Studies of Secondary Metabolites from endophytic fungi of *Terminalia chebula* Retz and *Aquilaria malaccensis* Lamk.

M. Phil. Thesis

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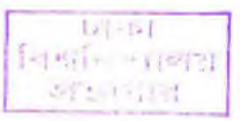
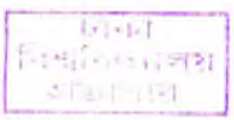
DEDICATED TO MY PARENTS



Abstract

Natural products derived from plants, endophytic fungi and marine fungi have been used for the treatment of various diseases for many years. An endophyte is a bacterial or fungal microorganism, which spends the whole or part of its life cycle colonizing inter and/or intra-cellularly inside the healthy tissues of the host plants. The word endophyte means "in the plant" and refers to all organisms that grow within plants. Fungal endophytes are fungi that live within their host plants without causing any noticeable symptoms of disease to the plant. Different types of secondary metabolites have been isolated from different endophytic fungi. Two medicinal plants, *Terminalia chebula* Retz and *Aquilaria malaccensis* Lamk were selected to separate endophytic fungi from them, and to isolate secondary metabolites from selective fungal strains. Each plant parts were separated and cut into small pieces followed by surface sterilized with 70% ethanol, 3% sodium hypochloride and sterile water. The surface sterilized plant materials were inoculated on autoclaved potato-carrot agar media on a sterilized petri dish. After 21 days of inoculation, fungi gave full growth. The strains IR-4, IR-6 and IR-7 from *T. chebula* and the fungal strain AL-2 from *A. malaccensis* were sub-cultured on semisolid potato-carrot agar media in a large scale for chemical and biological studies. Each fungal strain was extracted with ethyl acetate and fractionated by chromatographic techniques and the structure of isolated compounds were elucidated by spectroscopic techniques (UV, IR, 1D and 2D NMR). From the ethyl acetate extract of the strain IR-6 derived from *T. chebula* five compounds, pentadec-11-enoic acid methyl ester, 4-hydroxy hexadec-6-enoic acid methyl ester, octadec-6-enoic acid ethyl ester, octadec-6-enoic acid and a new compound terminotol were isolated. The fungal strain AL-2 from *A. malaccensis* yielded four compounds 1,7-dihydroxy-3-methoxyanthraquinone, nona-5-enoic acid, 4-methoxy propyl benzyl ether and a substituted scopoletin. The ethyl acetate extracts of the fungal strains IR-6 and AL-2 were assessed for antibacterial activities, and only AL-2 showed mild activities against *Bacillus cereus and Staphylococcus aureus.* These extracts showed low toxicity against brine shrimp lethality assay.



Certification

This is to certify that Shahanara Begum, M. Phil. Student of the Department of Chemistry, University of Dhaka, has been jointly supervised by us and now she is submitting her thesis entitled "Studies of Secondary Metabolites from endophytic fungi of *Terminalia chebula* Retz and *Aquilaria malaccensis* Lamk" with our consent.

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Contents

1. Introduction

2. Experimental

3. Results and Discussion

Schemes

List of Tables

List of Figures

(Figures 6-27 are in Appendix)

CHAPTER- ONE

INTRODUCTION

1. Introduction

1.1 General

Natural products derived from plants, endophytic fungi and marine fungi have been used for the treatment of various diseases for many years. Plants, on this earth have been utilized as medicines in Egypt, China, India and Greece from ancient time and an impressive number of modern drugs have been developed from them. The first records were written on the medicinal uses of plants appeared in about 2600 BC from the Sumerians and Akkaidians (Samuelsson, 1999). The best-known Egyptian pharmaceutical record, is the Ebers papyrus, which documented over 700 drugs, represents the history of Egyptian medicine dated from 1500 BC. The Chinese *Materia Medica*, has been well documented with the first record dating from about 1100 BC (Cragg et al, 1997). Documentation of the Ayurvedic system recorded in Susruta and Charaka dates from about 1000 BC (Kappor, 1990). In the last two centuries therapeutic agents have been extracted from plant sources and many drugs have found there way into the doctor's prescription all over the world. In the recent past bacteria, fungi and marine organisms have also been genuinely recognized as source of bioactive compounds. In fact with relatively less success with numerous plant sources, scientists are turning more and more towards alternative sources for biologically active chemical agents. Considering that 6 out of 20 of the most commonly prescribed medications are of fungal origin (Gloer, 1997) and only ~5% of the fungi have been described (Hawksworth 1991, 2001), fungi offer an enormous potential for new products.

Despite four decades of intense research, marine pharmacognosy is still considered a relatively young field compared to terrestrial pharmacognosy. Originally, pharmacognosy dealt exclusively with the study of drugs derived from terrestrial plants and animals. In the 1950s, marine organisms were identified as an excellent source of new biologically active compounds (Blunt et al. 2007). Marine-derived fungi have proven to be a rich source of structurally unique and biologically active secondary metabolites (Bugni and Ireland, 2004). Many significant new bioactive metabolites were isolated from marine-derived mangrove fungi from the South China Sea. (Yang et al., 2006; Chen et al., 2005, 2003; Lin et al., 2002, 2001, 2001a).

Natural products are naturally derived metabolites and/or byproducts from microorganisms, plants or animals (Baker et al, 2000). These products still play a major role in drug treatment either as the drug, or as a 'forebear' in the synthesis or design of the agent (Grifo et al, 1997). The world's best known and most universally used medicinal agent is aspirin, which is related to salicin, having its origins in the plant genera *Salix* sp. and *Populus* sp (Strobel et al, 2004). Over 50% of the new chemical products registered by the FDA as anticancer agents, antimigraine agents, and antihypertensive agents were natural products or derivatives in the time-frame of 1981-2002 (Newman et al, 2003). Between 1989 and 1995, 60% of approved drugs and pre-new drug application candidates were of natural origin (Grabely and Thiericke, 1999).

