	121.73	-	-	-
11	11.01, s	197.51	C-11	C-8	C-7
12	3.67, sept. 6.8	29.68	C-12	C-5, C-13	C-6
13	1.37, d, 6.8	23.11	C-13	C-12	C-5,
14	1.37, d, 6.8	23.11	C-14	C-12	C-5
15	2.45, s	16.25	C-15	C-3	C-2, C-4
1-OMe	3.78, s	59.60	59.60	-	C-1
2-OH	6.28, s	-	-	-	-
7-OH	13.87, s	-	-	C-7	C-8, C-6

*^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) in $CDCl_3$

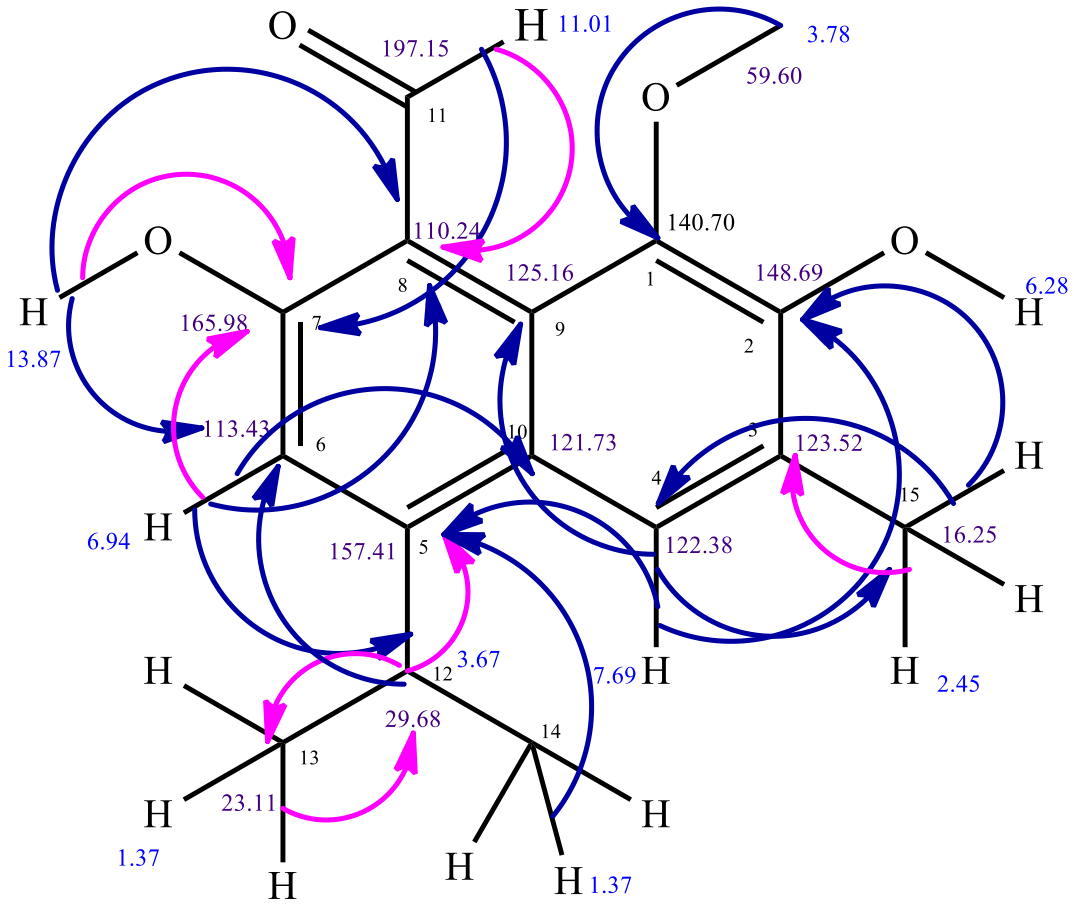


Figure 3.1: Structure of compound-1 showing ^{1}H NMR, ^{13}C NMR signals and HMBC (H⟶C) correlations

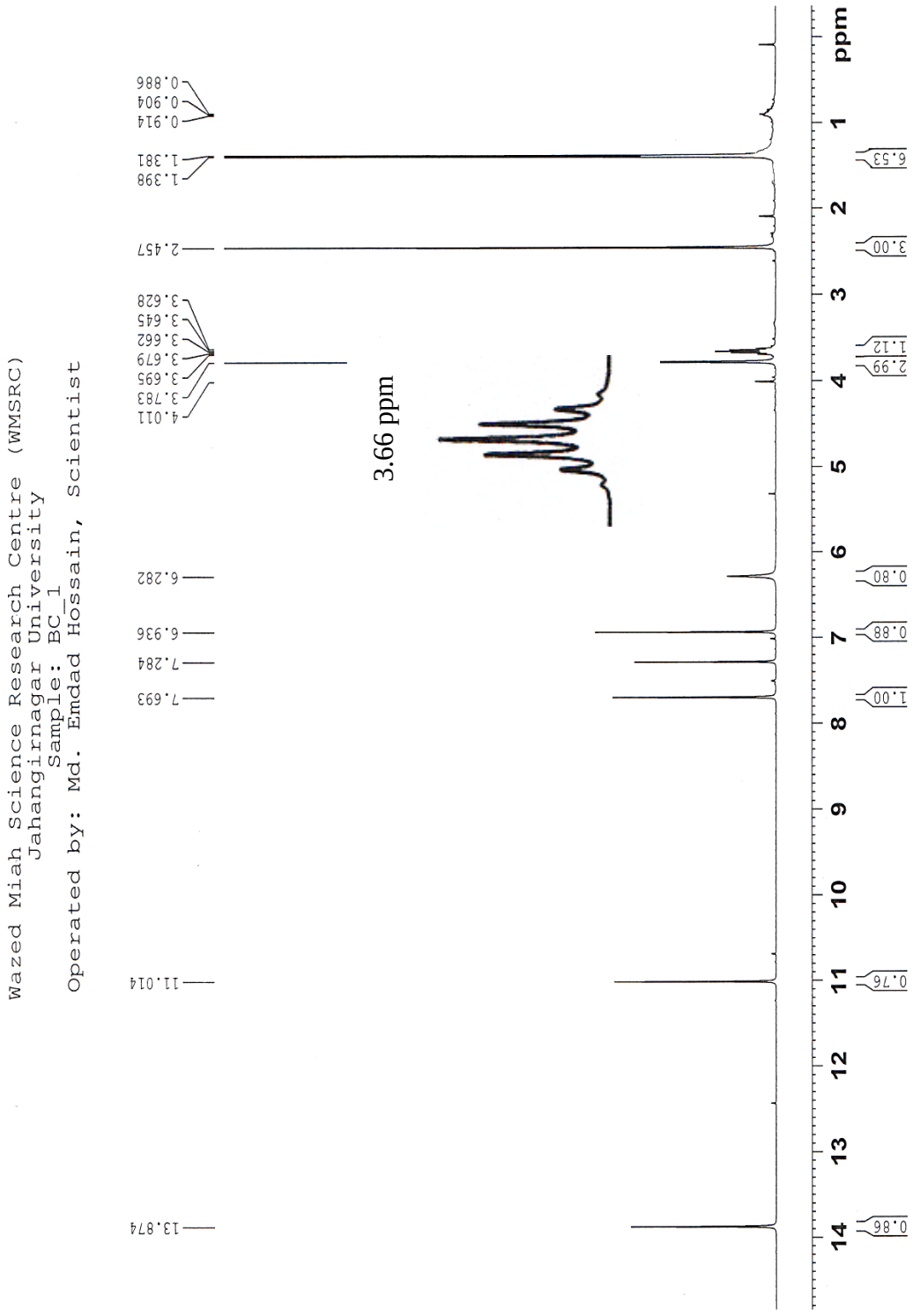


Figure 3.2: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of compound of compound-1

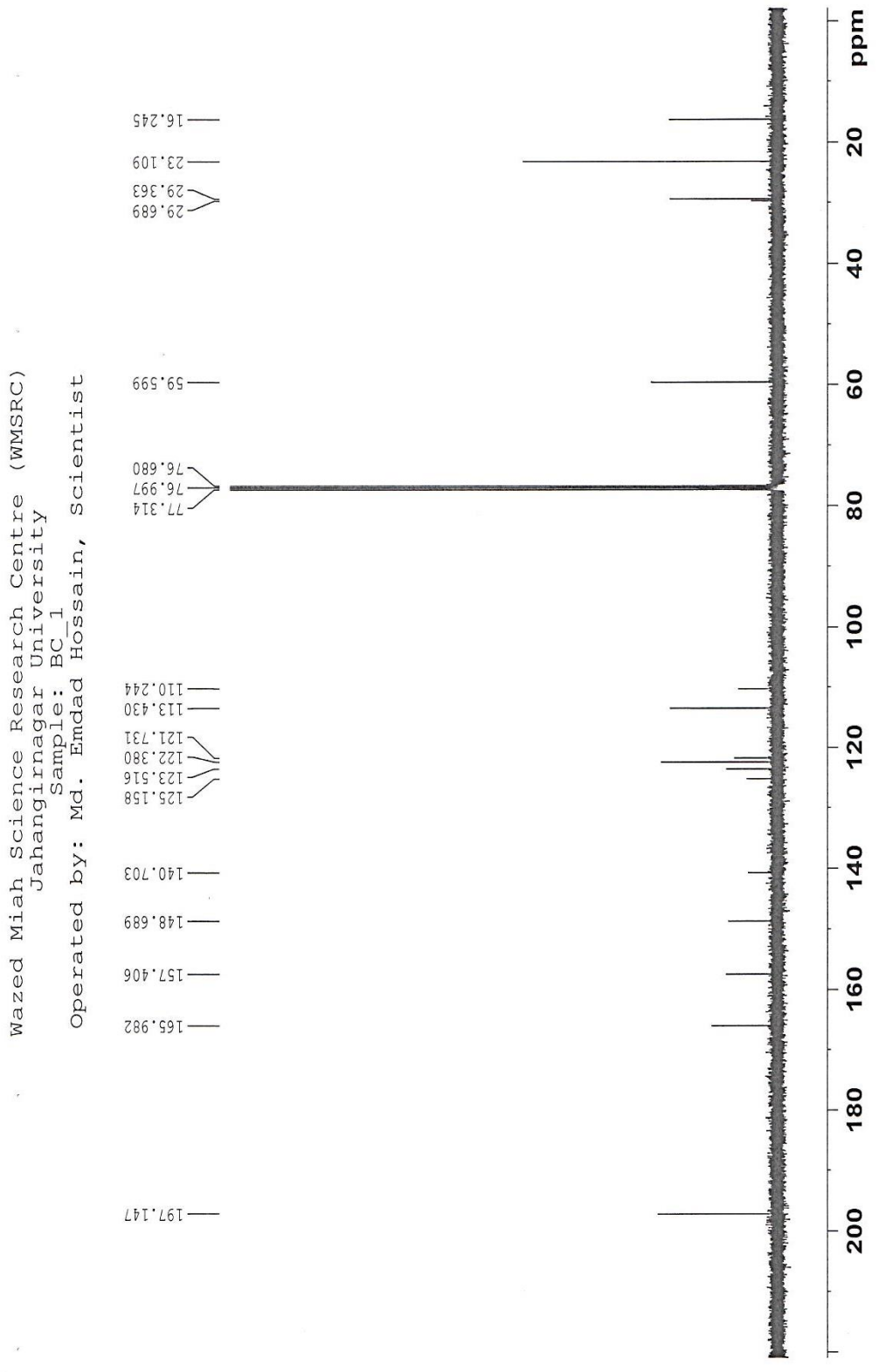


Figure 3.3: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of compound of compound-1

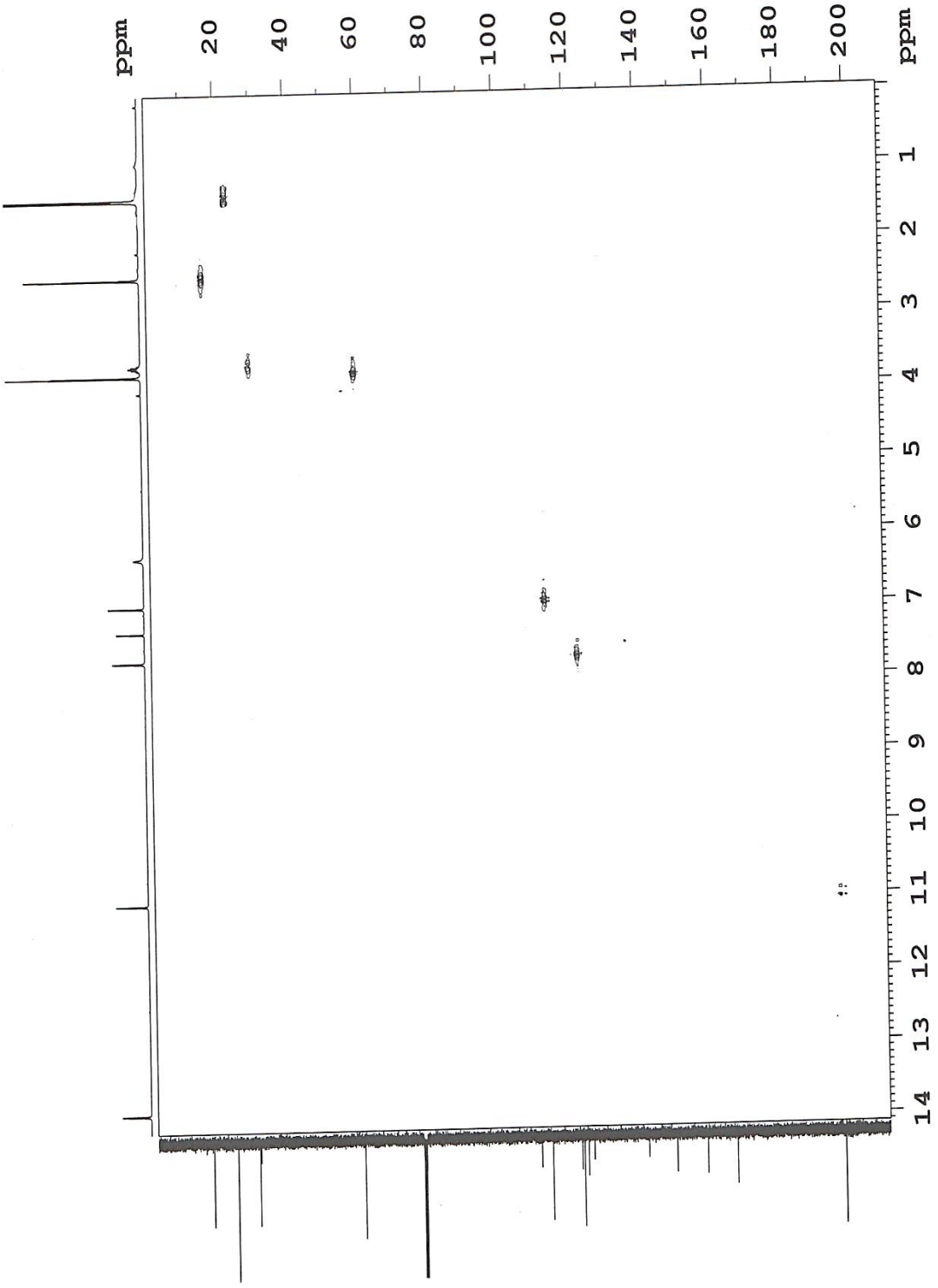


Figure 3.4: HSQC spectrum of compound -1

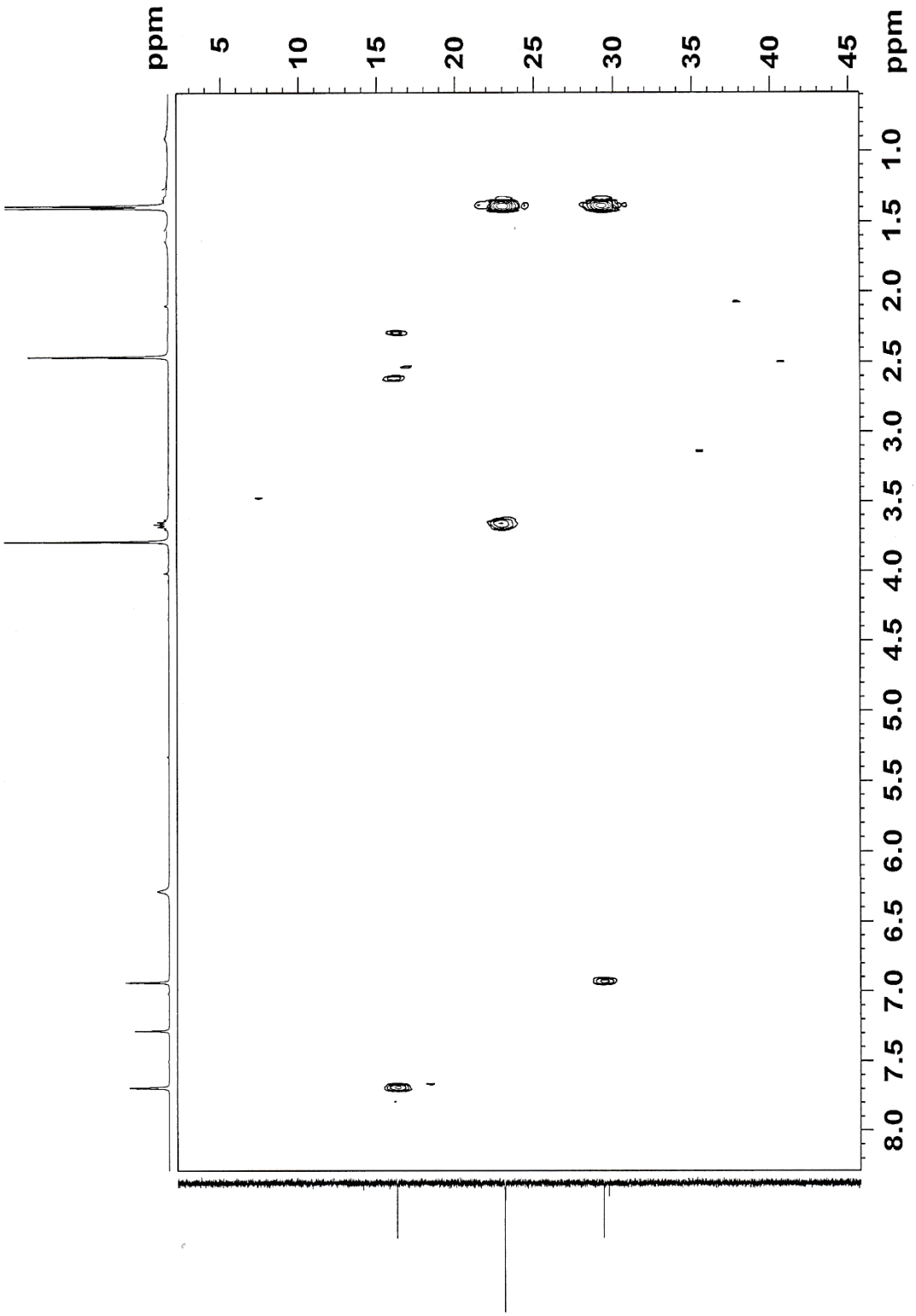


Figure 3.5: Partial HMBC spectrum of compound compound-1

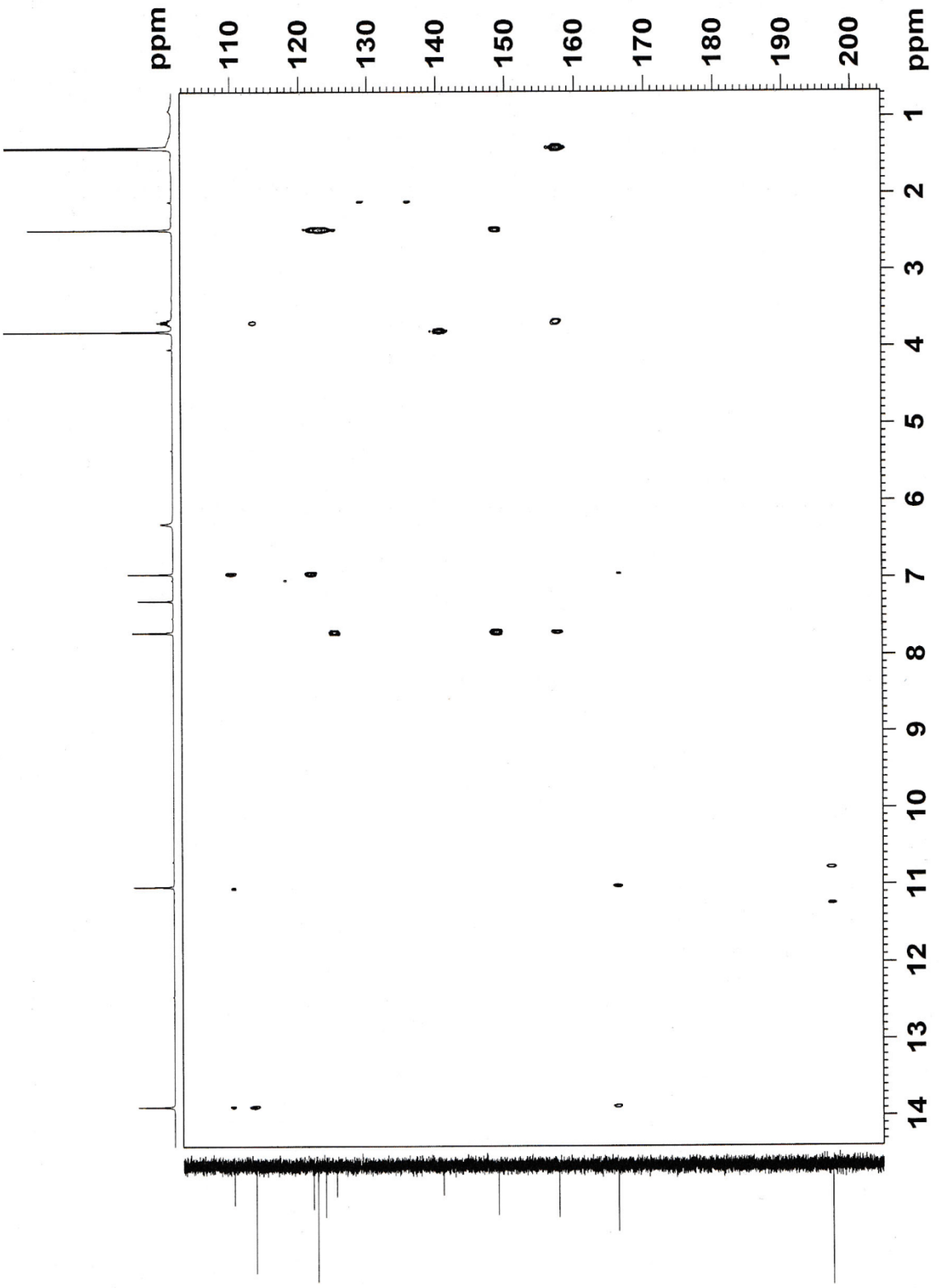


Figure 3.6: Partial HMBC spectrum of compound-1

From the ^{1}H NMR, ^{13}C-NMR data, HSQC and HMBC correlations the structure of the compound-1 **Ref. [17]** was suggested as-

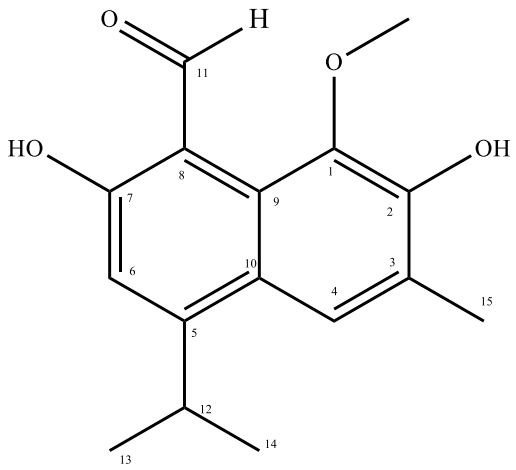


Figure 3.7: Structure of compound-1 (Isohemigossypol-1-methyl ether)

3.2. Characterization of compound-2

The compound **compound-2** (~ 7.5 mg) was a colorless crystalline solid having R_f value: 0.35 (in 100% DCM) and its melting point was found to be 210-211^{o}C. It was soluble in dichloromethane. On spraying with vanillin-sulfuric acid spray reagent, followed by heating at 105°C for several minutes, purple color appeared.