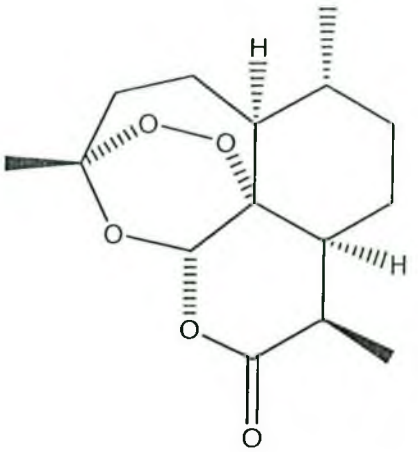
1.2 Plants: a source of biologically active secondary metabolites

A natural product is generally referred to as a secondary metabolite, in other words a metabolite that is not essential for the normal growth, development

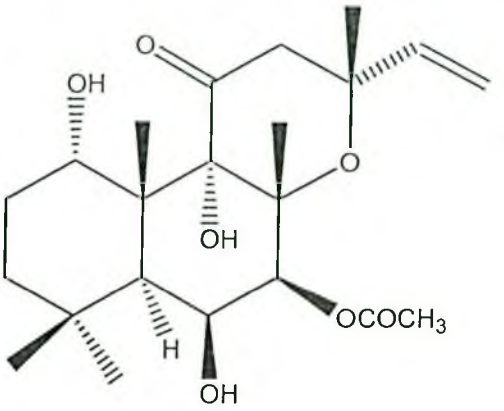
or reproducibility of an organism. Secondary metabolites usually have no effect on the producing organism, though in an indirect way a number of examples have poven to be essential for the survival of some organisms in that they have deterrent effects on predators, or competitors.

Plants have formed the basis for traditional medicine systems which have been used for thousands of years in countries such as China (Chang and But, 1986) and India (Dev, 1999; Kapoor, 1990). The use of plants in the traditional medicine of many other cultures has been extensively documented (Schultes and Raffauf, 1990). These plant-based systems continue to play an essential role in health care, and it has been estimated by the World Health Organization that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care Arvigo and Balick, 1993; Fransworth et al, 1985). Plant products also play an important role in the health care systems of the remaining 20% of the population, mainly residing in developed countries. In a study it has been shown at least 119 chemical substances, derived from 90 plant species, can be considered as important drugs that are in use in one or more countries (Arvigo and Balick, 1993). Of these 119 drugs, 74% were discovered as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine.

Artemisinine (**1**) is the antimalarial sesquiterpene from a Chinese medicinal herb *Artemisia annua* (Wormwood) used in herbal remedies since ancient times (Klaymann, 1985; Clark, 1996). Forskolin (**2**) is the antihypertensive agent from *Coleus forskohlii* Briq. (Labiatae), a plant whose use was described in ancient Hindu Ayurvedic texts (Bhat et al, 1977; Kinghorn, 1987).

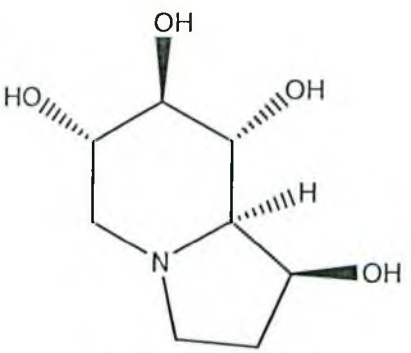


Artemisinine (**1**)



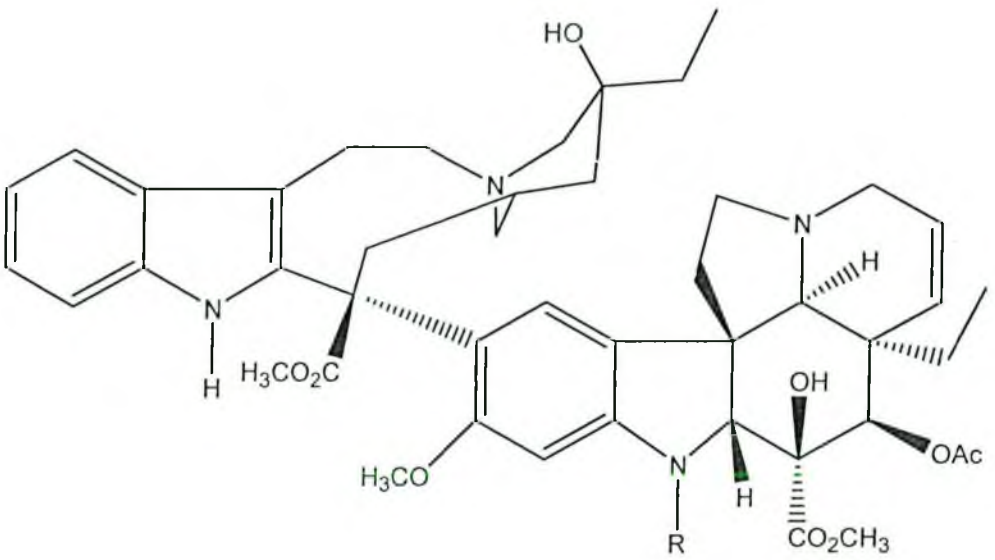
Forskolin (**2**)

The ginkgo tree, mentioned in Chinese medicinal books from 2800 B.C. and used in antiasthmatic and antitussive preparations, produces the ginkgolides, unusual diterpenes containing a tertiary butyl group. Their involvement in the clinical efficacy of ginkgo tree extracts was reported in 1985 (Hamburger et al, 1991). Castanospermine (**3**), a tetrahydroxyindolizidine alkaloid isolated from the Australian plant *Castanospermum austrate*, inhibits replication of the human immunodeficiency virus (HIV) (Fellows and Fleet, 1989).



Castanospermine (**3**)

Anticancer agents vincristine (**4**) and vinblastine (**5**) were isolated from *Catharanthus roseus*.