The ^{1}H NMR (400 MHz, in $CDCl_3$) spectrum (Table 3.2) of compound-2 displayed the aromatic proton signals at δ 7.01 and 7.53 ppm. The other signals from compound-2 at δ 1.41 (6H, d, J= 6.8 Hz), 2.42 (3H, s), 3.66 (1H, sept, J= 6.8Hz) and 4.34 (3H, s) were assigned to isopropyl methyl, isopropyl methine, aromatic methyl, methoxyl protons respectively. These assignments are in close agreement with the structure of isohemigossylic acid lactone-2-methyl ether (L.S. Puckhaber *et.al.* 2001).

The ^{13}C NMR (100 MHz) spectrum of compound-2 showed the presence of an ester group at δ 167.54; ten types of aromatic carbons at δ 156.97, 156.57, 140.25, 132.00,131.48, 130.09, 119.71, 118.19,113.63 and 98.85; one saturated oxygenated carbon at δ 59.85; and three saturated carbons at δ 29.69, 23.43 and 18.25. The signals at 23.43 and 18.25 are due to the presence of methyl carbons and the signals at δ 119.71, 113.63 and 29.69 are due to the presence of three methine carbons. The signals at δ 167.54, 156.97, 156.57, 140.25, 132.00, 131.48, 130.09 and 98.85 are due to the presence of quaternary carbons. These ^{13}C NMR data are very close agreement with the published data by **Ref. [20] L.S. Puckhaber *et.al*., 2001.**

The ^{1}H NMR and ^{13}C NMR data of **Compound-2** were tabulated and compared with the published data Ref. [20] of isohemigossylic acid lactone-2-methyl ether in **Table 3.2.**

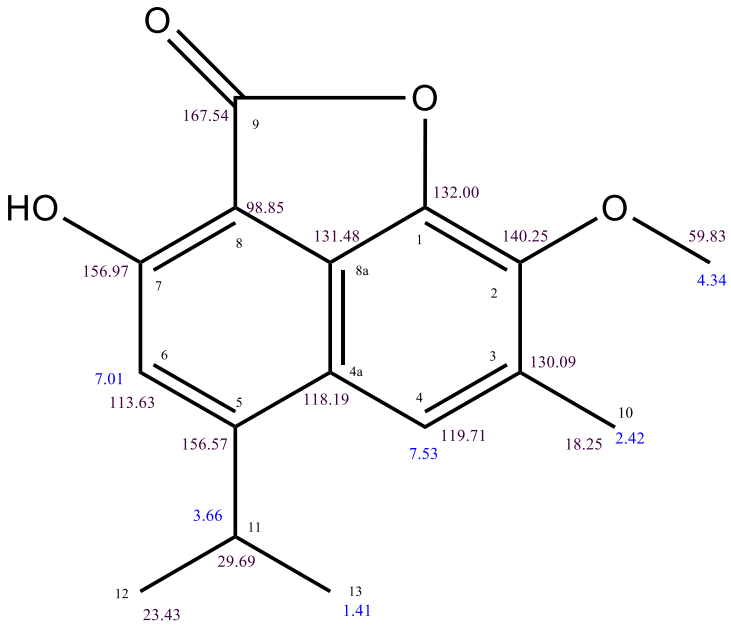


Figure 3.8: Structure of compound-2 showing ^{1}H and ^{13}C NMR signals

Table 3.2: ^{1}H NMR and ^{13}C NMR of compound compound-2 and Isohemigossylic acid lactone-2-methyl ether Ref. [20]

Position No.	Chemical shift δ in ppm*		Chemical shift δ in ppm of isohemigossylic acid lactone-2-methyl ether (L.S. Puckhaber *et.al.* 2001)	
	δ_H (mult., J in Hz)	δ_C	δ_H (mult., J in Hz)	δ_C
1	-	132.00	-	133.2
2	-	140.25	-	140.1
3	-	130.09	-	130.0
4	7.53, s	119.71	7.61, s	120.0
4a	-	118.19	-	118.9
5	-	156.57	-	156.8
6	7.01, s	113.63	7.08, s	115.2
7	-	156.97	-	158.4
8	-	98.85	-	99.5
8a	-	131.48	-	133.0
9	-	167.54	-	165.5
10	2.42, s	18.25	2.36, s	18.2
11	3.66, sept, 6.8	29.69	3.69, sept, 6.7	30.0
12	1.41, d, 6.8	23.43	1.36, d, 6.7	23.7
13	1.41, d, 6.8	23.43	1.36, d, 6.7	23.7
2-OMe	4.34, s	59.83	4.26, s	60.1
7-OH	-	-	10.14, s, exchanged with D_2O	-

*^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) in $CDCl_3$

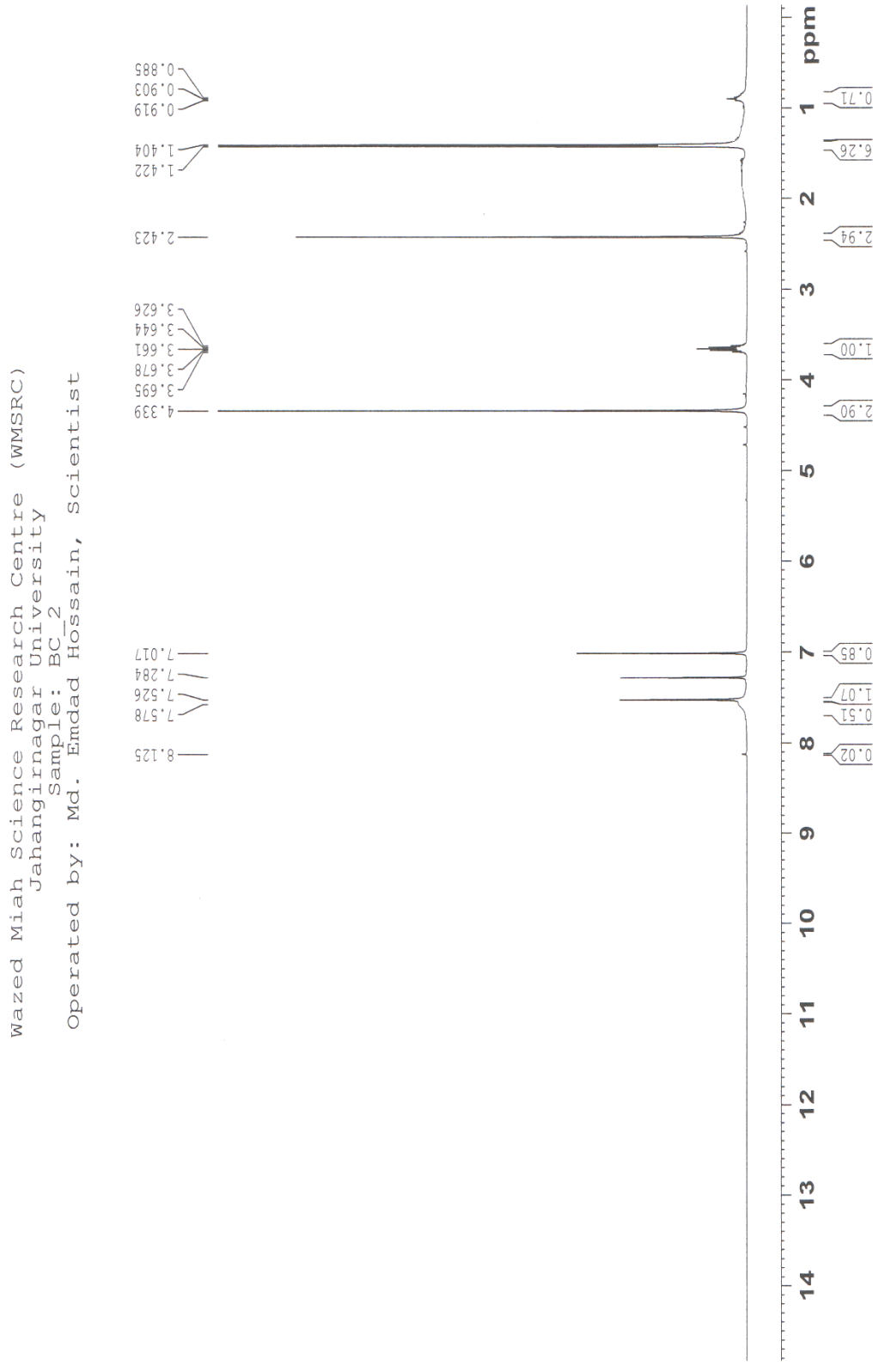


Figure 3.9: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of compound -2

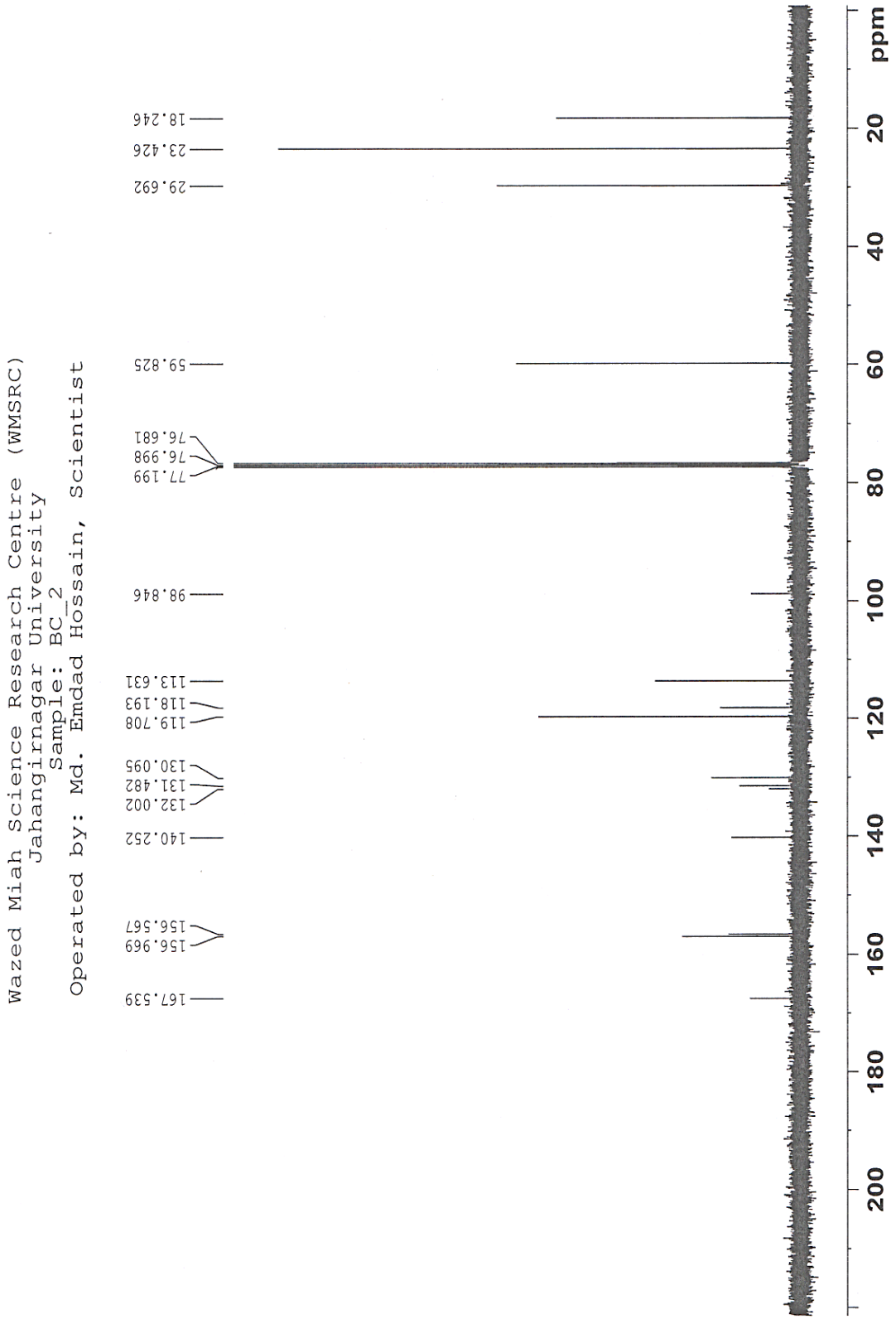


Figure 3.10: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of compound-2

Comparing the ^{1}H NMR and ^{13}C-NMR data of the compound **compound-2** with that of published data Ref. [20] ((L.S. Puckhaber *et.al.* 2001) of isohemigossylic acid lactone-2-methyl ether, the tentative structure of **compound-2** was suggested as-

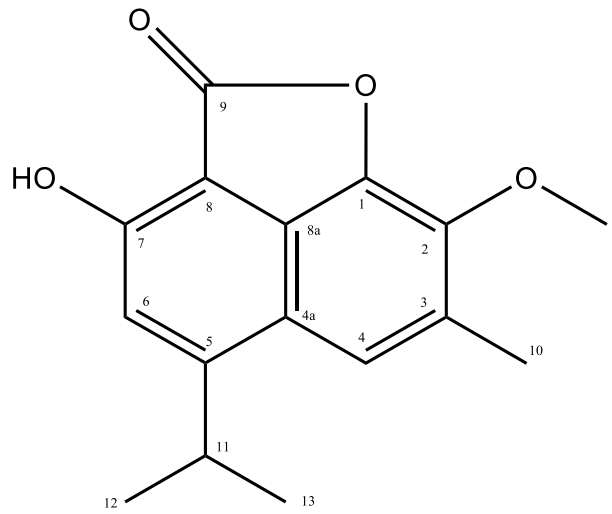


Figure 3.11: Structure of compound-2 (Isohemigossylic acid lactone-2-methyl ether)

3.3. Characterization of compound-3

The compound **compound-3** (~ 3.5 mg) was a colorless crystalline solid having R_f value: 0.57 (DCM: EtOAc = 5:1). It was soluble in dichloromethane plus a few drops of MeOH. On spraying with vanillin-sulfuric acid spray reagent, followed by heating at 105°C for several minutes, purple color appeared.

The ^{1}H NMR (400 MHz, in $CDCl_3$+ few drops of CD_3OD) spectrums (**Table 3.3**) of compound BC-3 displayed the aromatic proton signals at δ 6.87 and 7.45 ppm. The other signals from compound-3 at δ 1.34 (6H, d, J= 6.8 Hz), 2.40 (3H, s) and 3.59 (1H, sept, J= 6.8Hz) and 4.34 (3H, s) were assigned to isopropyl methyl, isopropyl methine, aromatic methyl protons respectively. These assignments were in close agreement with the structure of isohemigossylic acid lactone-2-methyl ether (L.S. Puckhaber *et.al.* 2001) with absence of a methoxyl at δ 4.23.

In the ^{13}C NMR spectrum15 carbon resonances were differentiated into 3 methyls, 3 methines and 10 quaternary carbons. An HSQC experiment showed that protons at δ 7.45, 6.87, 3.59, 2.40 and 1.34 ppm are directly attached to the carbons at δ 119.48, 114.07, 29.59, 17.32 and 23.4, respectively.

From the ESI-MS spectrum of the compound-3, the peaks at m/z, 259 and 281 were due to $[M+H]^+$ and $[M+Na]^+$ respectively. Thus, the molecular wight of compound-3 is 258 which is correctly matched with the calculated value of the molecular weight of the compound-3.

When all these data (^{1}H NMR, ^{13}C NMR, HSQC, HMBC and mass spectrum) were compared with those of isohemigossylic acid lactone-2-methyl ether (L.S. Puckhaber *et.al.* 2001), an absence of methoxy (δ_H 4.26 ppm; δ_C 60.1 ppm) was observed. Other NMR assignments (**Table 3.3**) were facilitated by comparison with the data of isohemigossylic acid lactone-2-methyl ether **Ref. [20]** (L.S. Puckhaber *et.al.* 2001). Accordingly, compound **compound-3** was identified as **2, 7-dihydroxy-5-isopropyl-3-methyl-2H-naphtho[1,8-bc]furan-2-one.**

Table 3.3: ^{1}H NMR, ^{13}C NMR , HSQC and HMBC data of compound-3

Position No.	Chemical shift δ in ppm*		HSQC (^{1}H⟶^{13}C)	HMBC (^{1}H ⟶ ^{13}C)	
	δ_H (mult, J in Hz)	δ_C	^{1}J	^{2}J	^{3}J
1	-	131.42	-	-	-
2	-	137.52	-	-	-
3	-	128.44	-	-	-
4	7.45, s	119.48	C-4	C-3	C-10, C-2
4a	-	117.12	-	-	-
5	-	157.21	-	-	-
6	6.86, s	114.07	C-6	-	C-8, C-4a
7	-	157.98	-	-	-
8	8	98.65	-	-	-
8a	-	130.86	-	-	-
9	-	168.20	-	-	-
10	2.40, s	17.32	C-10	C-3	C-2, C-4
11	3.59, sept, 6.8	29.59	C-11	C-12	-
12	1.34, d, 6.8	23.4	C-12	C-11	C-5
13	1.34, d, 6.8	23.4	C-13	C-11	C-5
2-OH	-	-	-	-	-
7-OH	-	-	-	-	-

*^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) in $CDCl_3$ + few drops of CD_3OD

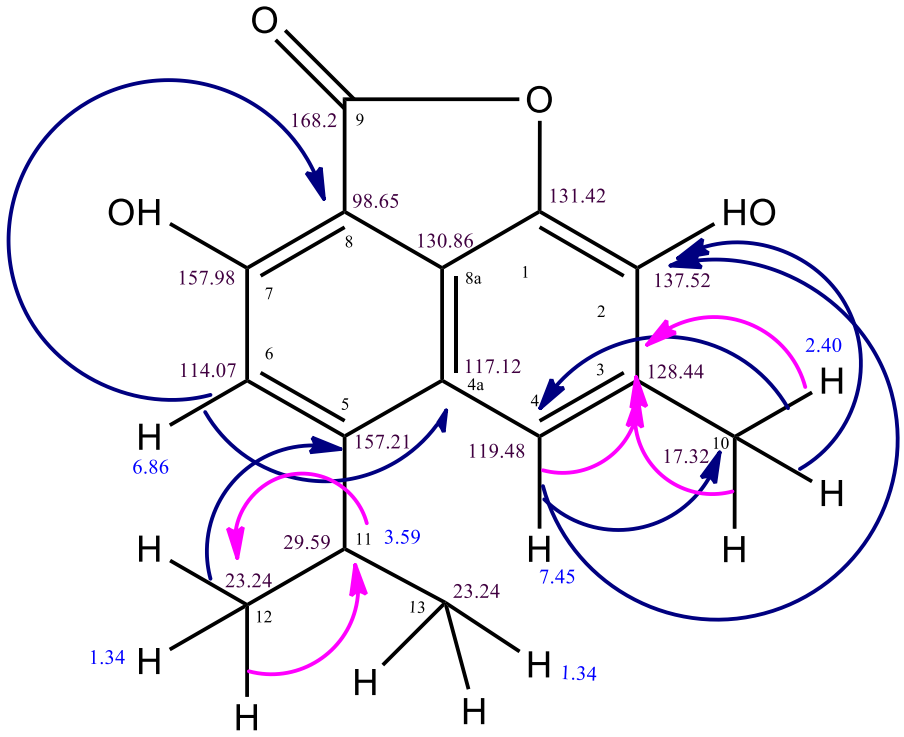


Figure 3.12: Structure of compound-3 showing ^{1}H NMR, ^{13}C NMR signals and HMBC (H ⟶ C) correlations

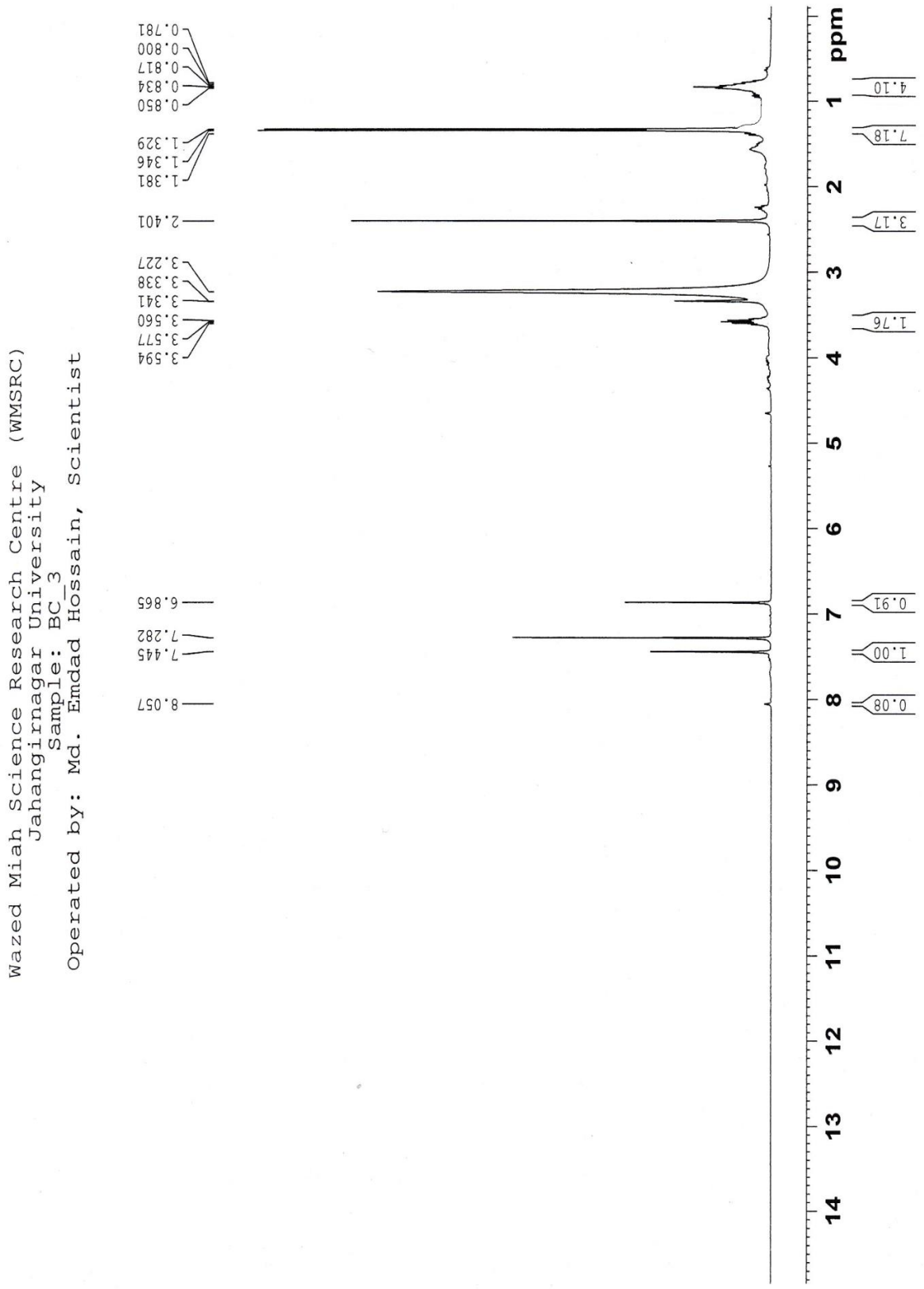


Figure 3.13: ^{1}H NMR (400 MHz, $CDCl_3$ + few drops of CD_3OD) spectrum of compound-3