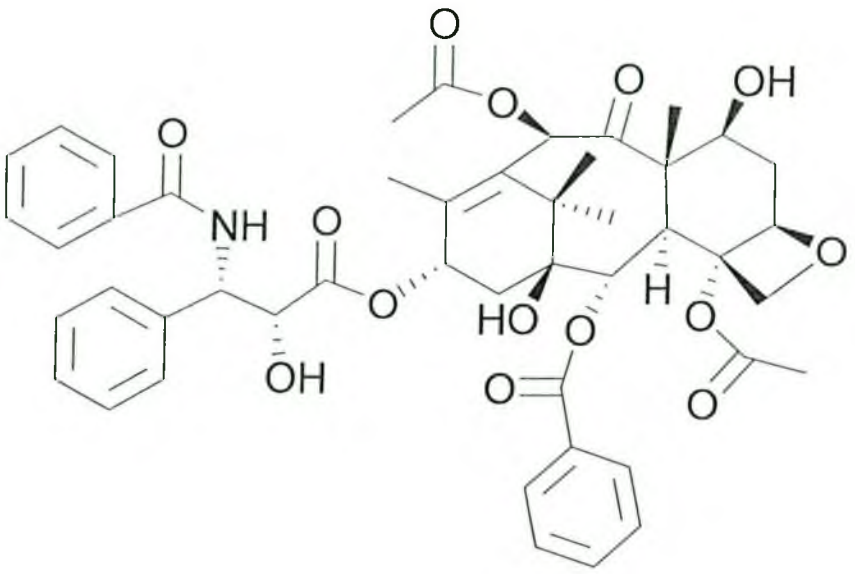


4 R=CHO

5 R=CH_3

Vincristine **(4)** and vinblastine **(5)**

Taxol (**6**), the world's first billion-dollar anticancer drug was originally isolated from *Taxus brevifolia* (Strobel et al 2004; Wani et al, 1971; Suffness, 1995). Taxol was being used for the treatment of ovarian and breast cancers, but now it is used to treat a number of other human tissue-proliferating diseases as well (Strobel et al 2004).



Paclitaxel (**6**)

1.3 General description of fungi

Fungi (singular**: Fungus**) is a term referring to biological organisms that are ranked in their own kingdom within the Domain *Eukaryota.* They have a diploid number of chromosomes and a nuclear membrane and have sterols in their plasma membrane. Genetic complexity allows morphologic complexity and thus these organisms have complex structural features that are used in speciation.

Fungi are plant-like organisms that lack chlorophyll. Fungi are one of the five kingdoms of life. Many fungi are good and useful (for example, edible mushrooms) while some cause problems. There are over 100,000 species of

fungi (Chang and Bennet, 1992). Medical mycologists study drugs to cure fungal infections, while agricultural and research mycologists study the industrial uses of fungi.

Fungi occur in all environments on the planet and include important decomposers and parasites. Parasitic fungi infect animals, humans, other mammals, birds and insects, with consequences varying from mild itching to death. Other parasitic fungi infect plants, causing diseases such as butt rot and making trees more vulnerable to toppling. The vast majority of vascular plants are associates with mutualistic fungi, called *mycorrhizae,* which assists their roots in absorption of nutrients and water.

Fungi are carbon heterotrophs, therefore they require preformed organic compounds as carbon sources. The great majority of fungi obtain their food from dead organic material and hence are known as saprophytes, a relatively small percentage derive their food directly from other living organisms and are known as parasites. There are two broad groups of fungi: yeasts and moulds. Since they don't use light to make food, fungi can live in damp and dark places.

Ainsworth (Ainsworth, 1973) has listed main characteristics of fungi. Some of them are given below (Table-1).

Table-1: Some common characteristics of fungi

Characteristic	Fungi
Cell type	Eucarytic
Optimum pH	3.8-5.8
Optimum temperature	22-30^{0}C
Oxygen requirement	Strictly aerobic(mold), Facultive(some yeast)
Light requirement	None
Sugar concentration in laboratory media	4-5%
Carbon requirement	Organic
Cell wall structural components	Chitin, cllulose,or hemi cellulose
Antibiotic susceptibility	Resistant to penicilins, tetracyclines, Chloramphenicol; sensitive to griseofulvin.
Sexuality	Asexual or sexual and homo- or heterothallic.
Habitat	Ubiquitous as saprobes, symbionts, parasites, or hyperparasites.
Distribution	Cosmopolitian

1.3.1 Medicinal value of fungi

Fungi have served as a source of important pharmaceuticals for more than fifty years. Today most major drug companies have research groups that focus on bioprospecting of fungi and other microorganisms for medically important compounds. Their use in medicine, however, goes back thousands of years in some cultures around the world. Many of us alive today have

antibiotics to thank for our existence. Some important examples of medical benefits from fungi are given below:

Penicillin: The first antibiotic discovered was **penicillin** from *Penicillium genera* and its discovery issued in a new age of medicine, providing the first effective treatment of bacterial infections.

Cephalosporins: Keflex was produced by a species of *Cephalosporium.*

Cyclosporin: Cyclosporin was produced by *Cylindrocarpon lucidum* and *Tolypocladium inflatum* fungi. Cyclosporin suppresses the activity of the immune system

Griseofulvin: Griseofulvin was produced by *P. griseofulvum.* It is an antifungal agent

Compactin and Lovastatin: Compactin is produced by fungi in the genera *Hypomyces, Paecilomyces* and *Trichoderma* and lovastatin is produced by *Aspergillus terreus*. Both drugs inhibit cholesterol synthesis and are major agents in the treatment of heart disease and atherosclerosis.

Antibiotic resistance in bacteria is a major problem in society today. Two major sources of origin of resistance are widespread use of antibiotics has selected for tolerant individuals in bacterial populations and people take antibiotics frequently fail to complete their treatment. So research on new drug is essential.

1.4 General description of endophytic fungi

1.4.1 The Endophytic Fungi

An endophyte is a bacterial or fungal microorganism, which spends the whole or part of its life cycle colonizing inter and/or intra-cellularly inside the healthy tissues of the host plants, typically causing no apparent symptoms of diseases. The word endophyte means "in the plant" and refers to all organisms that grow within plants. Fungal endophytes are fungi that live within their host plants without causing any noticeable symptoms of disease (Petrini, 1991; Carroll, 1988) to the plant. Endophytic fungi are found in all divisions of fungi so have presumably evolved the association independently on many occasions. Some species of endophytic fungi have been identified as sources of anticancer, antidiabetic, insecticidal and immunosuppressive compounds (Strobel and Daisy, 2003; Strobel et al 1999). Of the approximately 300,000 higher plant species that exist on earth, each individual plant is thought to play host to one or more endophytes then many interesting metabolites will be produced by endophytic fungi.