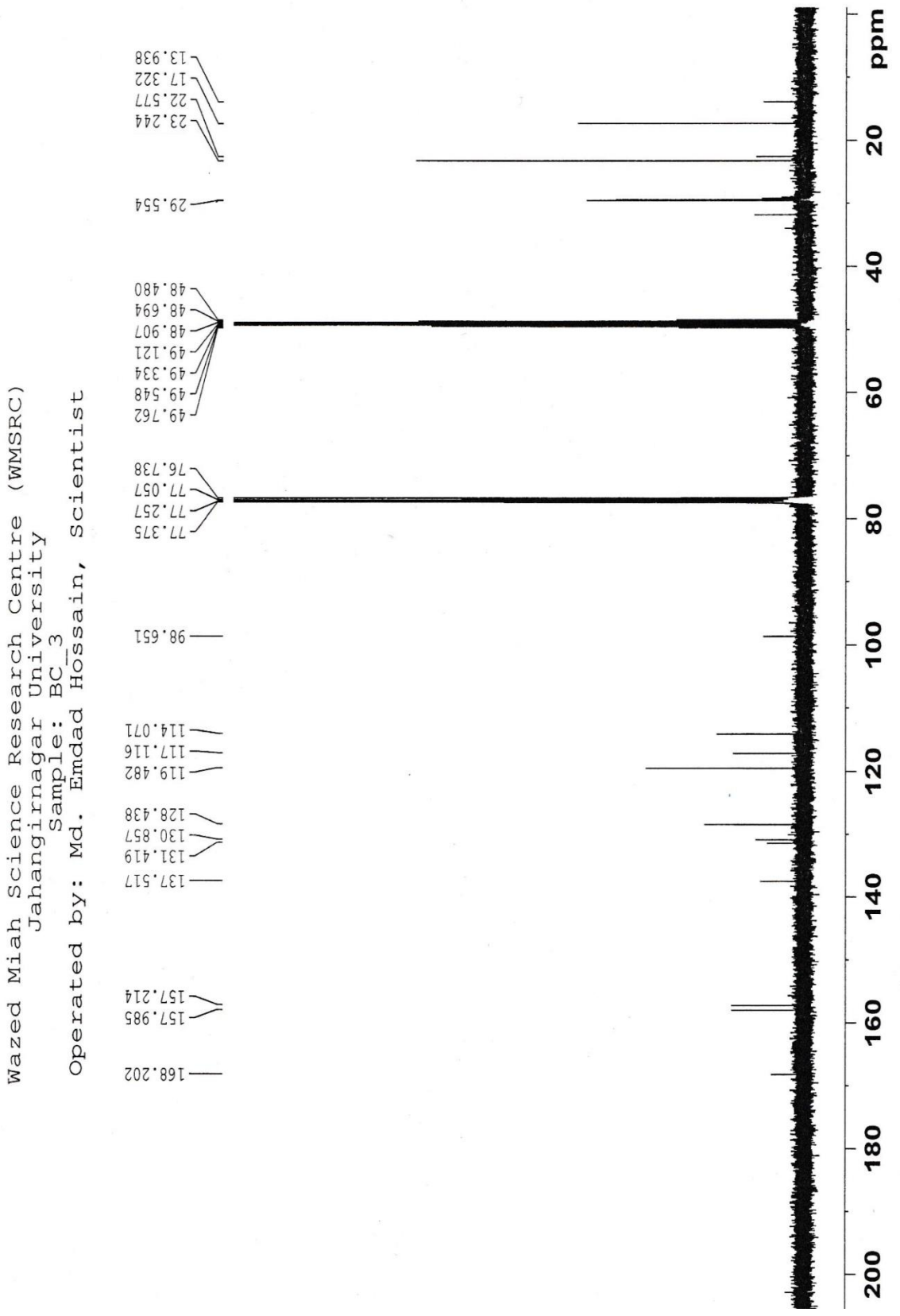


Figure 3.14: ^{13}C NMR (100 MHz, $CDCl_3$ + few drops of CD_3OD) spectrum of compound-3

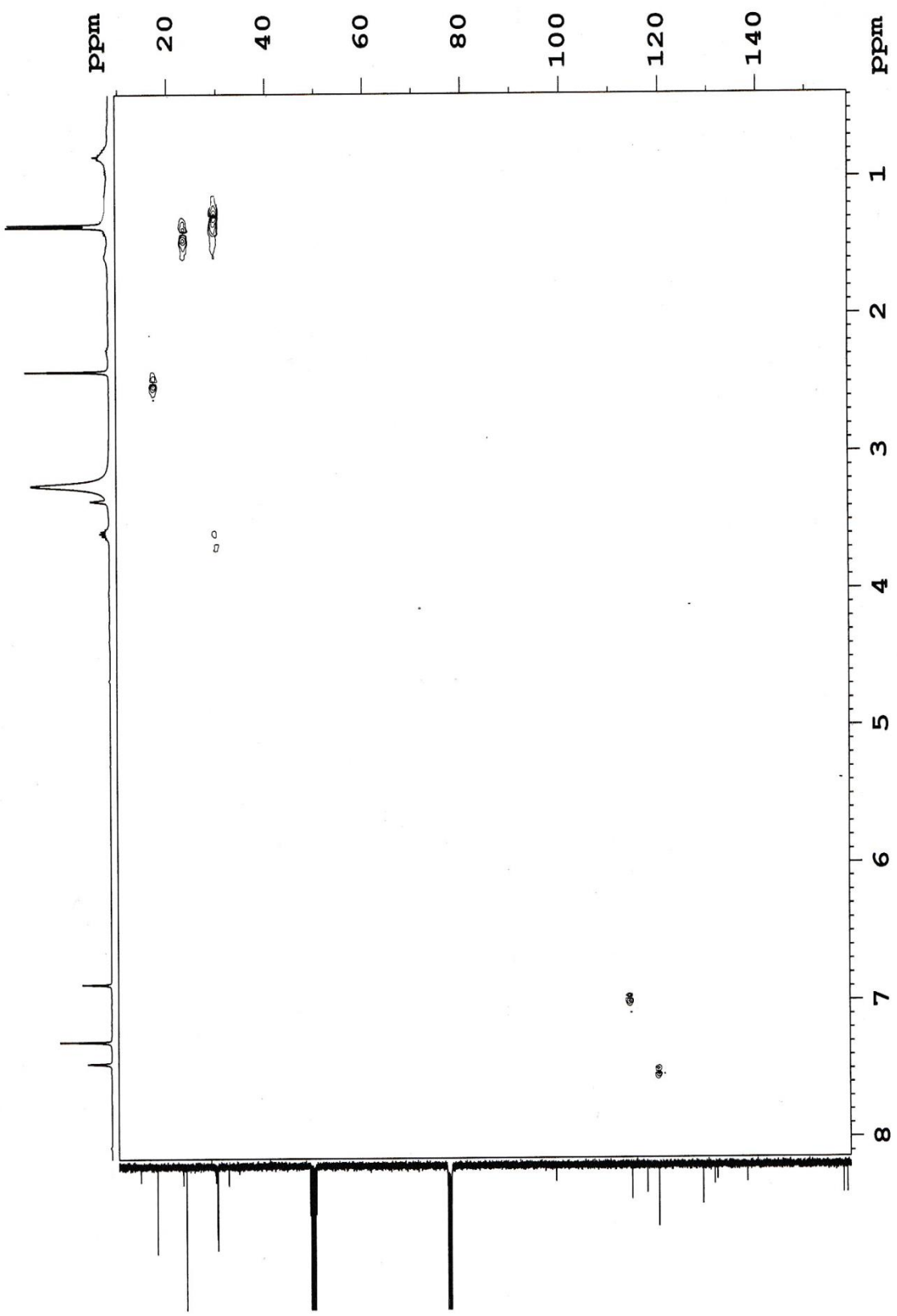


Figure 3.15: HSQC spectrum of compound-3

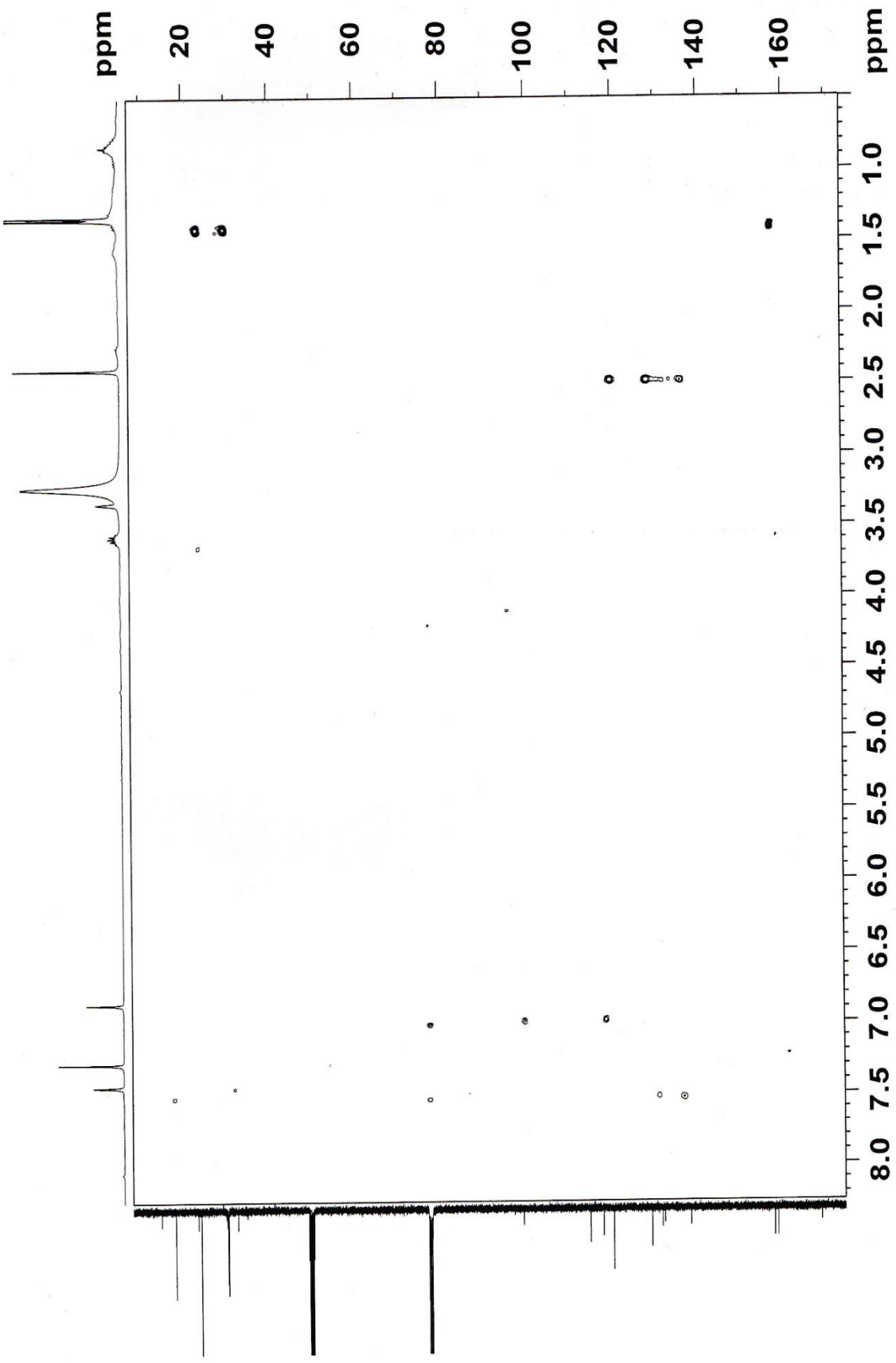
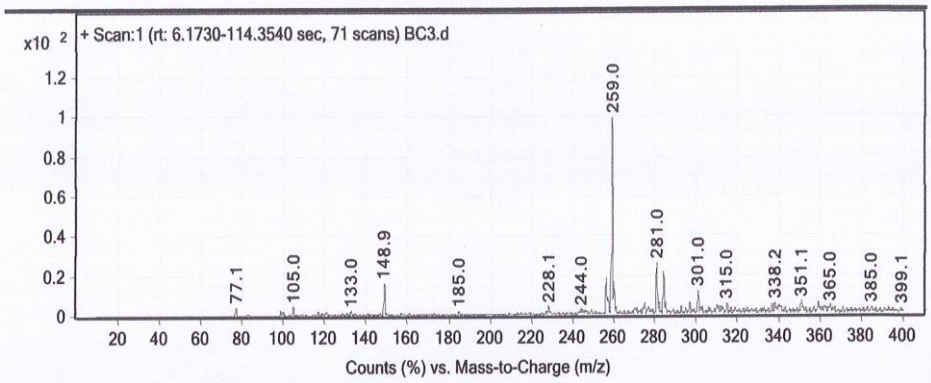


Figure 3.16: HMBC spectrum of compound-3

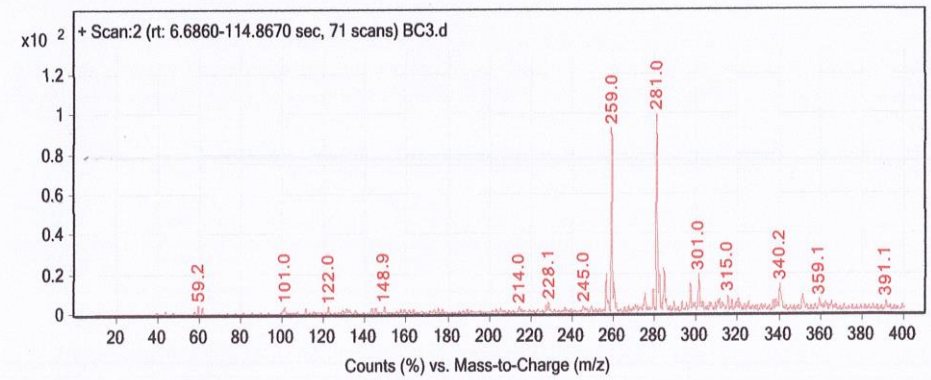
Qualitative Analysis Report



Peak List

m/z	z	Abund
148.9		630771.44
256.2		741289.25
257.1		347019.13
259	1	3862437
260	1	668005.25
275		260506.56
281	1	1015829.75
284.2	1	842942.06
301	1	467791.16
351.1		263468.16

Spectrum Source	Fragmentor Voltage	Collision Energy	Ionization Mode
Peak (1) in "+ TIC Scan"	70	0	ESI



Peak List

m/z	z	Abund
256.2	1	648526.44
259	1	2996587.5
260	1	507291.5
279		396416.09
281	1	3220343.75
282	1	612435.69
284.2	1	726007.75
297	1	468304.22
301	1	632911.5

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Figure 3.17: ESI-MS spectrum of compound-3

From the ^{1}H NMR, ^{13}C-NMR, HSQC and HMBC data of the **compound-3 Ref. [20]** was suggested as-

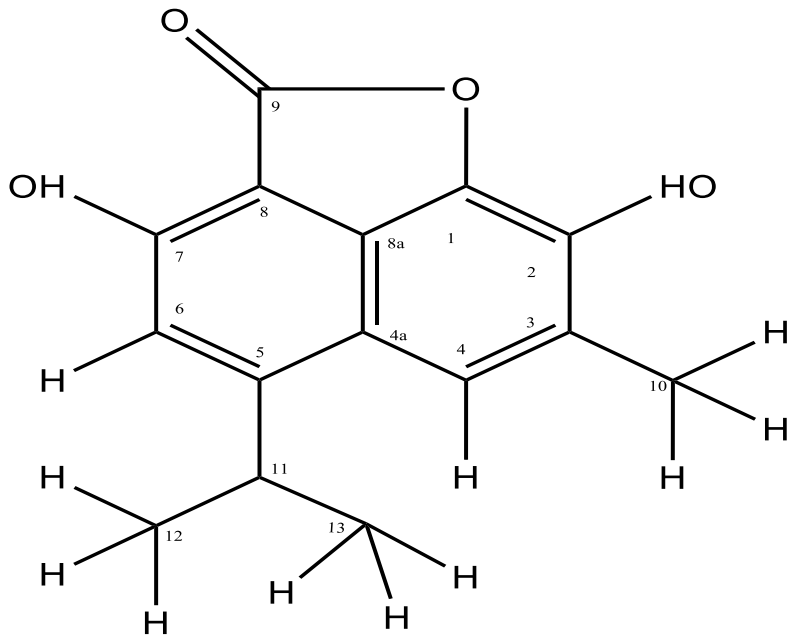

Figure 3.18: Structure of compound-3 (2, 7-dihydroxy-5-isopropyl-3-methyl-2H-naphtho [1, 8-bc]furan-2-one)

3.4. Characterization of compound-4

The compound **compound-4** (~ 4.5 mg) was a colorless crystalline solid having R_f value: 0.35 (in 100% EtOAc) and its melting point was found to be 199-201^{o}C. It was soluble in EtOAc, DMSO and MeOH.

The ^{1}H NMR (400 MHz, in DMSO-d_6) spectral data displayed three aromatic hydrogens (δ 6.78, 7.28 and 7.33 ppm) suggesting that other three positions of a benzene ring might be substituted. There was a doublet observed at δ 6.78 ppm (J=8.4 Hz) attributable to ortho-split of H-5 proton (on the C-5) by H-6.Another doublet observed at δ 7.33 ppm (J=2 Hz) was due to the meta-split of H-2 by H-6, while the doublet-doublet observed at δ 7.28 ppm (J=8.4, 2.0 Hz) was due to ortho-split of H-6 by H-5 and meta-split of H-6 by H-2. The protons of the OH groups and the carboxylic group were shifted downfield due to strong intramolecular hydrogen bonding.

The ^{13}C NMR spectral data revealed the presence of 7 carbon atoms in the molecule. The signal at δ 167.79 pm was due to the carbonyl carbon of carboxylic group. The C-1 resonated at δ 122.34 ppm; C-2 at 117.03 ppm; C-3 at145.34 ppm; C-4 at 150.45 ppm; C-5

at 115.61 ppm and C-6 at 122.19 ppm. The assignment of the carbons were in good agreement with that reported for protocatechuic acid by Ref. [23] G. Mrinmoy, M. Prasenjit, *et.al.*, 2013.

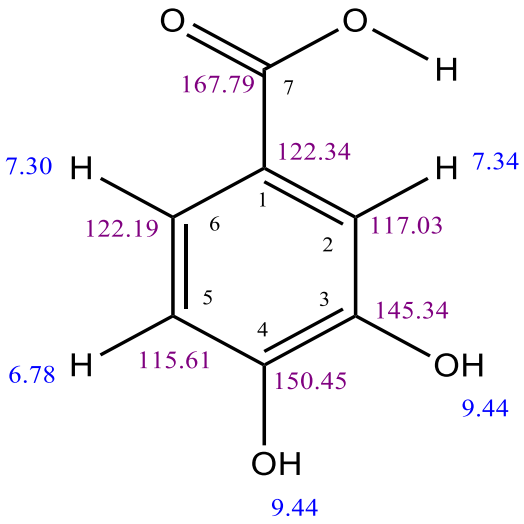

Figure 3.19: Structure of Compound-4 showing ^{1}H NMR and ^{13}C NMR signals

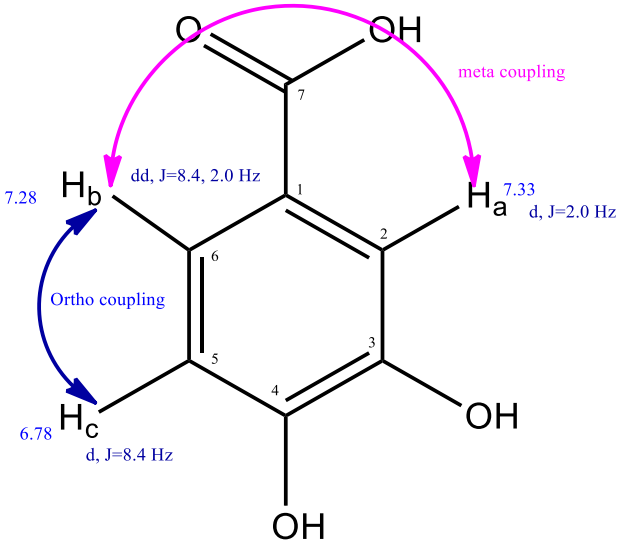

Figure 3.20: Spin-spin couplings between hydrogens in the structure of Compound-4

Table 3.4: ^{1}H NMR and ^{13}C NMR of compound compound-4 and 3, 4-dihydroxy benzoic acid Ref. [23]

Position No.	Chemical shift δ in ppm*		Chemical shift δ in ppm of protocatechuic acid (G. Mrinmoy, M. Prasenjit, *et.al.*,2013)	
	δ_H (mult., J in Hz)	δ_C	δ_H (mult., J in Hz)	δ_C
1	-	122.34	-	122.3
2	7.33, d, 2	117.03	7.36, d, 2.1	116.9
3	-	145.34	-	145.2
4	-	150.45	-	150.3
5	6.78, d, 8.4	115.61	6.80, d, 8.1	115.5
6	7.28, dd, 8.4, 2	122.19	7.31, dd, 8.1, 2.1	122.0
7	-	167.79	-	167.7
3-OH	9.44, br.s	-	9.30, br.s	
4-OH	9.44, br.s	-	9.67, br.s	
7-OH		-	12.36, br.s	