1.4.2 Endophytic microorganisms in the control of insects

In the early 80's the specialized literature published the first reports showing that endophytic microorganisms, in this case fungi, could play an important role inside plants. It was demonstrated that the presence of these microorganisms in their respective hosts could result in the reduction of insect attacks.

Webber (Webber, 1981) reported that the endophyte *Phomopsis oblonga* protected elm trees against the beetle *Physocnemum brevilineum.* It was suggested that the endophytic fungus *P. oblonga* was responsible for reducing the spread of the elm Dutch disease causal agent *Ceratocystis ulmi* by controlling its vector, the beetle *P. brevilineum.* The author associated the repellent effect observed towards the insect to toxic compounds produced by the fungi. This was confirmed four years later by Claydon et al. (1985), who showed that endophytic fungi belonging to the *Xylariacea* family synthesize secondary metabolites in hosts of the genus *Fagus* and that these substances affect the beetle larvae.

Recently, Miles et al. (1998) showed that endophytic isolates of *Neotyphodium* sp. produce N-formilonine and a paxiline analogous in the host *Echinopogum ovatus.* These compounds show insecticidal activity against *L. bonariensis* and other insects.

It is widely known that the existence of fungi and bacteria able to cause disease in insects. Fungal species like *Metarhizium anisopliae, Beauveria bassiana* and others are often used in the biological control of agriculture insects-pests. The potential of insect control by endophytic fungi, stressed that insect biocontrol may be improved by the development of artificial inoculation techniques, as those developed by Latch and Christensen (1988) and Leuchtman and Clay (1985).

As endophytic fungi, notably members of the genus *Acremonium*, are able to control some insects-pests, it is important to know if they can be inoculated in endophyte-free plants and, if they can trasmit to the host the capacity to resist certain pests. That was done with success by Koga et al. (1997), by infecting *F. arundinaceae* and *L. perenne* seeds with the endophytic fungus

Acremonium and, as a result, obtained plants resistant to the bluegrass webworm *P. teterrella.*

1.4.3 The plant/endophyte relationship

The endophyte/host relationship is believed to be complex and probably varies from host to host and microbe to microbe. An early, in-depth analysis of an endophyte fungus in the plant *Helianthemum chamaecistus* by boursnell showed that the relationship between the fungus and the plant is a hereditary one, and that the fungus passes from one generation to another through the seed (Boursnell, J.G., 1950). Some research workers have suggested that in certain instances, the host/guest relationship may be more akin to a balanced pathogen-host antagonism than a truly synergistic one (Strobel, 2002 and Schultz et al.1999). It has also been suggested that endophytic fungi might contribute to the process of nutrient recycling by initiating the biological degradation of the dying host plant (Strobel, 2002). There is some evidence that intercellular endophytes do elicit a chemical reaction from the host plant despite their largely benign relationship. For example, in Douglas fir seedlings, the plant produces the chemical resveratrol, which is a known anti-fungal agent, in response to endophyte infection (Sylvia and Sinclair, 1983).

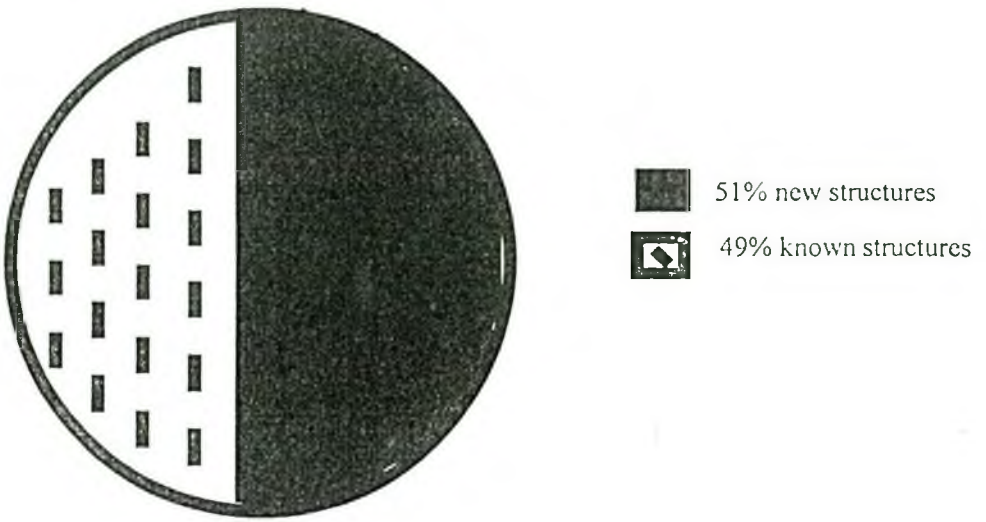
The host/endophyte relationship in abnormally developed plant tissues can be quite different from that of normal tissues. Galled plant sections and cysts may contain quite different fungal populations from the remainder of the plant (Stone et al, 2000). Endophyte-infected plants often grow faster than non-infected ones. This effect is at least in part due to the endophytes'

production of phytohormones such as indole-3-acetic acid (IAA), cytokines, and other plant growth-promoting substances, and/or partly owing to the fact that endophytes could have enhanced the hosts' uptake of nutritional elements such as nitrogen and phosphorus.

1.4.4 Secondary metabolites by an endophyte

Different types of secondary metabolites have been isolated from different endophytic fungi. Alkaloids, amines, amides, indole, pyrrolizidines, and steroids are quite common. Terpenoids, sesquiterpenes, diterpenes, isocoumarin derivatives, quinones, flavonoids, phenylpropanoids and lignans, peptides, phenols and phenolic acids, aliphatic compounds, chlorinated metabolites are also often been isolated from some endophytic cultures originiating from variety of host plants. A comparison of 135 isolated metabolites whose structures were determined shows that the proportion of novel structures produced by endophytes (51%) is considerably higher (Fig:1) than that produced by soil isolates (38%) (Schulz et al, 2002).