*^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) in DMSO-d_6

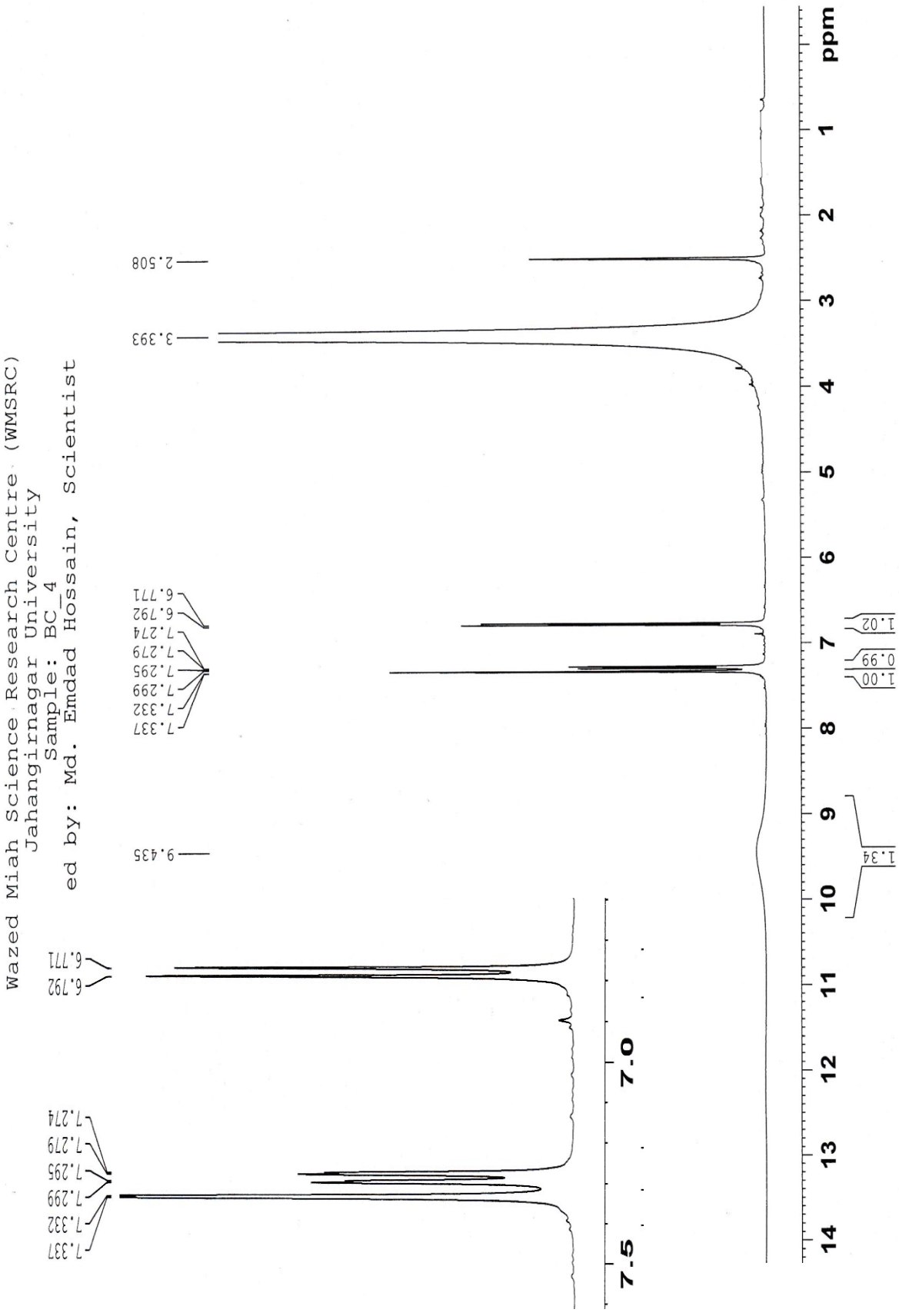

Figure 3.21: ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of compound-4

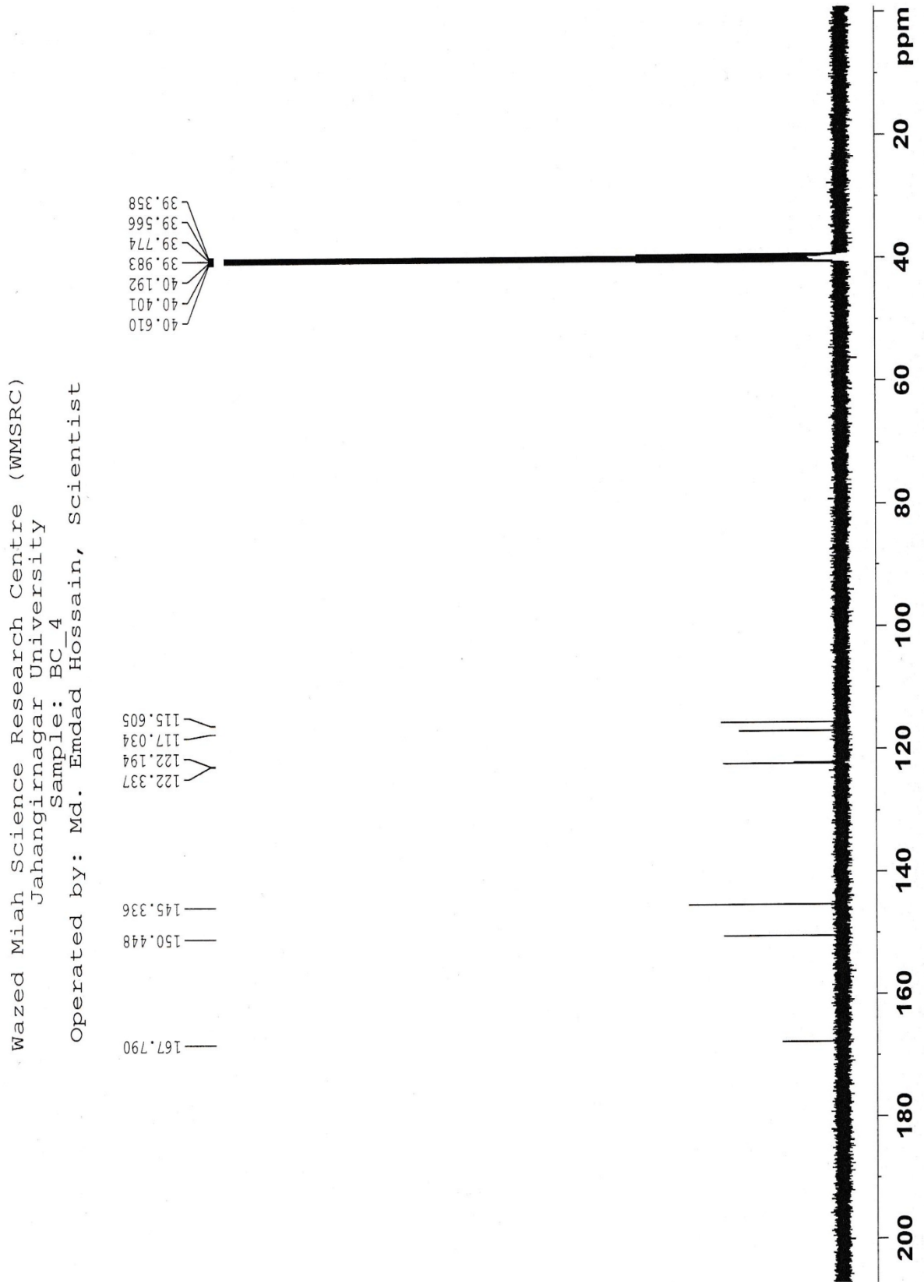

Figure 3.22: ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound-4

The ^{1}H NMR and ^{13}C-NMR data of the **compound-4** compared with the published data **Protocatechuic acid Ref. [23]** the structure **compound-4** was suggested as-

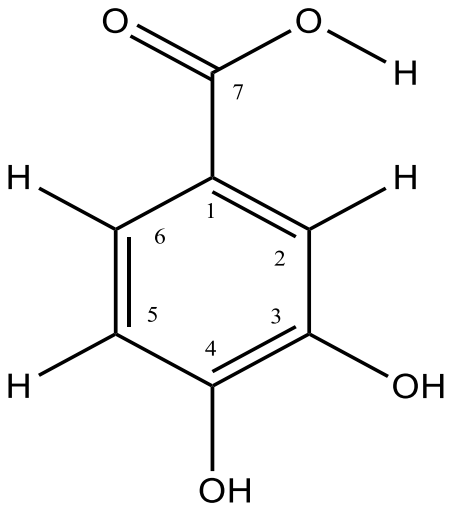

Figure 3.23: Structure of compound-4 (Protocatechuic acid)

3.5. Characterization of compound-5

The compound-5 (~ 3.5 mg) was a colorless crystalline solid having R_f value: 0.43 (in 100% EtOAc) and its melting point was found to be 210-212^{o}C. It was soluble in EtOAc, DMSO and MeOH.

The ^{1}H NMR (400 MHz in $CDCl_3$) spectral data compound-5 displayed four aromatic protons (δ 6.94, 6.97, 7.12 and 7.16 ppm) suggesting that other four positions of a benzene ring might be substituted. One proton triplet at 4.9 ppm due to (1H. t, H-1). Two multiplet at δ 2.15 and 2.45 due to methylene and two multiplet at δ 3.28, 3.45 due to (1H, m, H-5) and (1H, m, H-4) respectively. The spectrum showed significant chemical shift at δ 4.63 and 5.5 due to methylene and hydroxyl groups.

The ^{13}C NMR spectral data revealed the presence of 11 carbon atoms in the molecule. The signal at δ 163.06, 142.04 and 119.3 ppm indicates the compound consist three quaternary carbon. The signal at δ 71.03 for C-4 and 47.3 and 29.08 due to methylene carbon. The C-9 resonated at δ 122.8 ppm; C-8 at 127.9 ppm; C-7 at 124.1 ppm; C-6at 125.8 ppm. The assignment of the carbons were in good agreement with that reported for vasicine by **Ref. [27]: Bharat Singh.; R. et al. (2013)**

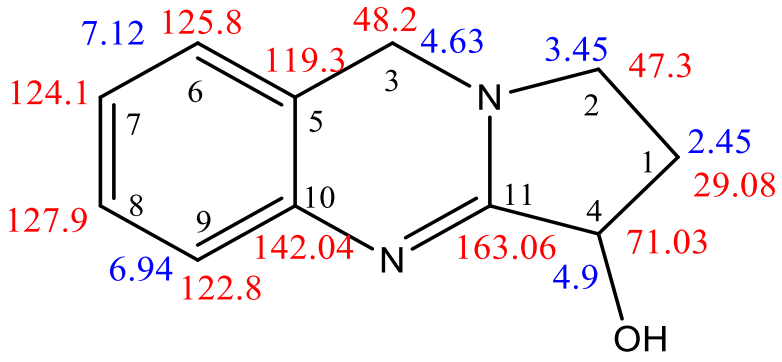

Figure 4.24: Structure of compound-5 showing ^{1}H NMR and ^{13}C NMR signals

Table 3.5: ^{1}H NMR and ^{13}C NMR of compound-5 and Vasicine **Ref. [27]**

Position No.	Chemical shift δ in ppm*		Ref. [Bharat Singh.; R. et al. 2013]	
	δ_H (mult., J in Hz)	δ_C	δ_H (mult., J in Hz)	δ_C
1	2.45, 2H, s	29.08	-	47.0
2	3.45	47.3	-	48.1
3	4.63, s	48.2	-	48.1
4	4.9, s	71.03	7.61, 1H, s	70.2
5	-	119.3	-	119.0
6	7.12, d	125.8	-	125.7
7	7.16, d	124.1	7.08, 1H, s	124.0
8	6.97, d	127.9	-	128.3
9	6.95, d	122.8	-	122.6
10	-	142.04	-	142.3
11	-	163.06	-	163.9

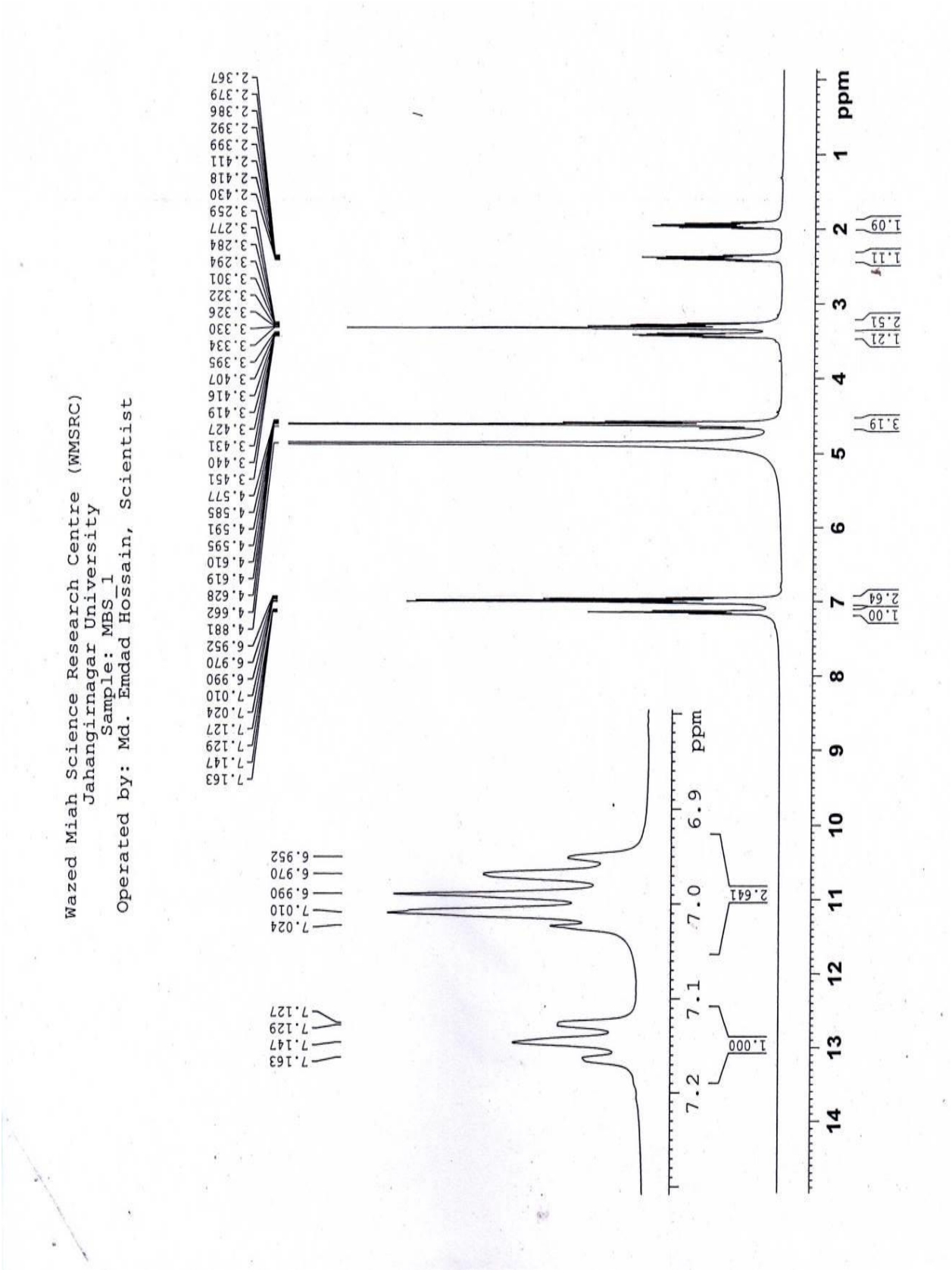

Figure 3.25: ^{1}H NMR (100 MHz, DMSO-d_6) spectrum of compound-5

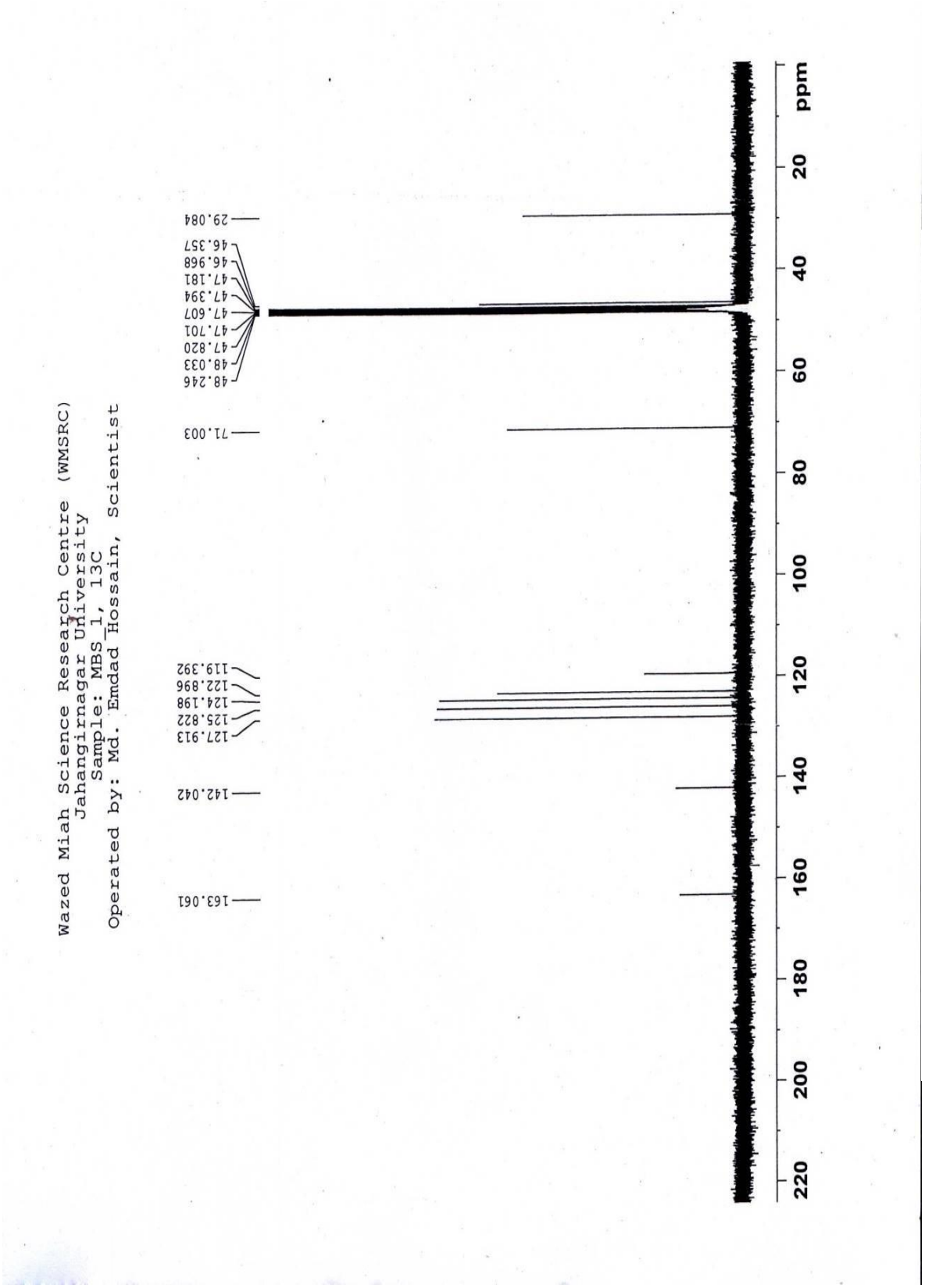

Figure 3.26: ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of compound-5

The ^{1}H NMR and ^{13}C-NMR data of the **compound-5** compared with the published data Vasicine. **Ref. [27]** the structure **compound-5** was suggested as-

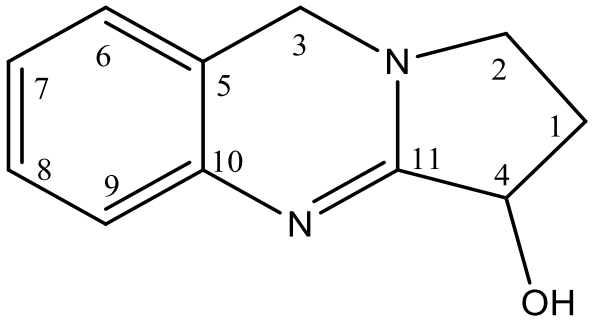

Figure 3.27: Structure of compound-5 (Vasicine)

3.6. Characterization of compound-6

The compound-6 (~ 4.9 mg) was a colorless crystalline solid having R_f value: 0.34 (in 5% EtOAc in DCM) and its melting point was found to be 203-204^{o}C. It was soluble in EtOAc, DMSO and MeOH. On spraying with vanillin-sulfuric acid spray reagent, followed by heating at 105°C for several minutes, purple color appeared.

The ^{1}H-NMR (400 MHz, in $CDCl_3$+CD_3OD) spectrum of compound-6 displayed the aromatic proton signals at 6.214 (1H, d, J=9.6Hz) and 6.818(s), 6. 865 (s) and 7.574 (1H, d, J=9.6). The other signals from compound-6 at δ 1.217, 2.211(q), 3.910 (s).

In the ^{13}C-NMR (100 MHz, in $CDCl_3$+CD_3OD) spectrum 10 different peaks were observed. Presence of O-methoxy (-OCH_3) observed at δ 56.40, carbonyl group (C=O) observed at δ 161.80 ppm. Five non-oxygenated aromatic carbons at δ 113.00, 143.53, 107.73, 103.26, 111.40. Three oxygenated aromatic carbon at δ 144.43, 150.22 and 150.09 ppm.

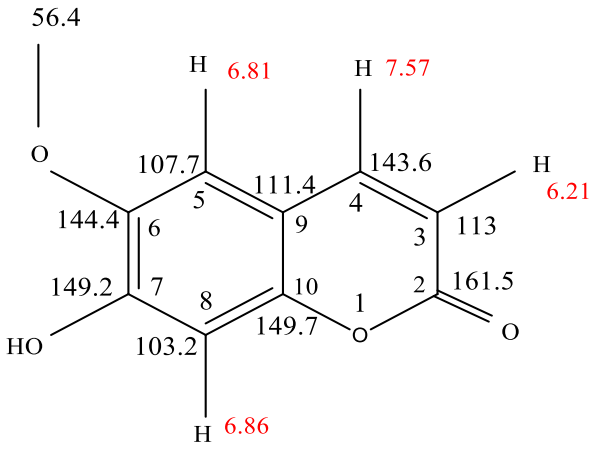

Figure 3.28: Structure of compound-6 showing ^{1}H NMR and ^{13}C NMR signals

Table 3.6: ^{1}H NMR and ^{13}C NMR of compound-6 and Scopoletin **Ref. [29]**

Position No.	Chemical Shift, δ (ppm)		Ref. [36] : N.M. Mofiz Uddin et al. (2015)	
	δ_H (Mult, J in Hz)	δ_C	δ_H	δ_C
O-Me	3.94 (s)	56.40	3.80	56.73
2	-	161.80	-	161.33
3	6.214 (d, 9.6)	113.00	6.16 (d)	113.30
4	7.574 (d, 9.6)	143.63	7.82 (d)	144.67
5	6.81 (s)	107.73	7.17 (s)	109.98
6	-	144.43	-	145.98
7	-	150.22	-	151.14
8	6.86 (s)	103.26	6.78 (8)	103.73
9	-	111.40	-	112.09
10	-	150.09	-	151.86

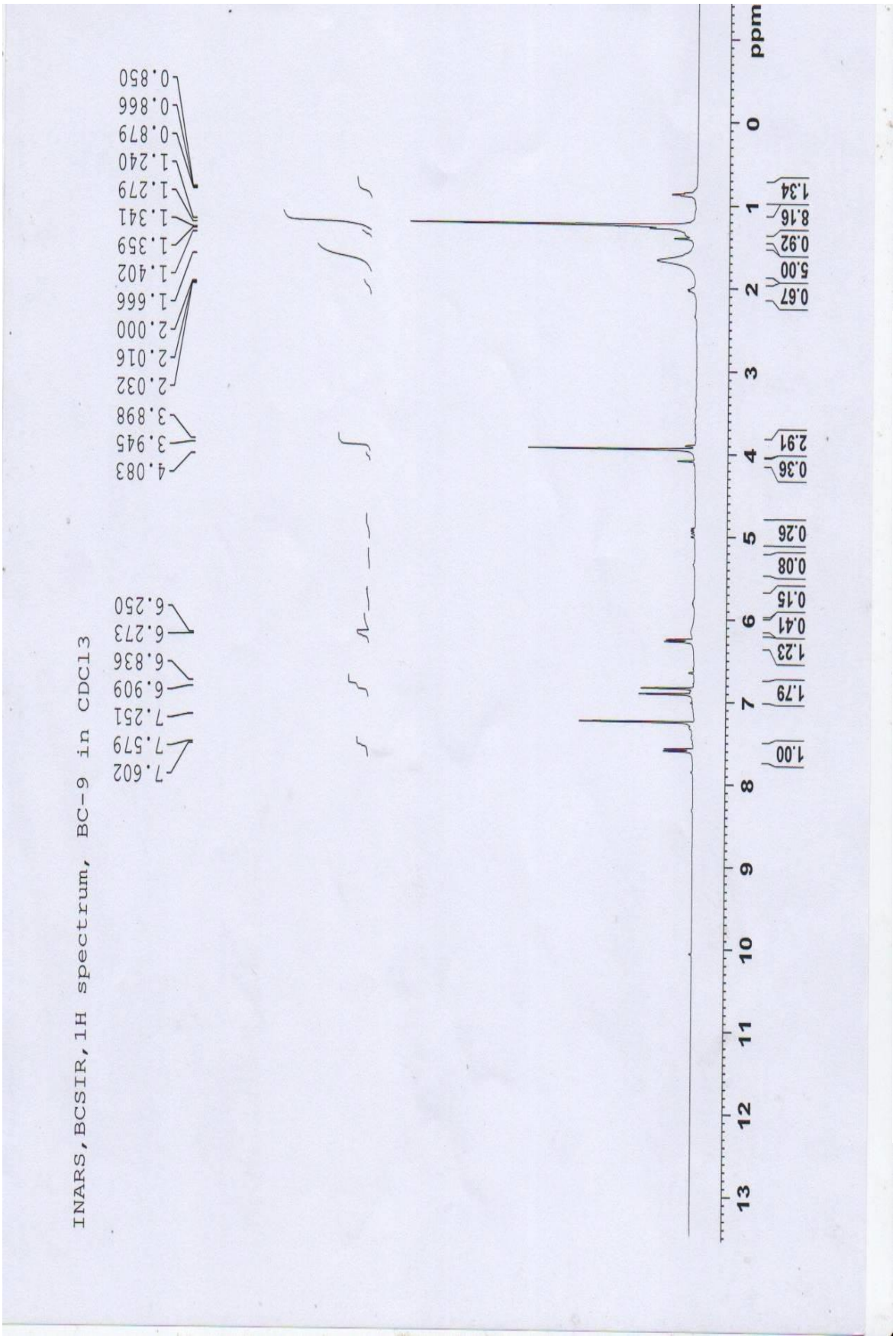

Figure 3.29: ^{1}H NMR (100 MHz, $CDCl_3$) spectrum of compound-6

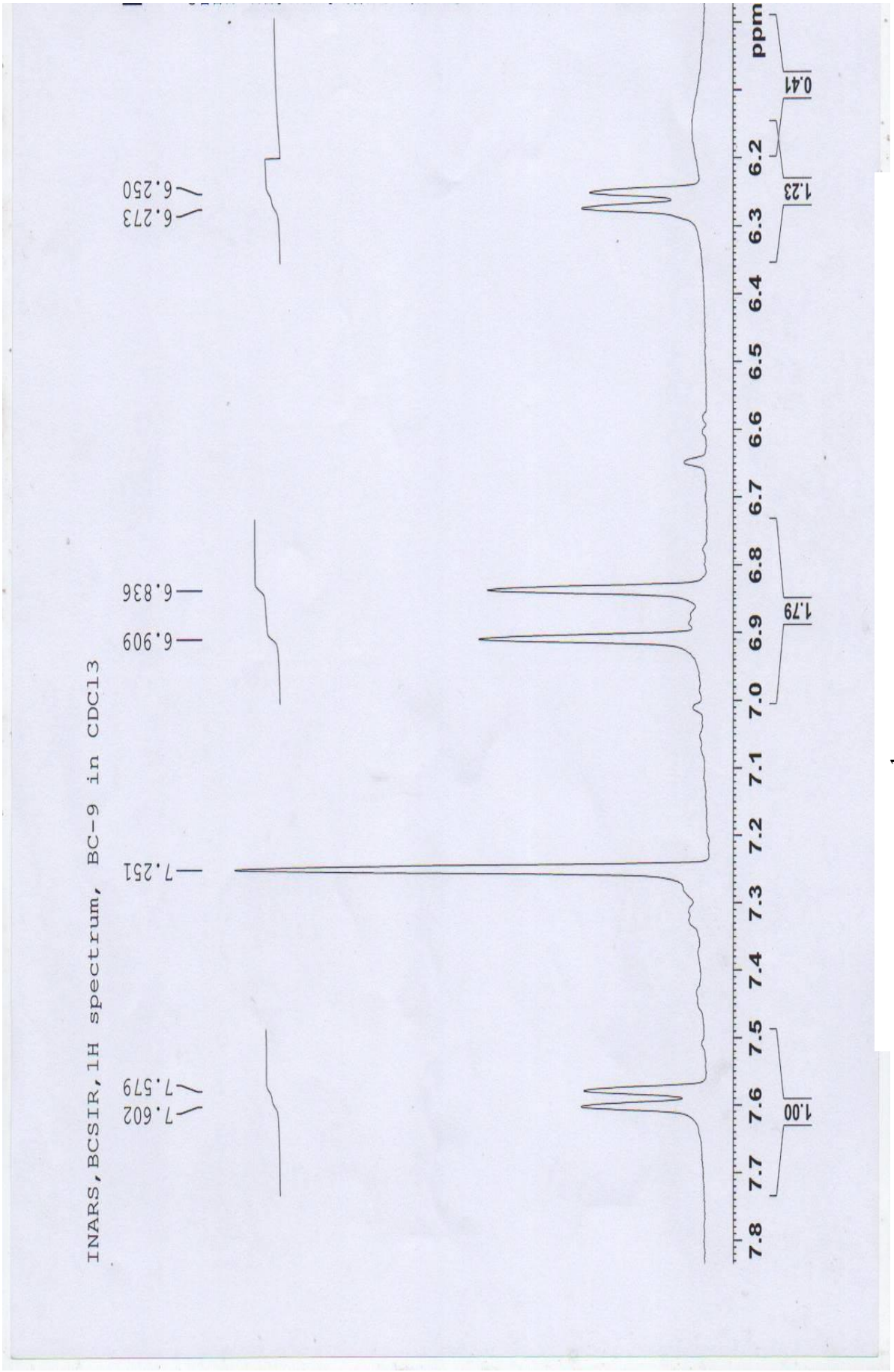

Figure 3.30: Expanded ^{1}H NMR (100 MHz, $CDCl_3$) spectrum of compound-6

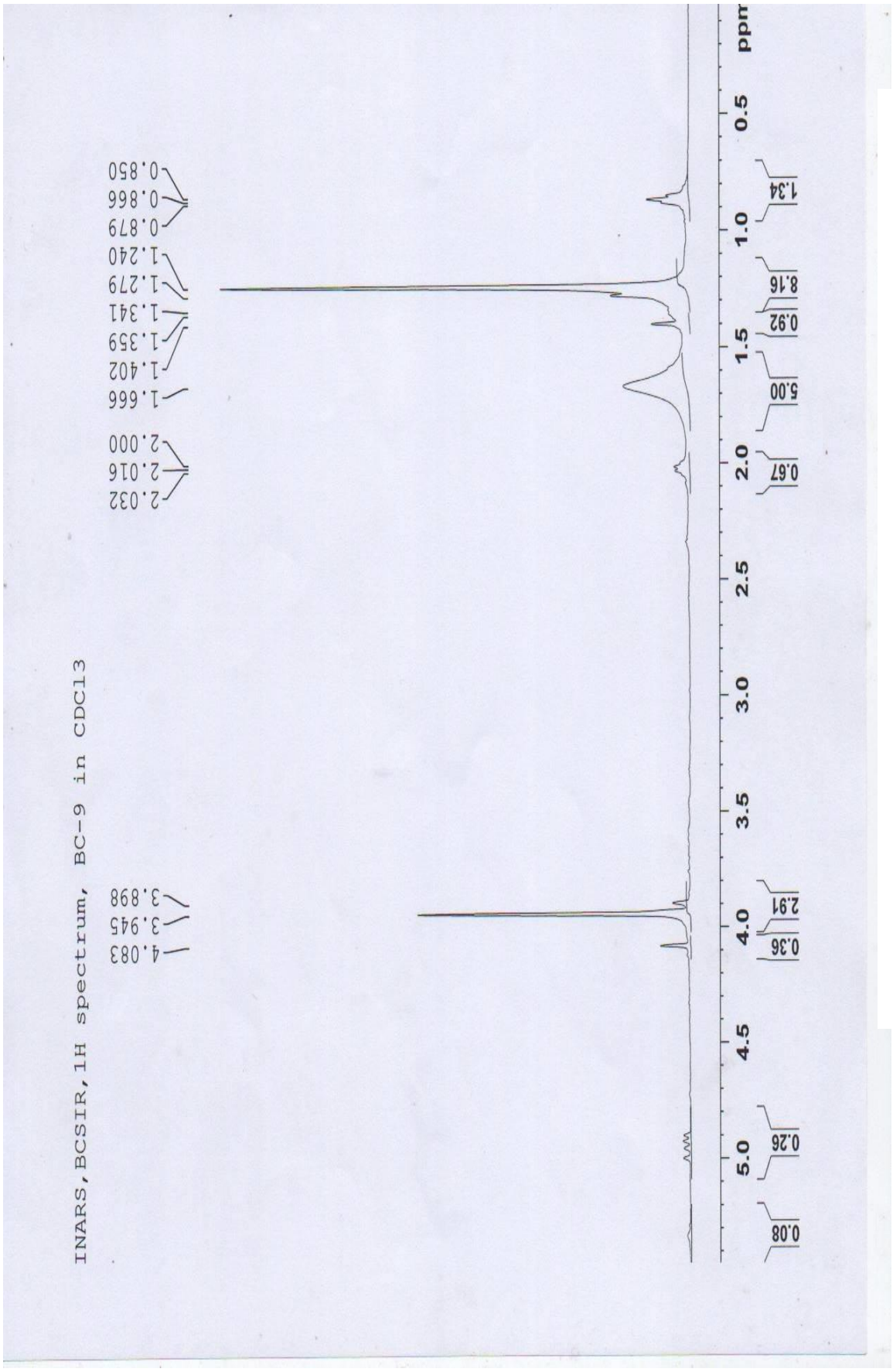

Figure 3.31: Expanded ^{1}H NMR (100 MHz, $CDCl_3$) spectrum of compound-6

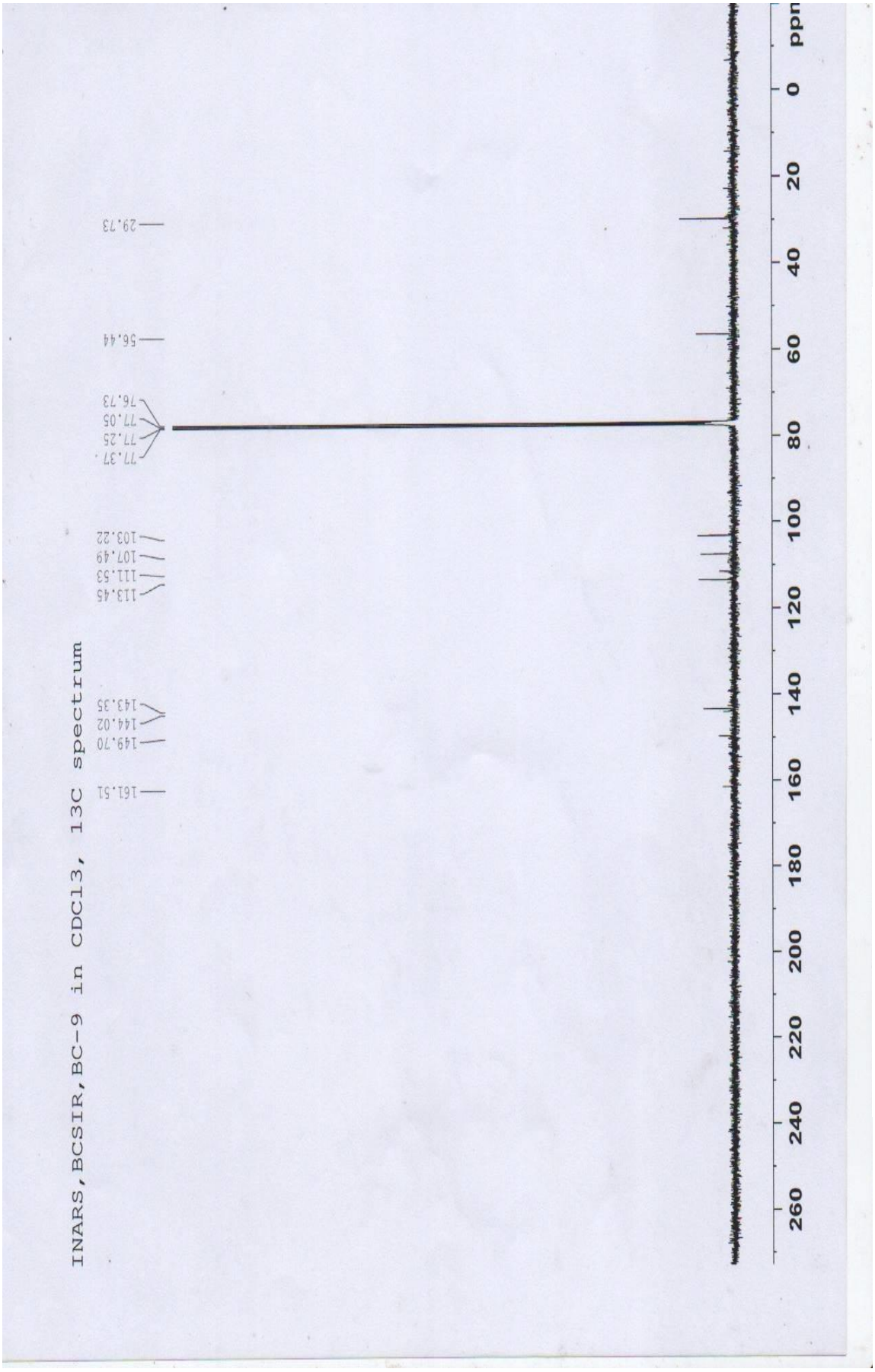

Figure 3.32: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of compound-6

The ^{1}H NMR and ^{13}C-NMR data of the **compound-6** compared with the published data Scopoletin. **Ref. [29]** the structure **compound-6** was suggested as-

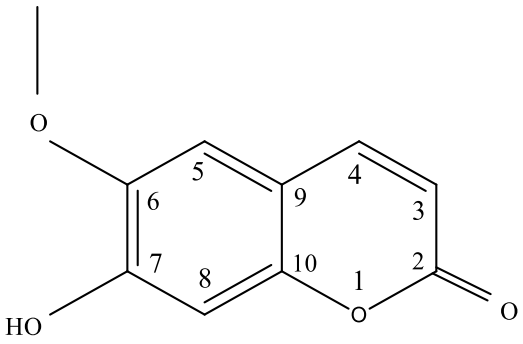

Figure 3.33: Structure of compound-6 (Scopoletin)

3.7. Characterization of compound-7

The ^{1}H NMR spectrum (400 MHz in $CDCl_3$) of compound-7 revealed the presence of a multiplet signal of one proton at δ 2.37 ascribable to 19β –H is characteristic of Lupeol. The H-3 proton showed a multiplet at δ 3.2 while a pair of broad singlets at δ 4.56 and δ 4.71 (IH, each) was indicative of olefinic protons at (H-29 a & b).. The singlet at 1.67 ppm was due to three protons at C-30. The signals between δ 0.69 - 1.54 ppm were due to several methylene and methane protons. These assignments were in good agreement for the structure of Lupeol. **Ref. [30]: Jain, P. S. and Bari, S. B., (2010)**

The ^{13}C NMR spectrum (100 MHz in $CDCl_3$) of compound-2 showed thirty signals indicating the presence of thirty carbons. Among them seven were methyl carbons, ten were methylene carbons, five quaternary carbons, five were methine carbons, twice were olefinic carbons and one oxymethine carbon. The seven methyl groups gave signals at δ 29.7 (C-23), 18.02 (C-28), 16.12 (C-25), 15.99 (C-26), 15.36 (C-24), 14.56 (C-27) and 19.31 (C-30) ppm. The signals were due to olefinic carbons at δ 109.32 (C-29) and 150.98 (C-20). The five quaternary carbons gave signals at δ 43.03 (C-17), 42.85 (C-14), 40.85 (C-8), 38.87 (C- 4) and 37.19 (C-17) ppm. The ten methylene carbons gave signals at δ 40.01 (C-22), 38.73 (C-1), 35.60 (C-16), 34.30 (C- 7), 29.87 (C-21), 28.0 (C-2), 27.45 (C-15), 25.17 (C-12), 20.95 (C-12) and 48.33 (C-6) ppm. The five methine carbons gave signals at δ 55.32 (C-5), 50.46 (C-9), 48.33 (C-18), 48.0 (C- 19) and 38.08 (C-17) ppm. The deshielded signal at δ 79.03 was due to C-3 with a hydroxyl group attached to it.

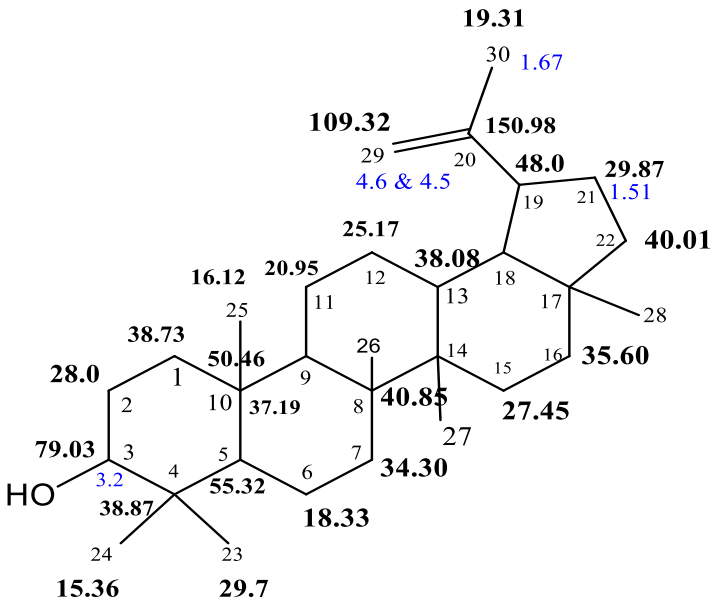

Figure 3.34: Structure of compound-7 showing ^{1}H NMR and ^{13}C NMR signals

Table 3.7: ^{1}H NMR and ^{13}C NMR of compound-7 and Lupeol. **Ref. [38] : Jain, P. S. and Bari, S. B., (2010)**

Carbon no.	Type of carbon	Compound-7 (Chemical shift in ppm)		Lupeol (Chemical shift in ppm)	
		δ_H	δ_C	δ_H	δ_C
1	CH_2	-	38.73	-	37.30
2	CH_2	-	28.0	-	28.72
3	CH-OH	3.2 (1H, d)	79.03	3.51 (1H, d)	79.85
4	C	-	38.87	-	38.36
5	CH	-	55.32	-	55.41
6	CH_2	-	18.33	-	18.74
7	CH_2	-	34.30	-	34.96
8	C	-	40.85	-	40.96
9	CH	-	50.46	-	50.24
10	C	-	37.19	-	36.56
11	CH_2	-	20.95	-	21.13
12	CH_2	-	25.17	-	25.83
13	CH	-	38.08	-	40.05
14	C	-	42.85	-	42.82
15	CH_2	-	27.45	--	27.34
16	CH_2	-	35.60	-	35.28
17	C	-	43.01	-	43.12
18	CH	-	38.33	-	38.08
19	CH	2.37 (1H, m)	48.0	2.36 (1H, m)	49.43
20	C	-	150.98	-	150.19
21	CH_2	1.91 (1H, m)	29.87	1.92 (1H, m)	28.82
22	CH_2		40.01	-	38.33
23	CH_3		29.60	-	29.31
24	CH_3	-	15.36	-	15.91
25	CH_3	-	16.12	-	16.23
26	CH_3	-	15.99	-	15.84
27	CH_3	-	14.56	-	14.08
28	CH_3	-	18.02	-	18.13
29	C	4.71 (1H, s), 4.56 (2H, s)	109.32	4.75 (1H, s) 4.50 (2H, s)	109.26
30	CH_3	1.67 (3H, s)	19.31	-	20.01

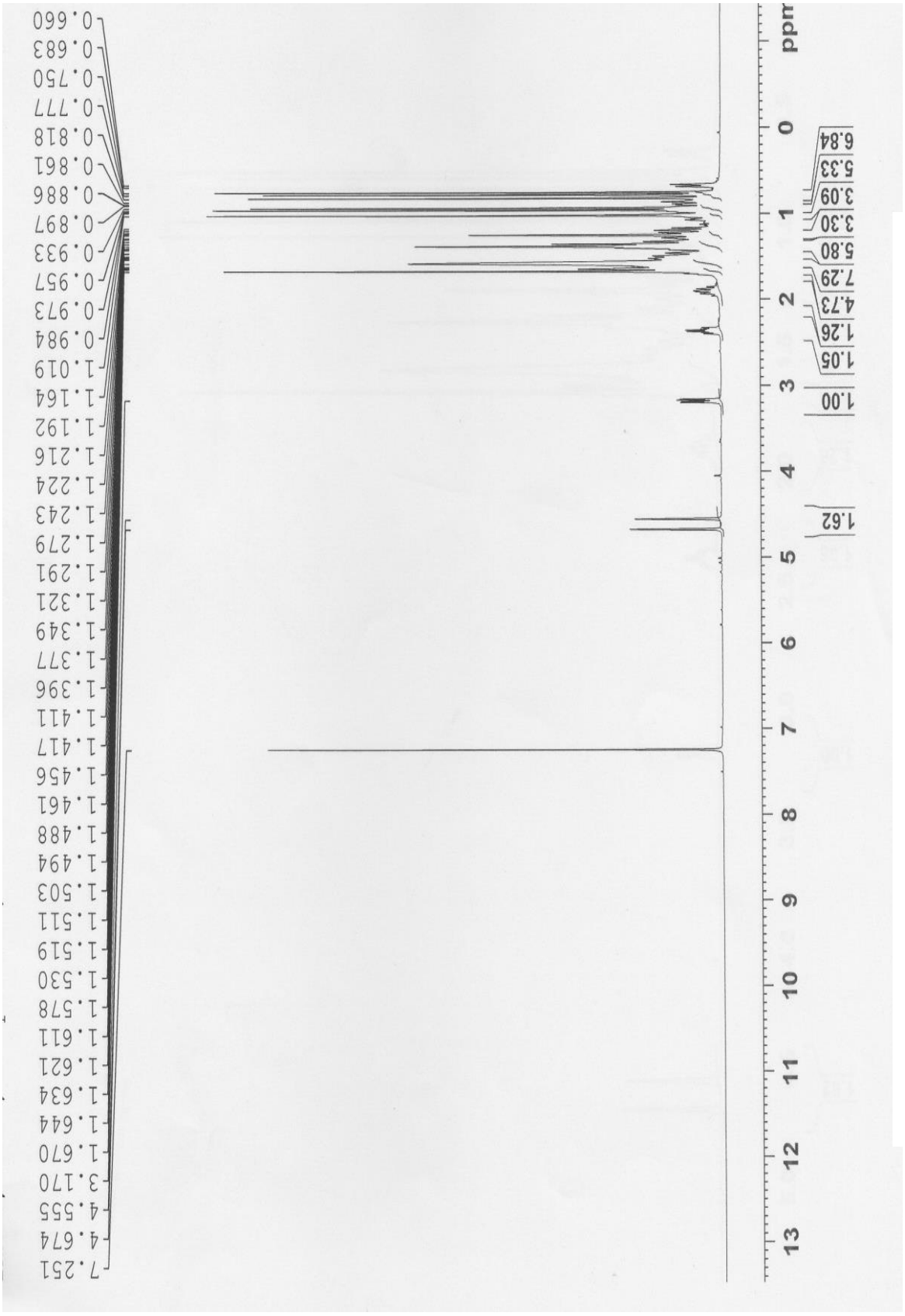

Figure 3.35: ^{1}H NMR (100 MHz, $CDCl_3$) spectrum of compound-7

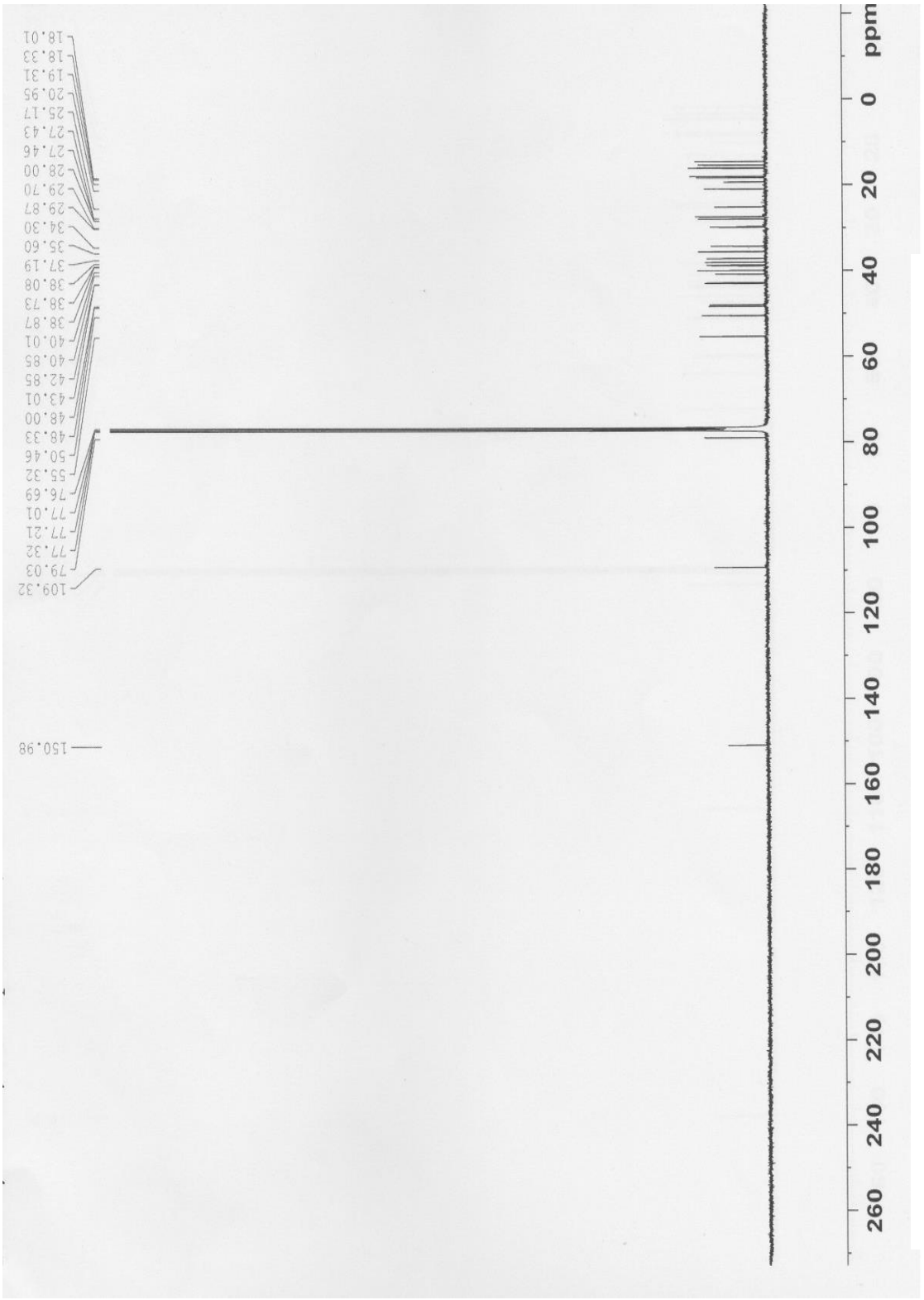

Figure 3.36: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of compound-7

The ^{1}H NMR and ^{13}C-NMR data of the **compound-7** compared with the published data Lupeol. **Ref. [30] : Jain, P. S. and Bari, S. B., (2010)** the structure **compound-7** was suggested as-

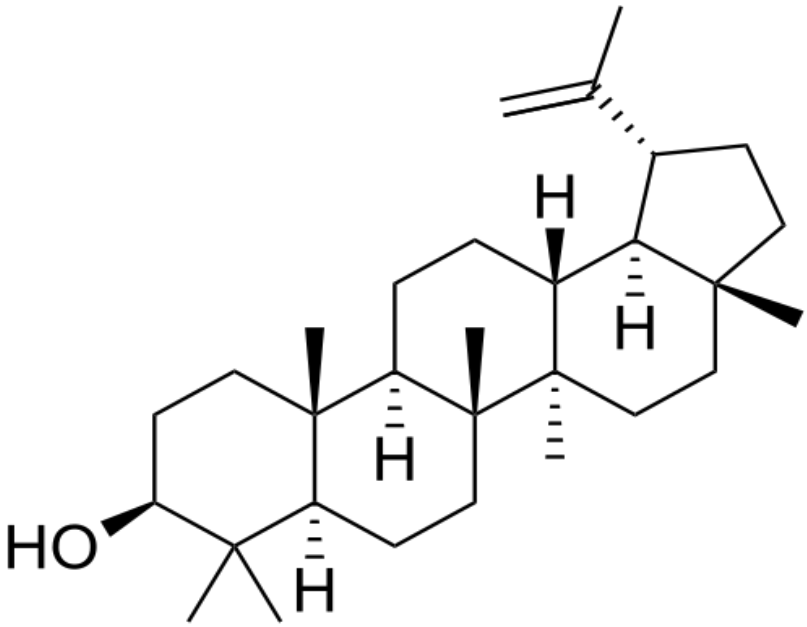

Figure 3.37: Structure of compound-7 (Lupeol)

ANTIOXIDANT ACTIVITY

4.1. Antioxidant activity: DPPH assay

Principle, Materials and methods are same and was discussed in previous section **Part-A page-97-98**

4.2 Results and discussion

4.2.1. Free radical scavenging activity (DPPH)

In this investigation, HEX showed the highest free radical scavenging activity with an IC_{50} value 65.27 µg/ml for the roots of *Bombax ceiba*. At the same time the ME, DCM, EAC and AQP also exhibited antioxidant potential having IC_{50} value 12.06, 22.04, 8.45 and 3.30 µg/ml respectively.