Endopytes



Soil isolates

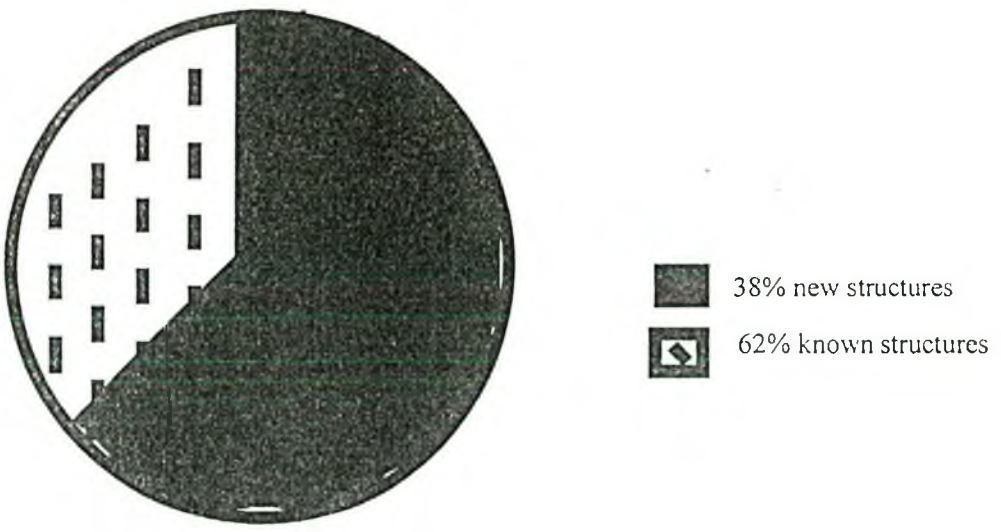
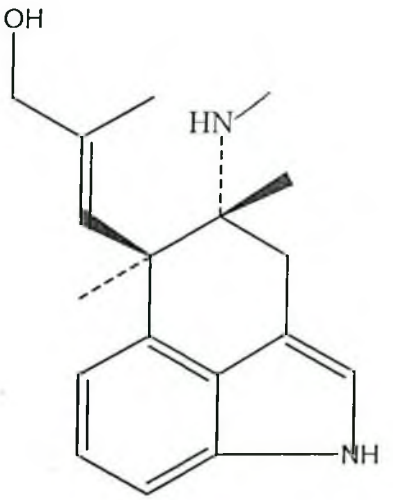


Figure- 1: Proportion of novel metabolite structures from soil isolates and endophytes

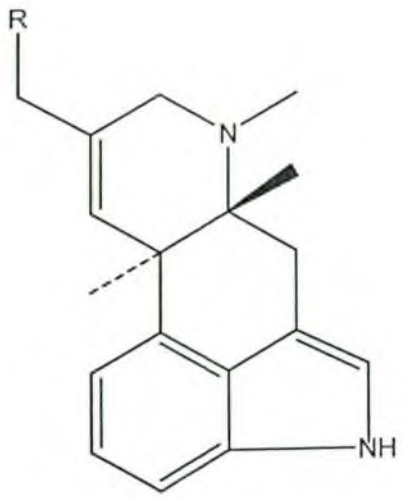
Structures of different compounds isolated from endophytic fungi are given below.

Indole derivatives were isolated from endophytic fungi. Indole alkaloids such as chanoclavine (**7**), agroclavine (**8**) and elymoclavine (**9**), previously characterized from ergot sclerotia, were reisolated from a culture of *Neotyphodium* endophytes (Powell and Petroski, 1992). They are toxic to some insects and mammals (Schardl and Phillips, 1997).

A indole derivative 6-isoprenylindole-3-carboxilic acid (**10**) was characterized from *A. annua* endophyte *Colletotrichum* sp. It shows moderate antibacterial activity against the Gram-positive bacteria *Bacillus subtilis, Staphylococcus aureus, Sarcina lutea* and the Gram-negative bacterium *Pseudomonas* sp. Furthermore, this product is also inhibitory to the growth of some crop phytopathogenic fungi *phytophthora capisici, Rhizoctonia cerealis* and *Gaeumannomyces graminis* var. *tritici* (Lu et al, 2000).

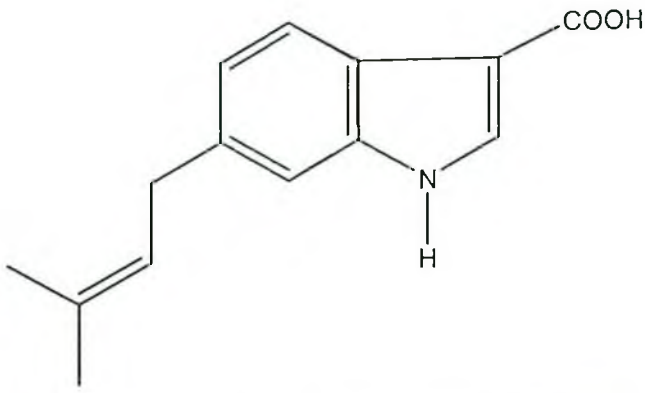


Chanoclavine (**7**)



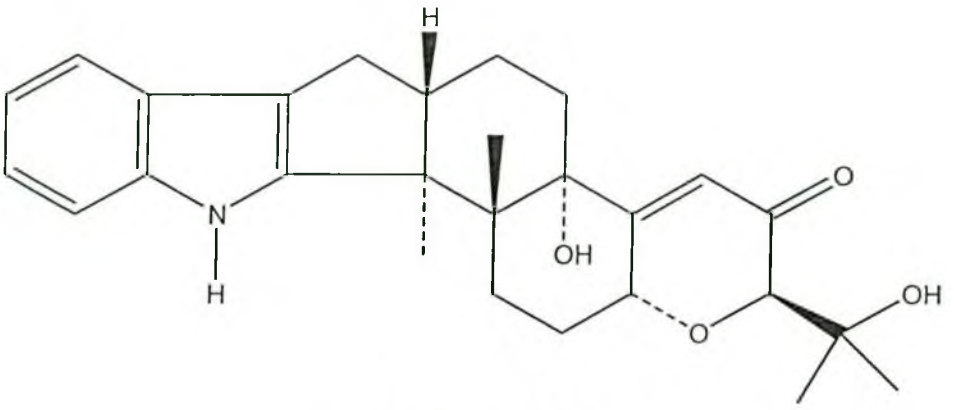
Agroclavine R=H (**8**)

Elymoclavine R=OH (**9**)



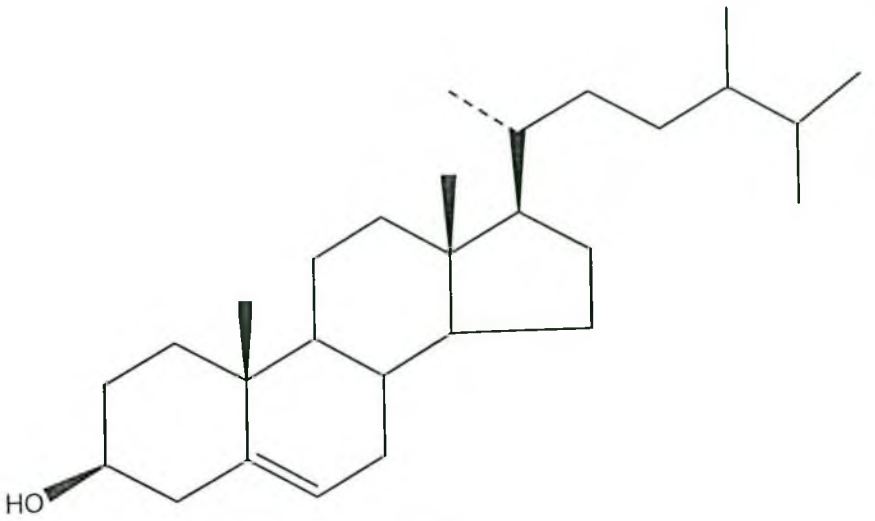
6-isoprenylindole-3-carboxilic acid (**10**)

A tremorgenic indolediterpenes paxilline **(11)** was detected in cultures of *N. lolli* and *E. festucae.*



Paxilline (**11**)

Steroids were isolated from endophytic fungi. Along with ergosterol, 3β,5α,6β-trihydroxyergosta-7,22-diene, 3β-hydroxyergosta-5-ene (**12**), 3-oxoergosta-4,6,8(14),22-tetraene (**13**), 3β-hydroxy-5α,8α-epidioxyergosta-6,22-diene, 3β-hydroxy-5α,8α-epidioxyergosta-6,9(11),22-triene and 3-oxoergosta-4-ene. Two steroids, 3β,5α-dihydroxy-6β-acetoxyergosta-7,22-diene (**14**) and 3β,5α-dihydroxy-6β-phenyl acetoxyergosta-7, 22-diene (**15**) were characterized from the liquid culture of an fungal endophyte *Colletotrichum* sp. of *A. annua.* Metabolites **12, 13, 14** and **15** were shown to be antifungal against some crop pathogens *Gaeumannomyces graminis* var. *tritici, Rhizoctonia cerealis, Helminthosporium sativum* and *Phytophthora capisici* (Lu et al,2000).



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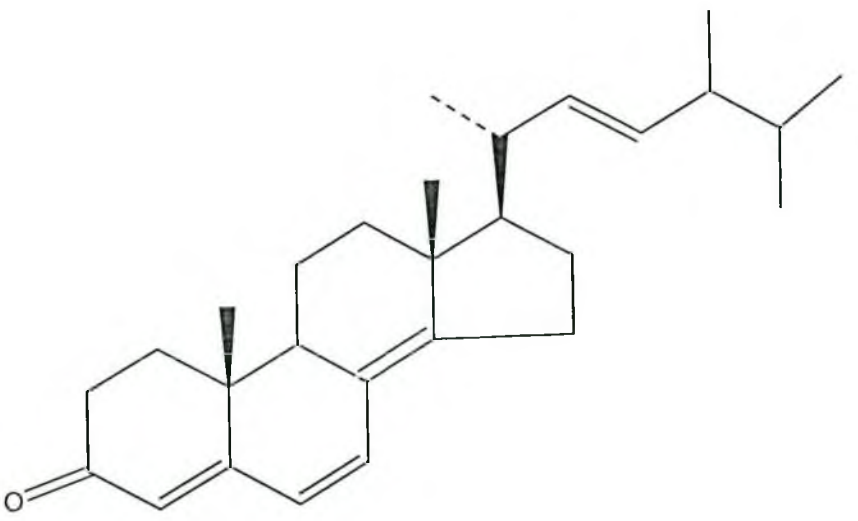

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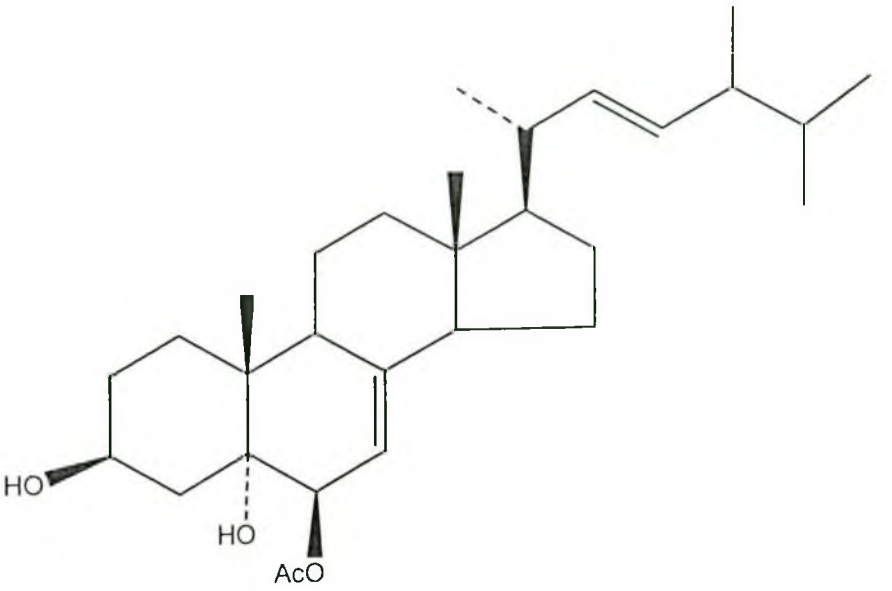