Table 4.1: IC_{50} values of the standard and partitionates of the roots of *Bombax ceiba*

Plant part	Sample code	Test Sample	IC_{50} (µg/ml)
Roots of	**ME**	Methanolic extract	12.06
Bombax	**HEX**	n-hexane soluble partitionate	65.27
ceiba	**DCM**	Dichloromethane soluble partitionate	22.04
	EAC	Ethyl acetate soluble partitionate	8.45
	AQP	Aqueous soluble partitionate	3.30
Standard	**BHT**	Tert-butyl-1-hydroxytoluene	62.45

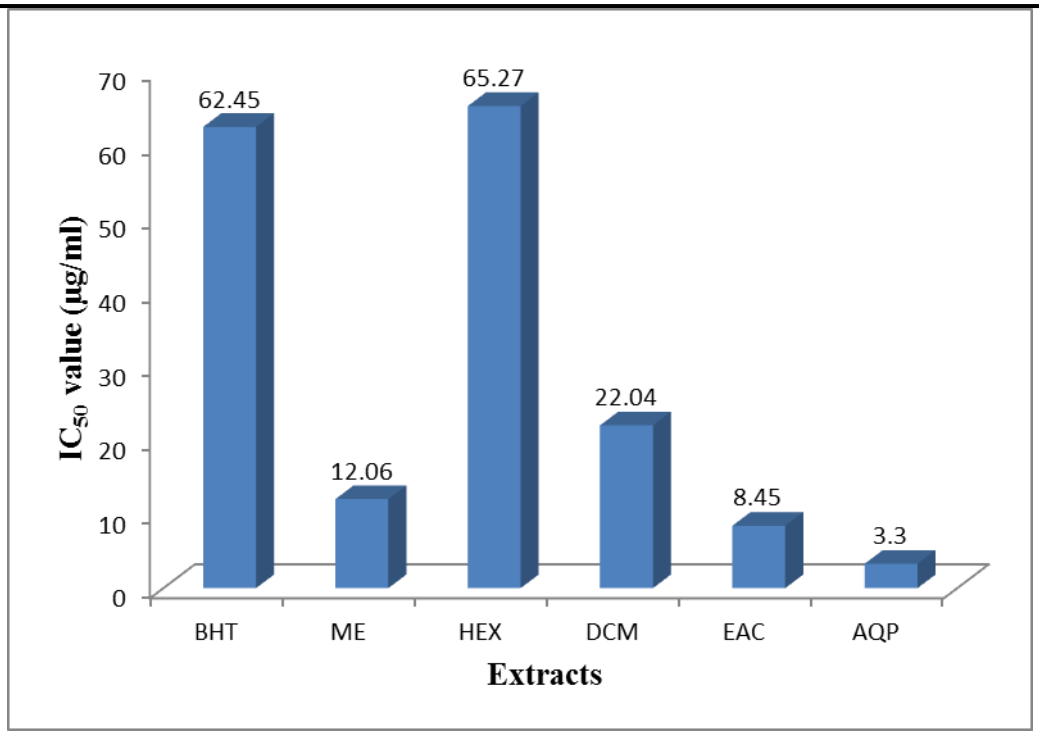

Figure 4.1 IC_{50} values of the standard and partitionates of roots of *Bombax ceiba*

Table 4.2: IC_{50} value of tert-butyl-1-hydroxytoluene (BHT)

Test tube No.	Abs. of Control	Conc. of sample (µg/ml)	Abs. of sample	% inhibition	IC_{50} (µg/ml)
1	0.269	500	0.011	95.91	62.45
2		250	0.048	82.16	
3		125	0.105	60.97	
4		62.5	0.149	44.61	
5		31.25	0.217	19.33	
6		15.625	0.226	15.98	
7		7.813	0.247	8.18	
8		3.906	0.254	5.58	
9		1.953	0.261	2.97	
10		0.977	0.277	0.74	

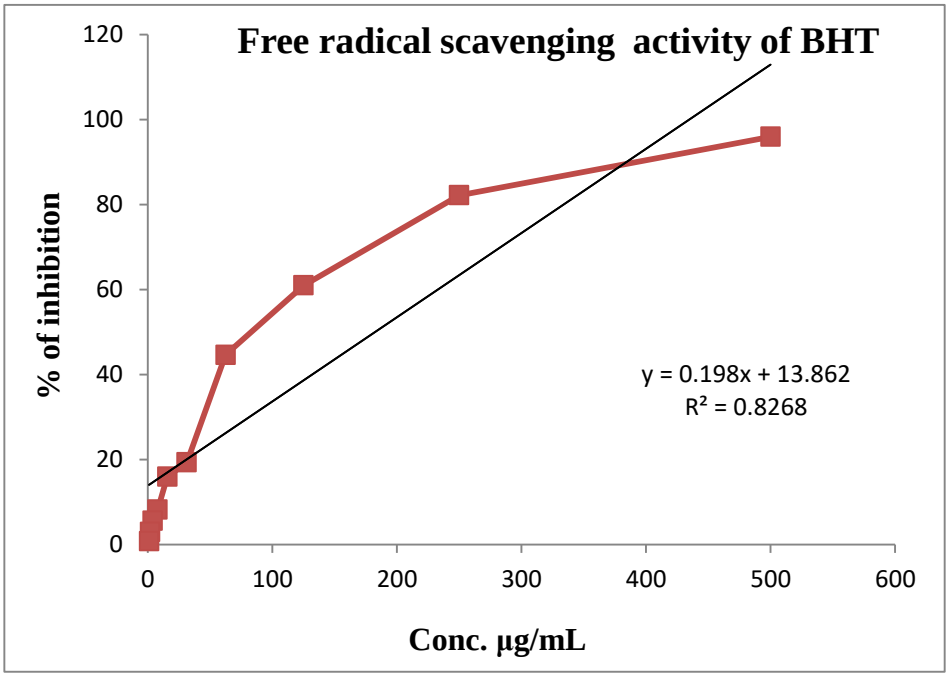

Figure 4.2: IC_{50} value of tert-butyl-1-hydroxytoluene (BHT)

Table 4.3: IC_{50} value of methanolic extract (ME) of roots of *Bombax ceiba*

Test tube No.	Abs. of Control	Conc. of sample (µg/ml)	Abs. of sample	% inhibition	IC_{50} (µg/ml)
1	0.269	500	0.011	95.91	12.06
2		250	0.013	95.17	
3		125	0.010	96.28	
4		62.5	0.002	99.26	
5		31.25	0.027	89.96	
6		15.625	0.123	54.28	
7		7.813	0.150	44.24	
8		3.906	0.227	15.61	
9		1.953	0.248	7.81	
10		0.977	0.248	7.81	

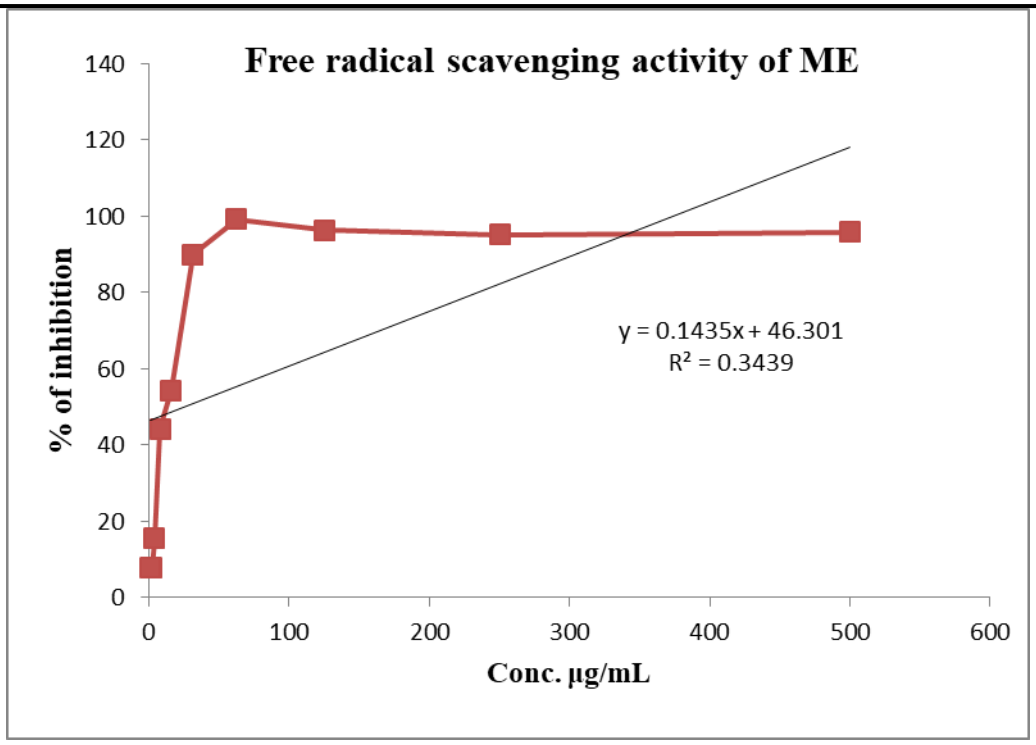

Figure 4.4: IC_{50} value of methanolic extract of roots of *Bombax ceiba*

Table 4.4: IC_{50} value of n-Hexane soluble partitionate (HEX) of roots of *Bombax ceiba*

Test tube No.	Abs. of Control	Conc. of sample (µg/ml)	Abs. of sample	% inhibition	IC_{50} (µg/ml)
1	0.269	500	0.032	88.10	65.27
2		250	0.028	89.59	
3		125	0.089	66.91	
4		62.5	0.162	39.77	
5		31.25	0.208	22.68	
6		15.625	0.255	5.20	
7		7.813	0.255	5.20	
8		3.906	0.258	4.09	
9		1.953	0.248	7.81	
10		0.977	0.267	0.74	

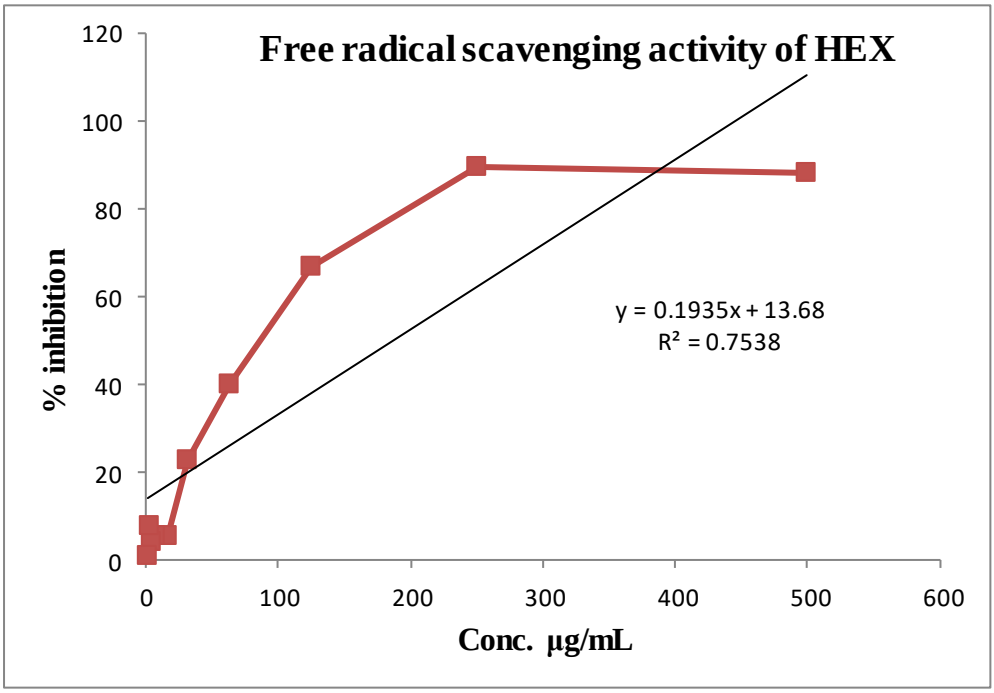

Figure 4.5: IC_{50} value of n-hexane soluble partitionate of roots of *Bombax ceiba*

Table 4.5: IC_{50} value of Dichloromethane soluble partitionate (DCM) of roots of *Bombax ceiba*

Test tube No.	Abs. of Control	Conc. of sample (µg/ml)	Abs. of sample	% inhibition	IC_{50} (µg/ml)
1	0.269	500	0.082	69.52	22.04
2		250	0.036	86.62	
3		125	0.018	93.31	
4		62.5	0.026	90.33	
5		31.25	0.094	65.06	
6		15.625	0.155	42.38	
7		7.813	0.203	24.53	
8		3.906	0.234	13.01	
9		1.953	0.246	8.55	
10		0.977	0.250	7.06	

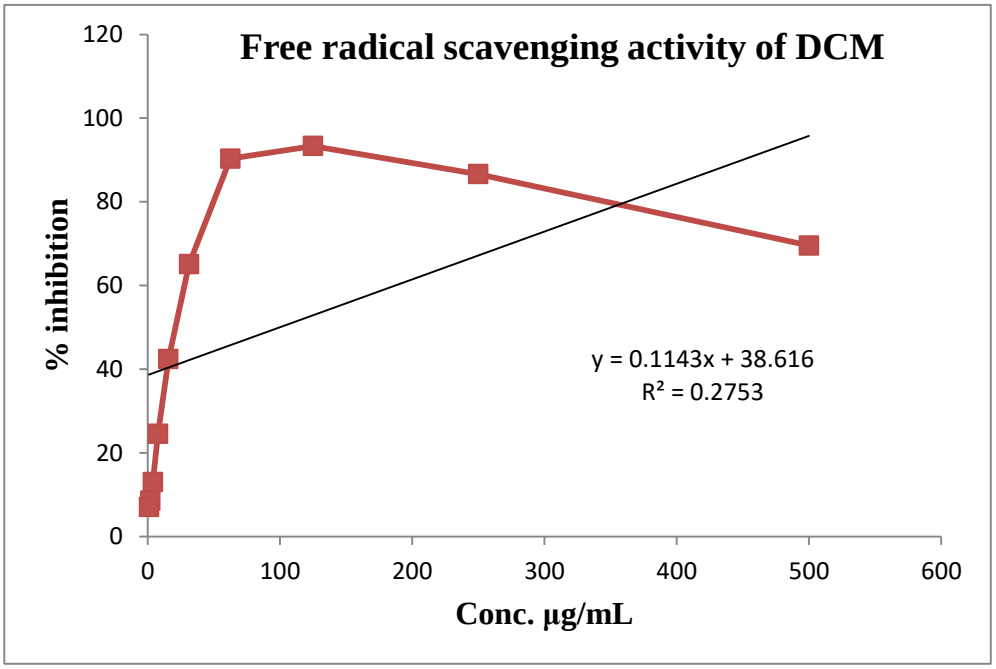

Figure 4.6: IC_{50} value of DCM soluble partitionate of roots of *Bombax ceiba*

Table 4.6: IC_{50} value of ethylacetate soluble partitionate (EAC) of roots of *Bombax ceiba*

Test tube No.	Abs. of Control	Conc. of sample (µg/ml)	Abs. of sample	% inhibition	IC_{50} (µg/ml)
1	0.269	500	0.016	94.05	8.45
2		250	0.013	95.17	
3		125	0.009	96.65	
4		62.5	0.001	99.63	
5		31.25	0.007	97.40	
6		15.625	0.077	71.38	
7		7.813	0.138	48.70	
8		3.906	0.190	29.37	
9		1.953	0.232	13.75	
10		0.977	0.244	9.29	

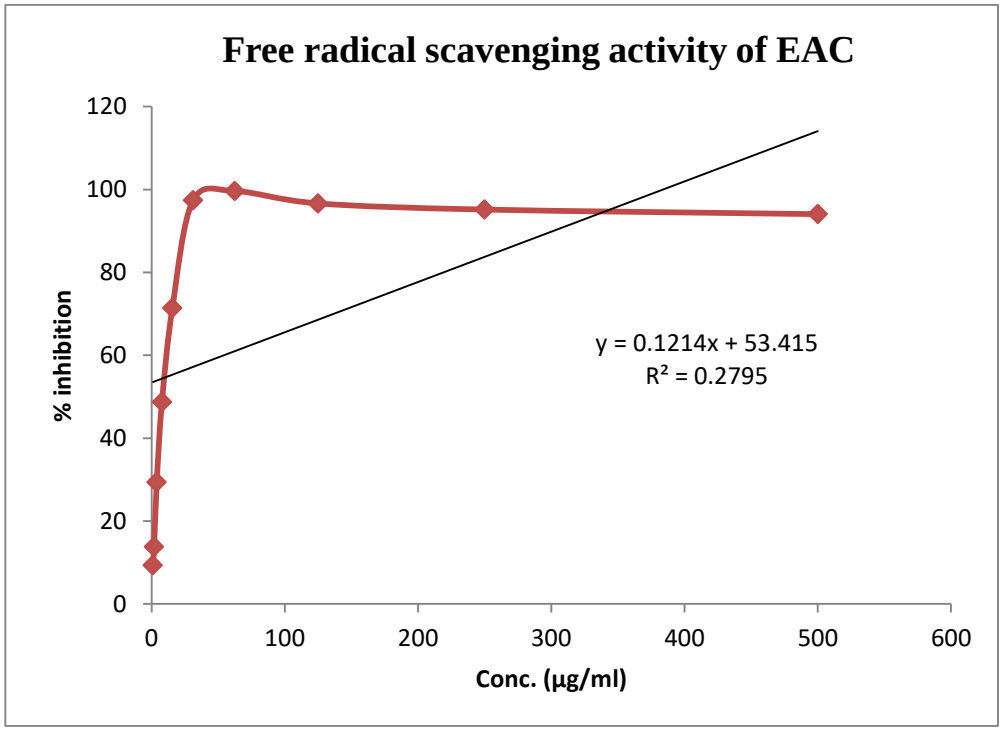

Figure 4.7: IC_{50} value of EtOAc soluble partitionate of roots of *Bombax ceiba*

Table 4.7: IC_{50} value of aqueous soluble partitionate (AQP) of roots of *Bombax ceiba*

Test tube No.	Abs. of Control	Conc. of sample (µg/ml)	Abs. of sample	% inhibition	IC_{50} (µg/ml)
1	0.269	500	0.014	94.80	3.33
2		250	0.012	95.54	
3		125	0.008	97.03	
4		62.5	0.007	97.40	
5		31.25	0.006	97.77	
6		15.625	0.001	99.63	
7		7.813	0.042	84.39	
8		3.906	0.136	49.44	
9		1.953	0.202	24.91	
10		0.977	0.229	11.15	

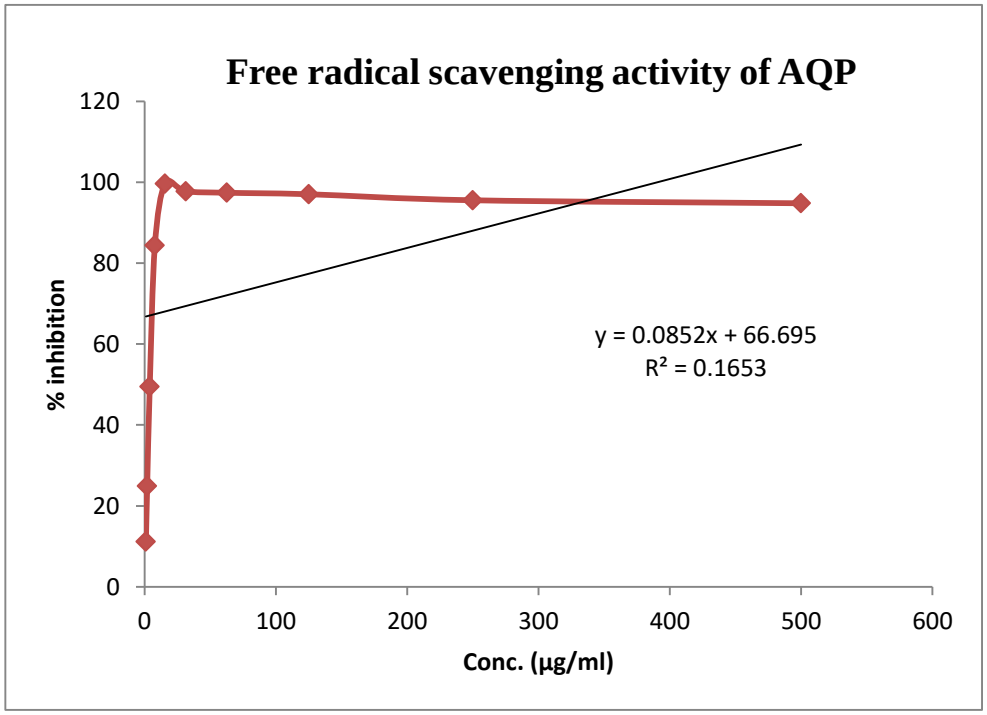

Figure 4.8: IC_{50} value of aqueous soluble partitionate of roots of *Bombax ceiba*

ANTIMICROBIAL SCREENING

5.1 Principle of disc diffusion method and Experimental procedure are same and was illustrated in previous section **Part-A page-105-106**

5.2 Results and discussion

The Methanolic extract of roots of *B. ceiba* (ME) and different partitionates i.e. aqueous (AQP), hexane (HEX), dichloromethane (DCM), and ethylacetate (EAC) soluble partitionate of the methanolic extract were subjected to antimicrobial screening with a concentration of 400 μg/disc in every case. The results are given in the Table 8.3.