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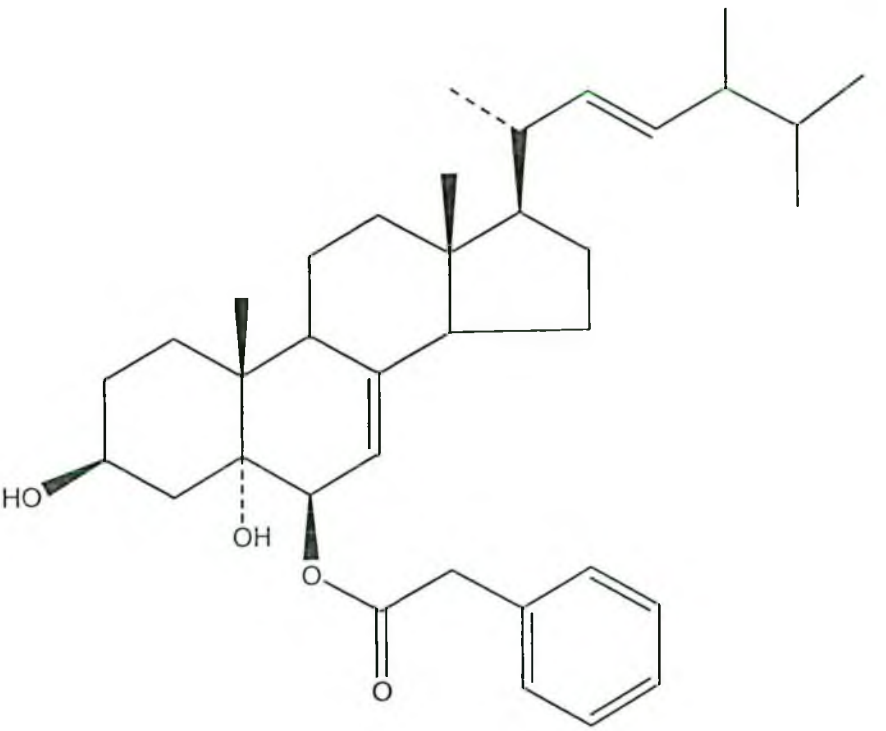
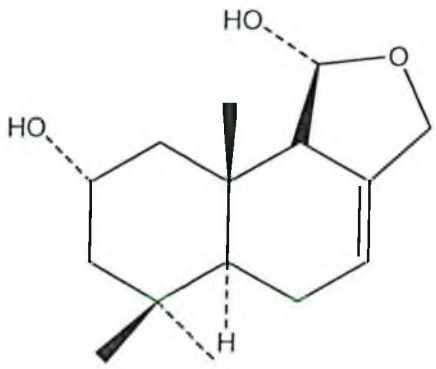
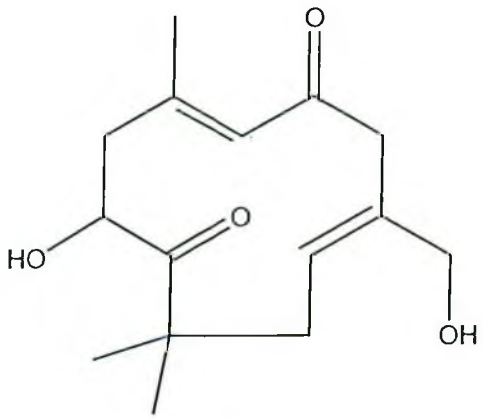


15

Sesquiterpenes were isolated from endophyte. 2α-Hydroxydimeninol (**16**), the compound (**17**) were isolated from endophytic *Pestalotiopsis* spp. The sesquiterpene (**17**) is a highly functionalized humulane derivative. Heptelidic acid (**18**) and hydroheptelidic acid (**19**) isolated from *Phyllosticta* sp., an endophytic fungus of *Abies balsamea,* have been shown to be toxic to spruce bud worm (*Choristoneura fumiferana)* larvae (Calhoun et al, 1992).



2α-hydroxydimeninol (**16**)



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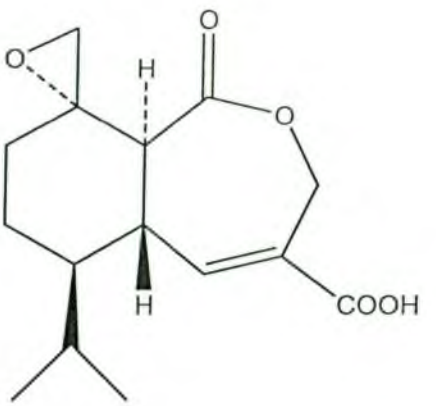
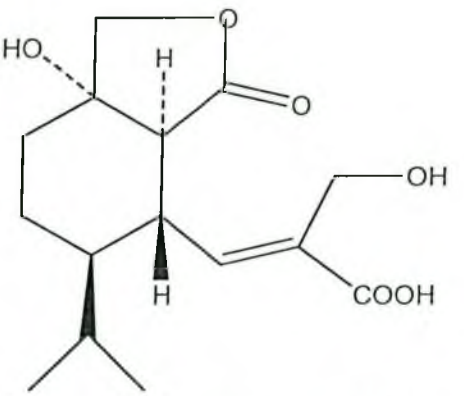


heptelidic acid (**18**)