The DCM partitionate showed the growth of inhibition specially against gram negative bacteria including *Shigella dysenteriae*, *S. paratyphi*, *Pseudomonas aeruginosa*, *Shigella boydil* and against fungi *Aspergillus niger*.The other fractions including methanol (ME), hexane (HEX), ethylacetate (EAC) and aqueous partitionates (AQP) did not show any antimicrobial activity.

The results of In vitro microbial screening of roots of *B. ceiba* indicated that DCM partitionate of *B. ceiba* roots possess better antimicrobial activity and these can be further studied to explore potent antimicrobial agents.

Table 5.1: Antimicrobial activity of test samples of *Bombax ceiba*

Test organism	ME /mm	HEX /mm	DCM /mm	EAC /mm	AQP /mm	STD /mm
Gram positive bacteria						
Bacillus cereus	0	0	0	0	0	43
B. megaterium	0	0	0	0	0	42
B. subtilis	0	0	0	0	0	39
Staphylococcus aureus	0	0	0	0	0	42
Sarcina lutea	0	0	0	0	0	40
Gram negative bacteria						
Escherichia coli	0	0	0	0	0	45
Pseudomonas aeruginosa	0	0	8	0	10	41
Salmonella paratyphi	0	0	8	0	10	44
***S.* Typhi**	0	0	0	0	0	45
Shigella boydii	0	0	8	0	0	45
Sh. dysenteriae	0	0	14	0	0	48
Vibrio mimicus	0	0	0	0	0	43
V. parahemolyticus	0	0	0	0	0	43
Fungi						
Candida albicans	0	0	0	0	0	44
Aspergillus niger	0	0	9	0	0	44
Sacharomyces cerevacae	0	0	0	0	0	41

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BRINE SHRIMP LETHALITY BIOASSAY

6.1. Principle

Brine shrimp eggs are hatched in simulated sea water to get nauplii. By the addition of calculated amount of dimethylsulphoxide (DMSO), desired concentration of the test sample is prepared. The nauplii are counted by visual inspection and are taken in vials containing 5 ml of simulated sea water. Then samples of different concentrations are added to the premarked vials through micropipette. The vials are then left for 24 hours. Survivors are counted after 24 hours.

6.2. Experimental procedure

The experiment was done by following the method illustrated by Meyer *et al*., 1982.

6.3. Test samples of experimental plant

Table 6.1: Test samples of *B. ceiba*

Plant part	Sample code	Test Sample	Calculated amount (mg)
Roots of *Bombax ceiba*	ME	Methanolic extract	4.0
	HEX	n-hexane partitionate	4.0
	DCM	Dichloromethane partitionate	4.0
	EAC	Ethyl acetate partitionate	4.0
	AQP	Aqueous soluble partitionate	4.0

6.4. Results and discussion

In the present bioactivity study methanolic extract and its different partitionate of *B. ceiba* i.e. n-hexane, dichloromethane, ethylacetate and aqueous soluble fractions showed positive results indicating that the test samples are biologically active.

Vincristine sulfate (VS) was used as positive control and the LC_{50} was found 9.02 µg/ml for VS. Compared with the negative control VS (positive control) gave significant mortality. The LC_{50} values of the different extractives 0f *Bombax ceiba* were compared to this positive control.

The LC_{50} values of ME, HEX, DCM, EAC and AQP were found to be 44.37 µg/ml, 1.02 µg/ml, 10.59 µg/ml 102.03 µg/ml and 775.19 µg/ml respectively. HEX revealed significant lethality whereas ME and DCM revealed moderate activity. EAC and AQP revealed very low activity.

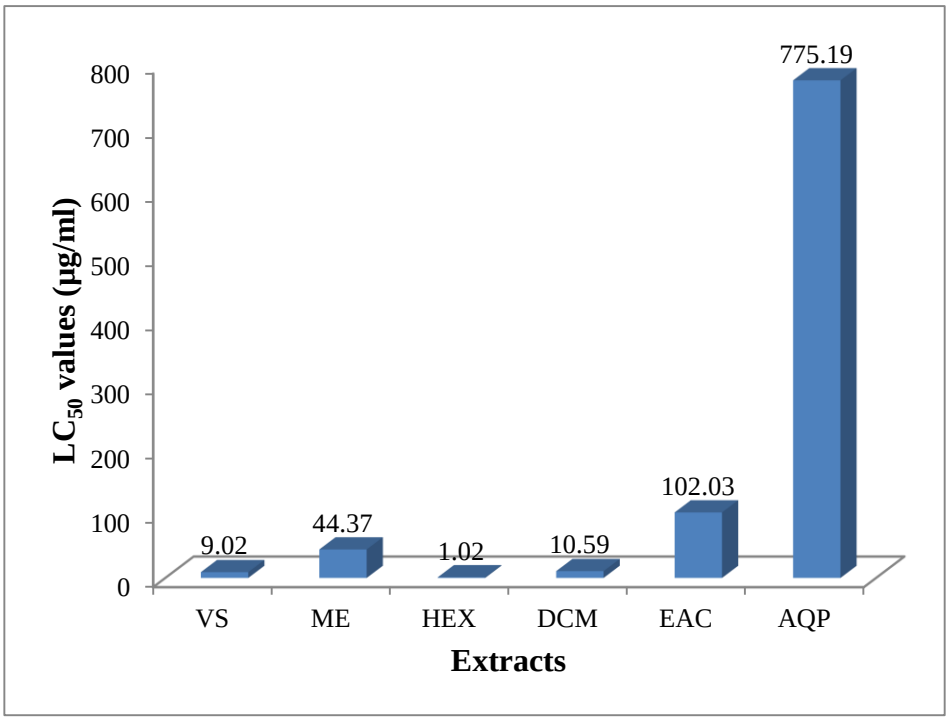

Figure 7.1: LC_{50} values of the different extractives of the roots of *Bombax ceiba*

Table 6.2: Effect of Vincristine sulphate (positive control) on shrimp nauplii

Test tube No.	Conc. (µg/ml)	No. of nauplii taken	No. of nauplii dead	% of mortality	LC_{50} (µg/ml)
1	400	10	10	100	9.02
2	200	10	9	90	
3	100	10	8	80	
4	50	10	8	80	
5	25	10	7	70	
6	12.5	10	5	50	
7	6.25	10	4	40	
8	3.125	10	3	30	
9	1.5625	10	3	30	
10	0.78125	10	2	20	

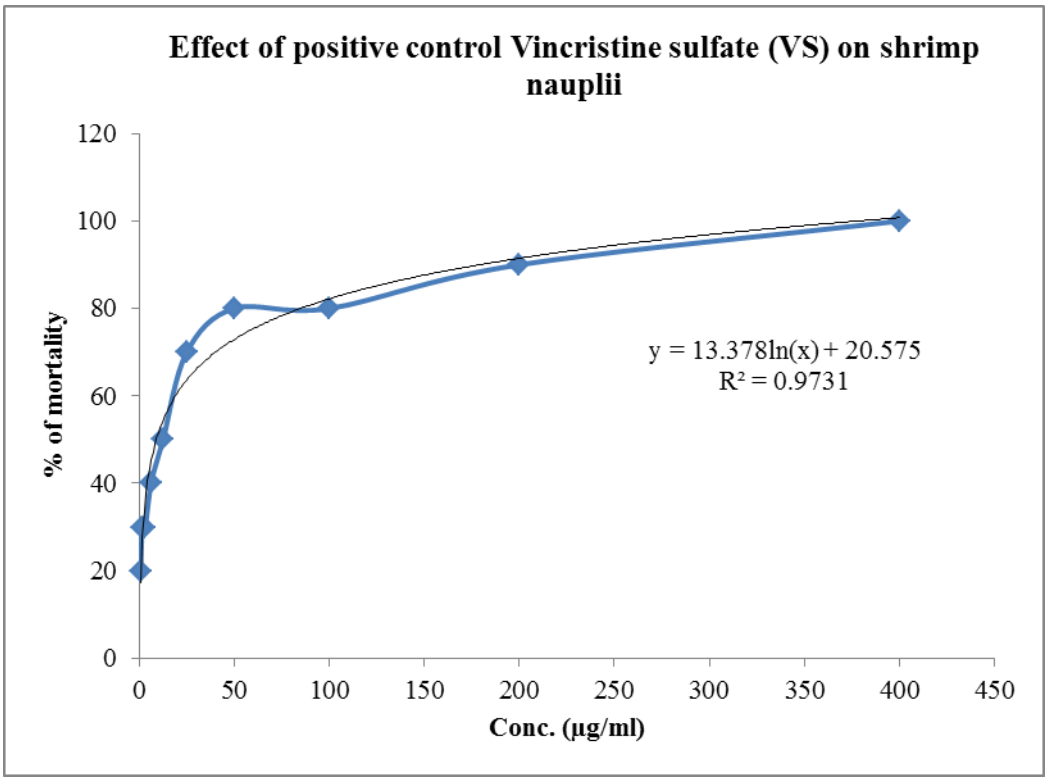

Figure 7.2: LC_{50} value of positive control, VS

Table 6.3: Effect of methanolic extract of roots of Bombax ceiba on shrimp nauplii

Test tube No.	Conc. (µg/ml)	No. of nauplii taken	No. of nauplii dead	% of mortality	LC_{50} (µg/ml)
1	400	10	8	80	
2	200	10	8	80	
3	100	10	6	60	
4	50	10	6	60	
5	25	10	4	40	44.37
6	12.5	10	3	30	
7	6.25	10	1	10	
8	3.125	10	0	0	
9	1.5625	10	0	0	
10	0.78125	10	0	0	

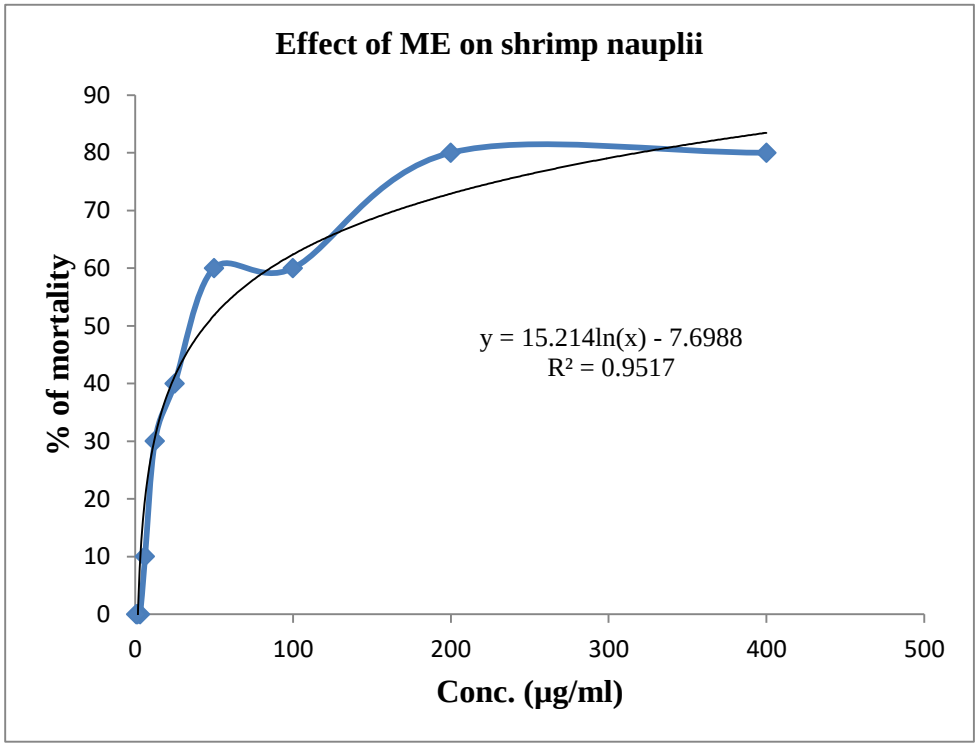

Figure 7.3: LC_{50} value of ME of *Bombax ceiba*

Table 6.4: Effect of n-Hexane extract of roots of *Bombax ceiba* on shrimp nauplii

Test tube No.	Conc. (µg/ml)	No. of nauplii taken	No. of nauplii dead	% of mortality	LC_{50} (µg/ml)
1	400	10	10	100	
2	200	10	10	100	
3	100	10	10	100	
4	50	10	10	100	
5	25	10	9	90	1.02
6	12.5	10	8	80	
7	6.25	10	8	80	
8	3.125	10	6	60	
9	1.5625	10	4	40	
10	0.78125	10	4	40	

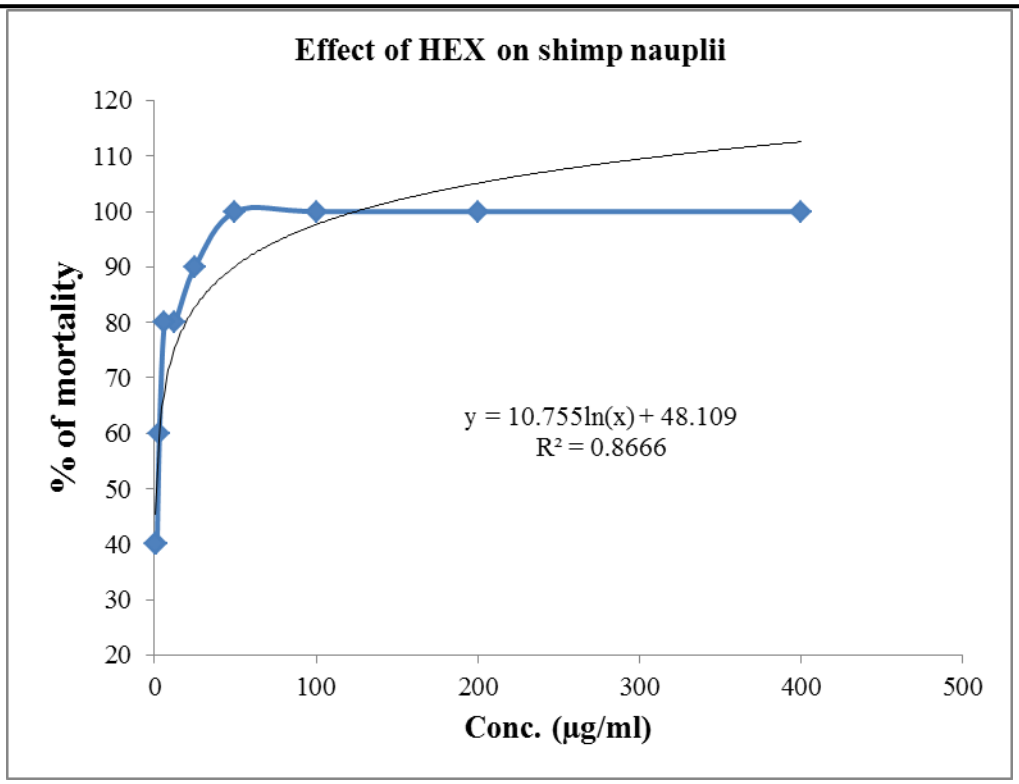

Figure 7.4: LC_{50} value of HEX of *Bombax ceiba*

Table 6.5: Effect of Dichloromethane extract of roots of Bombax ceiba on shrimp nauplii

Test tube No.	Conc. (µg/ml)	No. of nauplii taken	No. of nauplii dead	% of mortality	LC_{50} (µg/ml)
1	400	10	10	100	10.59
2	200	10	9	90	
3	100	10	8	80	
4	50	10	7	70	
5	25	10	4	40	
6	12.5	10	4	40	
7	6.25	10	4	40	
8	3.125	10	4	40	
9	1.5625	10	3	30	
10	0.78125	10	3	30	

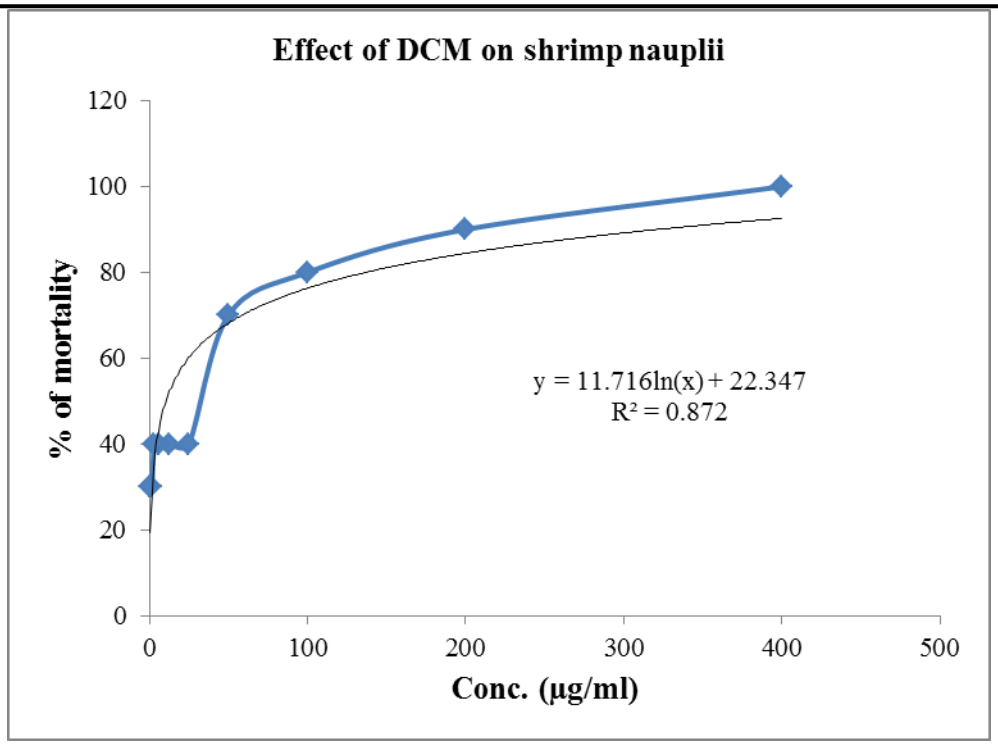

Figure 7.5: LC_{50} value of DCM of *Bombax ceiba*

Table 6.6: Effect of Ethylacetate extract of roots of Bombax ceiba on shrimp nauplii

Test tube No.	Conc. (µg/ml)	No. of nauplii taken	No. of nauplii dead	% of mortality	LC_{50} (µg/ml)
1	400	10	8	80	102.03
2	200	10	7	70	
3	100	10	5	50	
4	50	10	3	30	
5	25	10	2	20	
6	12.5	10	1	10	
7	6.25	10	1	10	
8	3.125	10	0	0	
9	1.5625	10	1	10	
10	0.78125	10	1	10	

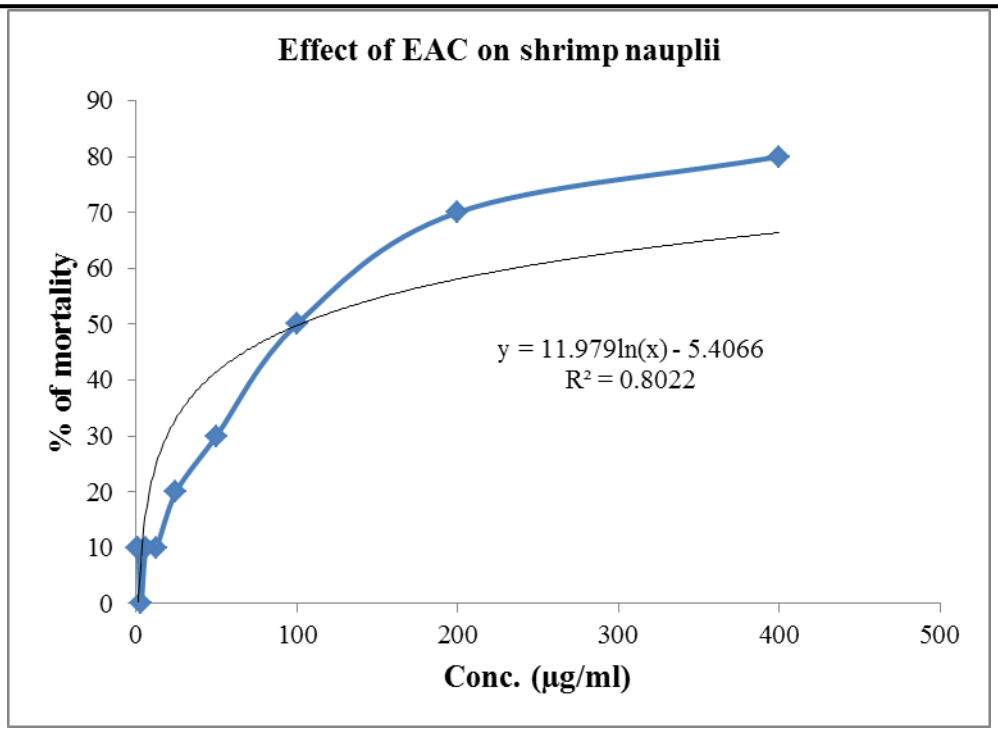

Figure 7.6: LC_{50} value of EAC of *Bombax ceiba*

Table 6.7: Effect of aqueous extract of roots of *Bombax ceiba* on shrimp nauplii

Test tube No.	Conc. (µg/ml)	No. of nauplii taken	No. of nauplii dead	% of mortality	LC_{50} (µg/ml)
1	400	10	3	30	
2	200	10	2	20	
3	100	10	2	20	
4	50	10	1	10	
5	25	10	1	10	775.19
6	12.5	10	0	0	
7	6.25	10	0	0	
8	3.125	10	0	0	
9	1.5625	10	0	0	
10	0.78125	10	0	0	

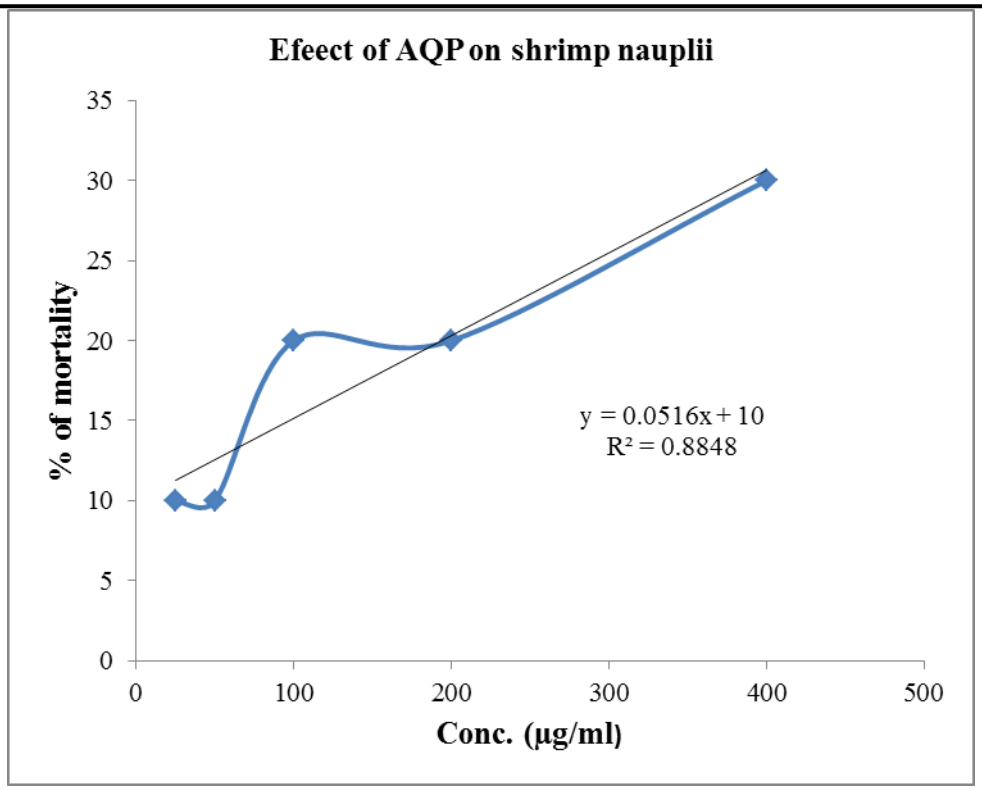

Figure 7.7: LC_{50} value of AQP of *Bombax ceiba*

EVALUATION OF ANTIDIRRHOEAL ACTIVITY

7.1. Principle

The antidiarrheal activity of the methanolic extract of *Bombax ceiba* roots was evaluated using the method of castor oil induced diarrhea in mice (Shoba & Thomas, 2001). Each mouse was fed with 1ml of highly pure analytical grade of castor oil. The numbers of fecal stools were recorded for each individual mouse. The observations of the experimental groups were compared against that of the control to evaluate the anti-diarrheal activity of the samples.

7.2. Procedure

The method, described by Shoba and Thomas (2001), was followed for this study with slight modification.

7.3. Results and discussion

Table 7.1: Test materials used in the evaluation of antidiarrheal activity of methanol extract of *Bombax ceiba*

Code no.	Test Samples	Identification	Dose (mg/kg)	Route of administration
CTL	1% Tween-80 in normal saline	Control Group	0.1 ml/10 g of body wt	Oral
STD	Loperamide	Standard Group	50	Oral
ME 1	Methanolic extract *Bombax ceiba*	Test Sample	200	Oral
ME 2		Test Sample	400	Oral

Table 7.2: Total No. of diarrheal feces stool given by each mouse

Code No.	Mice No.	No. of diarrheal feces				Total No. of diarrheal feces	Average	% reduction of diarrheal feces
		1 hr	2 hrs	3 hrs	4 hrs			
CTL	1	1	3	2	2	8	7.33	-
	2	1	2	2	2	7		
	3	1	1	2	3	7		
STD	1	0	1	1	1	3	2.33	68.21
	2	0	1	0	1	2		
	3	0	0	1	1	2		
ME 1	1	0	0	1	1	3	4.00	45.43
	2	1	2	1	0	4		
	3	0	2	1	2	5		
ME 2	1	0	1	1	1	3	3.33	54.57
	2	1	1	1	1	4		
	3	0	0	1	1	3		

Table 7.3: Effect of methanolic extract of *roots of* Bombax ceiba on castor oil (1ml/mice) induced diarrhoea in mice

Code No.	t-Test value	P value	Standard deviation	Standard error	Level of significance
STD	10.6066	0.0004	0.5774	0.3333	statistically significant
ME 1	5.0000	0.0075	1.0000	0.5774	statistically significant
ME 2	8.4853	0.0011	0.5774	0.3333	statistically significant

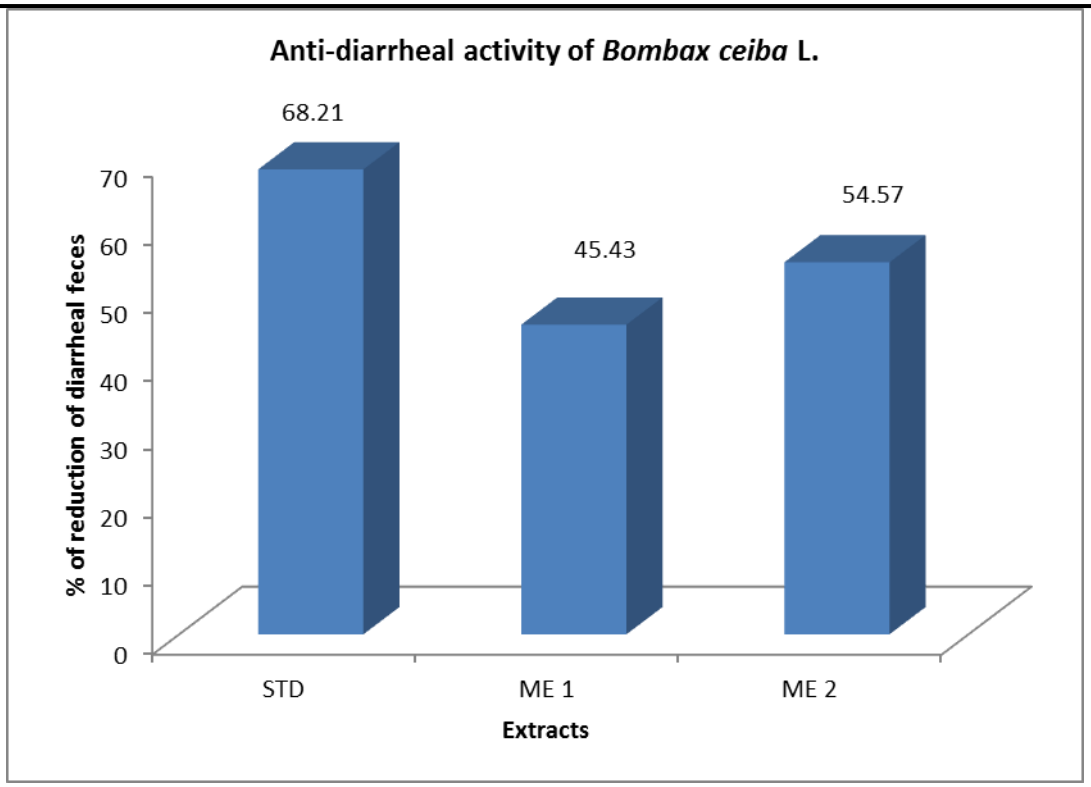

Figure 7.1: Antidiarrhoeal effect of methanolic extract of *Bombax ceiba*

From the above discussions, it can be concluded that the methanolic extract of roots of Bombax ceiba at the dose of 200 and 400 mg/kg body weight showed highly antidiarrhoeal activity and it was statistically significant. This can be related to the plant's tradition use in GIT problems. Further research can be performed in this area to identify an antidiarrhoeal lead compound.

EVALUATION OF THROMBOLYTIC ACTIVITY

8.1. Introduction

Since ancient times, herbal preparations have been used for the treatment of several diseases. The leaves and/or twigs, stem, bark and underground parts of plants are most often used for traditional medicines. Herbal products are often perceived as safe because they are "natural" (Gesler, 1992). Thrombolytic drugs like tissue plasminogen activator (t-PA), urokinase, streptokinase etc. play a crucial role in the management of patients with CVST (Baruah, 2006). Thus, the aim of the present study was to investigate the thrombolytic activity of methanolic extracts and its different fractions of roots of *Bombax ceiba.*

8.2. Materials and methods

8.2.1. Preparation of sample

The thrombolytic activity of all extractives was evaluated by a method using streptokinase (SK) as standard substance. 10 mg of methanolic extracts and its different fractions of roots of *Bombax ceiba* were taken in different vials to which 1ml distilled water was added.

8.2.2. Streptokinase (SK)

Commercially available lyophilized Altepase (Streptokinase) eppendorf tube of 15, 00,000 I.U., was collected and 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100µl (30,000 I.U) was used for *in vitro* thrombolysis.

8.2.3. Blood sample

1ml of blood was collected and transferred to the previously weighed sterile eppendorf tubes and was allowed to form clots.

8.2.4. Thrombolytic activity

Aliquots (5 ml) of venous blood were drawn from healthy volunteers who were distributed in ten different pre weighed sterile eppendorf tubes (0.5 ml/tube) and incubated at 37 °C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and each eppendorf tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone).

To each eppendorf tube containing pre-weighed clot, 100 µL aqueous solutions of different partitionates along with the crude extracts was added separately. As a positive control, 100 µl of streptokinase (SK) and as a negative non thrombolytic control, 100 µl of distilled water were separately added to the control eppendorf tubes. All the eppendorf tubes were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, the released of fluid was removed and eppendorf tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

% of clot lysis = (Weight of the lysis clot / Weight of clot before lysis) × 100

8.3. Results and discussion

Addition of 100µl SK, a positive control (30,000 I.U.), to the clots and subsequent incubation for 90 minutes at 37°C, showed 64.22% lysis of clot. On the other hand, distilled water was treated as negative control which exhibited a negligible percentage of lysis of clot (3.80%). The mean difference in clot lysis percentage between positive and negative control was found very significant.

In this study, the methanolic extract of roots of *Bombax ceiba* (ME) exhibited thrombolytic activity 21.09%, the hexane soluble fraction (HEX) exhibited 9.56%, dichloromethane soluble fraction (DCM) exhibited 40.67%, ethylacetate soluble fraction (EAC) exhibited 29.55% and the aqueous soluble fraction (AQP) exhibited 44.55% thrombolytic activity.

Table 8.1: Thrombolytic Activity of the extractives of *Bombax ceiba*

Fractions	Weight of empty eppendorf tube	Weight of clot containing eppendorf tube before clot disruption	Weight of clot containing eppendorf tube after clot disruption	Weight of clot before lysis	Weight of lysis clot	% of lysis
	W1 mg	W2 mg	W3 mg	W4=W2-W1 mg	W5=W2-W3 mg	(W5/W4) * 100%
ME	5299.4	5450.2	5418.4	150.8	31.8	21.09
HEX	4842.3	5047.3	5027.7	205	19.6	9.56
DCM	4828.8	5044.7	4957.2	215.9	87.5	40.67
EAC	4727.2	4871.7	4826.0	144.5	42.7	29.55
AQP	4796.1	5074.7	4935.6	251.6	112.1	44.55
Blank	4747.0	4984.0	4975.2	237.0	9.0	3.80
SK	4885.0	5387.5	5064.8	502.5	322.7	64.22

Here,

ME = Methanolic extract of the roots of *Bombax ceiba*

HEX = n-Hexane soluble fraction of the methanolic extract of *Bombax ceiba*

DCM = Dichloromethane soluble fraction of the methanolic extract of *Bombax ceiba*

EAC = Ethylacetate soluble fraction of the methanolic extract of *Bombax ceiba*

AQP = Aqueous soluble fraction of the methanolic extract of *Bombax ceiba*

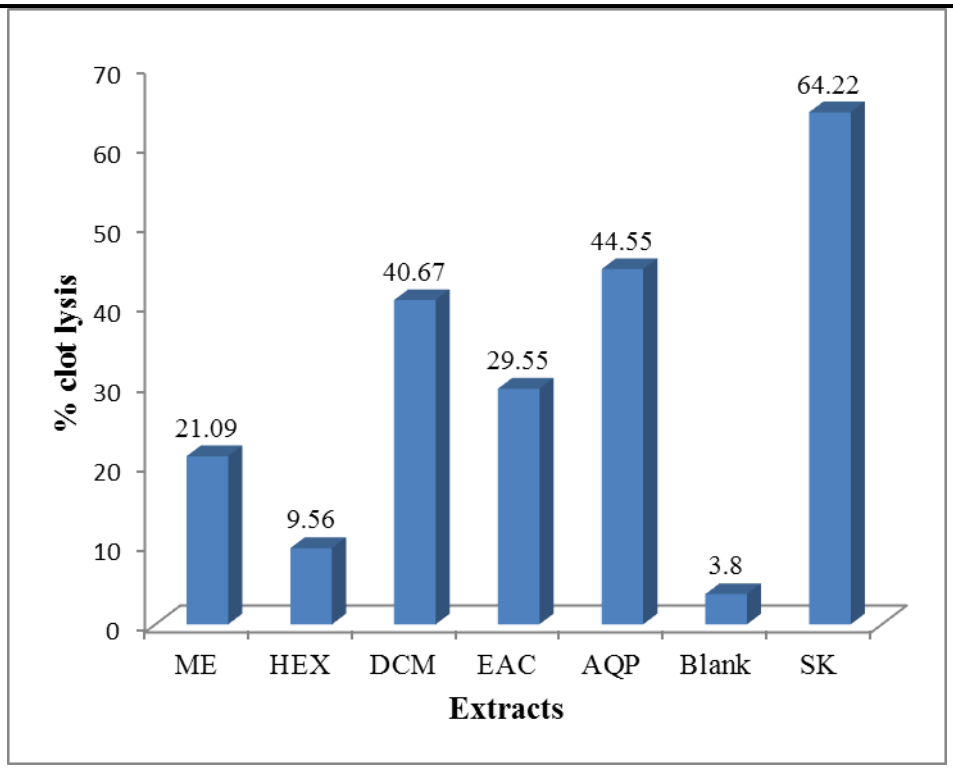

Figure 8.1: Thrombolytic activity of the different extractives of *Bombax ceiba*

From this experiment, it can be concluded that the few of the extractives of *Bombax ceiba* showed significant clot lysis activity. Once found these herbal preparations may be incorporated as a thrombolytic agent for the improvement of the patients suffering from Atherothrombotic diseases. This is only a preliminary study and to make final comment the extract should thoroughly investigated phytochemically and pharmacologically to exploit their medicinal and pharmaceutical potentialities.

EVALUATION OF CENTRAL ANALGESIC ACTIVITY

9.1. Principle

Evaluation of central analgesic activity was carried by tail immersion method using Diclofenac as a positive control. The changes in sensitivity due to analgesic activity of drugs were measured in this method. A constant heat stress was applied to rat tail, which acts as pain stimulus. When the stimulus exceeded the threshold, rats showed a quick withdrawal of its tail. Time taken by the rat to withdraw the tail is termed as tail immersion time. Analgesic compounds elongate this responding time. By this test discrimination was done between centrally acting morphine –like analgesics and non-opiate analgesics. The test rats were orally fed with test materials whereas the positive control received diclofenac subcutaneously. From 1-2 cm of the tail of mice was immersed in warm water kept constant at 55^0 C .The reaction time is the time required by the mice to deflect their tails. The time required to withdraw the tail was recorded.

9.2 Experimental animal

Swiss-albino mice of either sex, aged 4-5 weeks, were used for the experiment.

9.3 Experimental Procedure

9.3.1 Preparation of Test Materials

In order to administer the extract at doses of 200 mg/kg body wt and 400 mg/kg body wt of mice, the exactly weighed extracts were measured respectively and triturated in unidirectional way by adding of small amount of Tween-80 (a suspending agent). After proper mixing of extract and suspending agent, normal saline was slowly added. The final volume of the suspension was made up to 3.0 ml. To stabilize the suspension, it was stirred well by vortex mixture.

Diclofenac sodium (Diclofenac sodium 75 mg) was administered subcutaneously in the form of solution. The solution was prepared by diluting the supplied Diclofenac sodium (75 mg in 2 ml) with saline water.0.4 ml Diclofenac sodium solution was taken from the ampoule and it was then diluted up to 180 ml with normal saline. Then each 0.6 ml contained 0.05 mg of morphine which ensured a dose of 2mg/kg for 25 mg mice.

Table 9.1: Test materials used in the evaluation of central analgesic activity of crude extract of *Bombax ceiba*

Code no.	Test Samples	Identification	Dose (mg/kg)	Route of administration
CTL	1% Tween-80 in normal saline	Control Group	30 ml	Oral
STD	Diclofenac	Standard Group	2 mg	Subcutaneous
ME 1	Methanolic extract of *Bombax ceiba*	Test Sample	200	Oral
ME 2		Test Sample	400	Oral

9.4 Methodology

Test samples and control were given orally by means of a feeding needle to the mice at zero hour. At zero hour, 1-2 cm of the tail of mice was immersed in warm water kept constant at 55^0 C. The reaction time is the time required by the mice to deflect their tails. A latency period of 20 seconds was defined as complete analgesia and the measurement was stopped to avoid injury to mice. The latent period of tail-flick response was determined before and 0, 30, 60 and 90 minutes after the administration of drugs. A 30 minutes interval was given to ensure proper absorption of the administered substances. Then morphine solution was administered subcutaneously to the mice. After 30 minutes, 60 minutes and 90 minutes, the tail immersion time was measured.

9.5 Results and discussion

The time for tail immersing of each mouse was recorded and the average immersing time of each group was calculated. The % time elongation of tail immersing was calculated in respect to the control. The higher the elongation percentage of the group the greater is the group's central analgesic activity. The central analgesic activity of the test samples were compared with respect to Diclofenac.

$$\% \text{ Time elongation} = \frac{\text{Average time of tail flicking of test samples} - \text{Average time of tail immersing of the control group}}{\text{Average time of tail flicking of control group}}$$

Table 9.2: Analysis of the data obtained after 30 minutes

Code No.	Immersion time count (seconds)			Average time of immersion	Standard deviation	Standard error	% Elongation
	M-1	M-2	M-3				
CTL	2.02	2.64	1.82	2.16	0.4276	0.2468	-
STD	5.82	6.97	5.74	6.18	0.6882	0.3973	186.11
ME 1	3.88	4.13	3.95	3.99	0.1290	0.0745	84.72
ME 2	3.01	3.79	4.07	3.62	0.5493	0.3171	67.59

Table 9.3: Statistical evaluation of the data obtained after 30 minutes

Code No.	t-Test value	Degree of freedom	P value	Level of significance
STD	8.5867	4	0.0010	Statistically extremely significant
ME 1	7.0847	4	0.0021	Statistically extremely significant
ME 2	3.6412	4	0.0219	Statistically significant

Table 9.4: Analysis of the data obtained after 60 minutes

Code No.	Immersion time count (seconds)			Average time of immersion	Standard deviation	Standard error	% Elongation
	M-1	M-2	M-3				
CTL	2.08	1.76	1.78	1.87	0.1793	0.1035	-
STD	9.64	8.96	8.96	9.19	0.3926	0.2267	391.44
ME 1	7.64	6.51	7.30	7.15	0.5797	0.3347	282.35
ME 2	6.7	8.15	7.31	7.39	0.7280	0.4203	295.19

Table 9.5: Statistical evaluation of the data obtained after 60 minutes

Code No.	t-Test value	Degree of freedom	P value	Level of significance
STD	29.3500	4	0.000008	Statistically extremely significant
ME 1	15.0612	4	0.00011	Statistically extremely significant
ME 2	12.7363	4	0.00022	Statistically extremely significant

Table 9.6: Analysis of the data obtained after 90 minutes

Code No.	Immersion time count (seconds)			Average time of immersion	Standard deviation	Standard error	% Elongation
	M-1	M-2	M-3				
CTL	2.59	2.14	1.97	2.23	0.3204	0.1850	-
STD	13.30	13.19	12.95	13.15	0.1790	0.1033	489.68
ME 1	7.37	7.42	10.04	8.28	1.5273	0.8818	271.30
ME 2	8.76	9.67	11.4	9.94	1.3411	0.7743	345.74

Table 9.7: Statistical Evaluation of the data obtained after 90 minutes

Code No.	t-Test value	Degree of freedom	P value	Level of significance
STD	51.5096	4	0.0000008	Statistically extremely significant
ME 1	6.7075	4	0.00257	Statistically extremely significant
ME 2	9.6854	4	0.00063	Statistically extremely significant

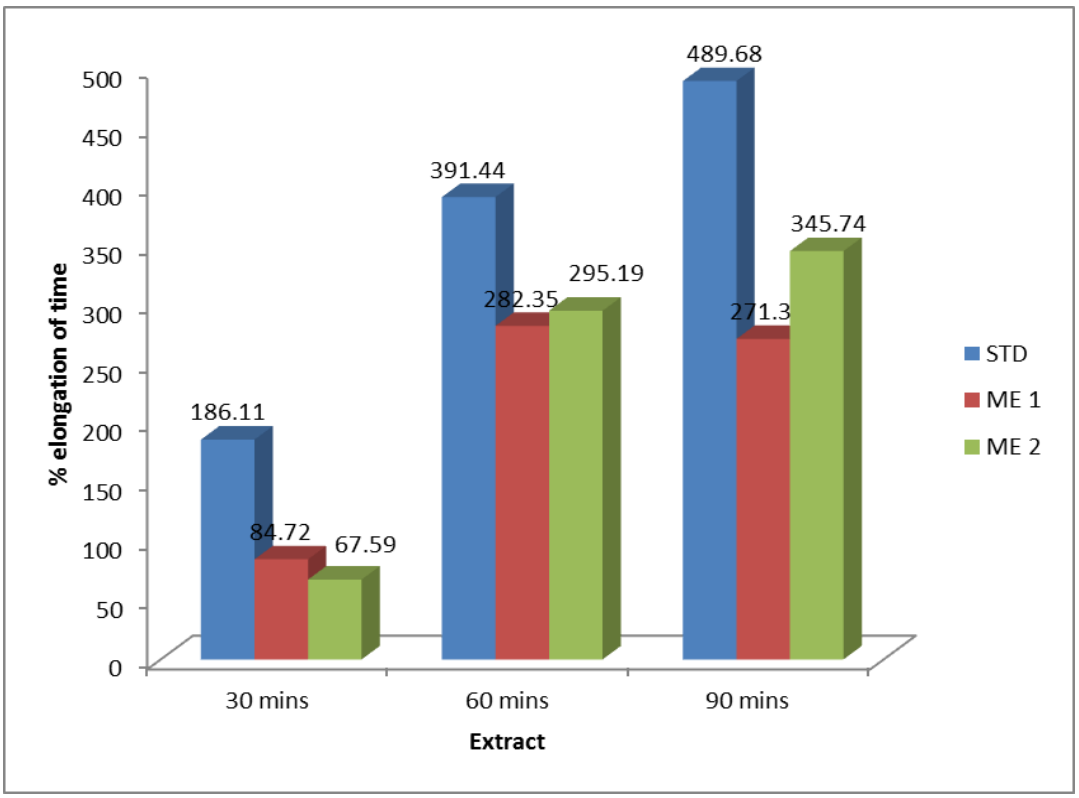

Figure 9.1: Comparison of percent time elongation of tail immersion of methanolic extract of *Bombax ceiba*

The analysis and statistical evaluation of the data leads to the following conclusion that the methanol extract of *Bombax ceiba* showed significant central analgesic activity at both doses of 200 mg/kg and 400 mg/kg body weight after 60 and 90 minutes of administration. So they can be further investigated for the development of central analgesic drug.

CONCLUSION

Piper chaba H. (Part-A) belongs to the genus piper and family piperaceae and is locally known as Chui Jhal and *Bombax ceiba* belongs to the genus bombax and family Bombacaceae locally known as shemul. Successive separation and purification of the column chromatographic fractions of ethyl acetate extract of the roots of *Piper chaba* afforded five compounds as stigmasterol, Piperine, 5, 6-dihydroxy- 7, 8-dimethyl isoflavan-4-one, two new compound [1-(4''-chlorophenyl)-3-(4'-methoxyphenyl) prop-2-en-1-one] and 1-(4''-chlorophenyl)-3-(p-tolyl)prop-2-en-1-one and the ethyl acetate extract of the roots of *Bombax ceiba* afforded seven compounds as Isohemigossypol-1-methyl ether, Isohemigossylic acid lactone-2-methyl ether, 2, 7-dihydroxy-5-isopropyl-3-methyl-2H-naptho[1,8-bc]furan-2-one (new compound), protocatechuic acid, Scoplotein, Vasicine and Lupeol.

The test materials of *Piper chaba* were subjected to several biological screenings such as antimicrobial and antioxidant. The portioned n-Hexane, dichloromethane, ethyl acetate and methanol extract were found to have high anti-oxidant property. In addition, Two compound Piperine, stigmasterol and two new compounds; 1-(4''-chlorophenyl)-3-(4'-methoxyphenyl)prop-2-en-1-one, and 1-(4''-chlorophenyl)-3-(p-tolyl)prop-2-en-1-one and four extracts, n-hexane, ethyl acetate, methanol and ethanol has also anti-microbial activity.

The test materials of *Bombax ceiba* L. (Part-B) were subjected to several biological screenings such as antioxidant, brine shrimp lethality, antimicrobial, anti-diarrheal, peripheral and central analgesic activities. Biological activities showed by different partitionates were statistically evaluated. Here the antioxidant, anti-diarrheal, brine shrimp lethality and central analgesic activities of the plant extractives showed promising results.

Therefore, considering the potential bioactivity, the plants materials can be further studied extensively to find out their unexplored efficacy, to rationalize their uses as traditional medicines and hopefully helping in the discovery of a new lead compound. So it may be concluded that this both plants might play a vital role as a medicinal plant.

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