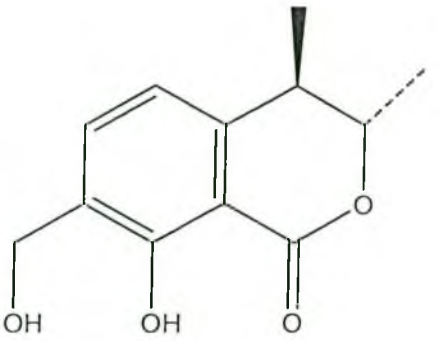


hydroheptelidic acid (**19**)

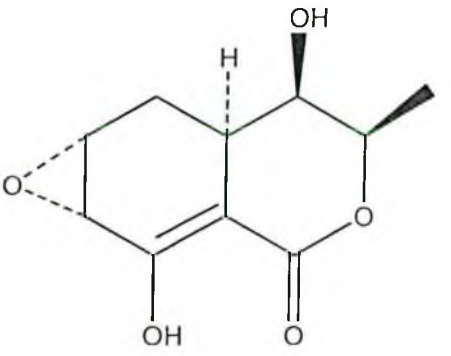
Diterpenes were also isolated from endophytic fungus. Subglutinol A and B, immunosuppressive but noncytotoxic, were produced by *Fusarium subglutinans,* an endophytic fungus from the perennial twining vine

Tripterygium wilfordii (Lee et al, 1995). Taxol (**6**) originally characterized from the inner bark of the Pacific yew, *Taxus brevifolia,* is an efficacious anticancer diterpene found in extremely small quantities in slowly growing *Taxus* species (Wani et al, 1971; Georg et al, 1994). In 1993, Taxol was reported to be produced in *in vitro* culture by a new endophytic fungus, *Taxomyces andreanae,* which was isolated from a Pacific yew *T. brevifolia* in Montana, USA (Stierle et al, 1993). Since then, a variety of endophytic fungi belonging to different categories isolated from *T. brevifolia* (Stierle et al, 1995), *T. wallachiana* (Strobel et al, 1996), *T. yunnanensis* (Qiu et al, 1994), *T. baccata* (Caruso et al, 2000), *T. mairei* (Wang et al, 2000), *Taxodium distichum* (Li et al, 1996), *Torreya grandifolia* (Li et al, 1998), *and Wallemia nobilis* (Strobel et al, 1997) have been reported to be capable of producing Taxol and/or taxane derivatives in some endophyte cultures.

Isocoumarin derivatives were isolated from endophytes. (*R*)-Mellein, an isocoumarin isolated from *Pezicula* spp. (Schulz et al, 1995), is strongly fungicidal, herbicidal and algicidal. Gamahorin (**20**) is a isocoumarin from stromata of *E. typhina* on *P. pratense* (Koshino et al, 1992). Isocoumarin-related metabolites, as for example (**21**) was isolated from the conifer endophyte cultures (Findlay et al, 1995).



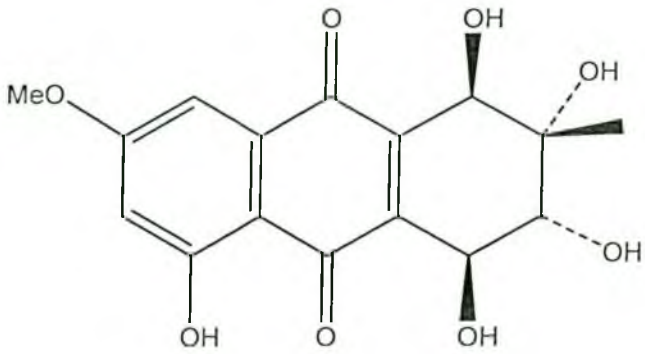
Gamahorin (**20**)



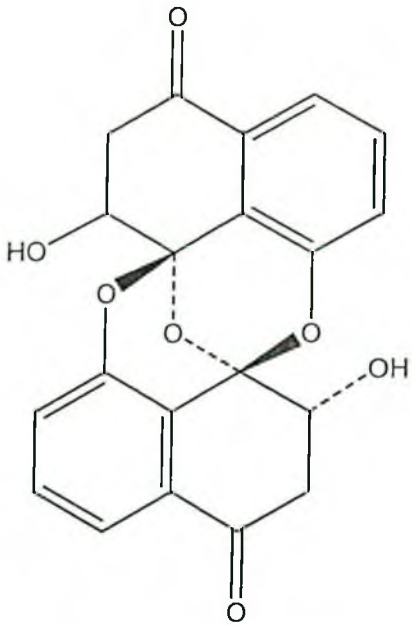
21

Quinones were isolated from endophytic fungus. Insecticidal rugulosin was characterized from *Hormonema dematioides,* an endophytic fungus of balsam fir (Calhoun et al, 1992). A highly hydroxylated quinone altersolanol A **(22),** characterized from phytopathogenic *Alternaria* spp., was isolated from an endophytic *Phoma* sp. with its antibacterial activity (Yang et al,

1994). Preussomerin N_1 **(23)** was ras farnesyl-protein transferase inhibitors produced by an endophytic *Conithyrium* sp (Krohn et al, 2000).

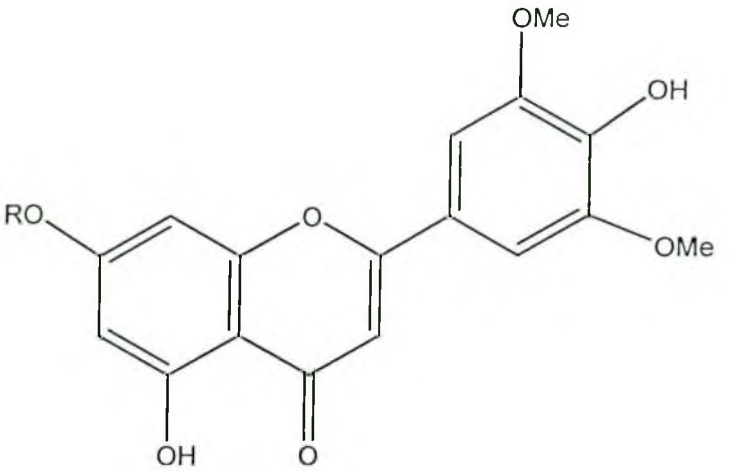


Altersolanol A (**22**)



Preussomerin N_1 (**23**)

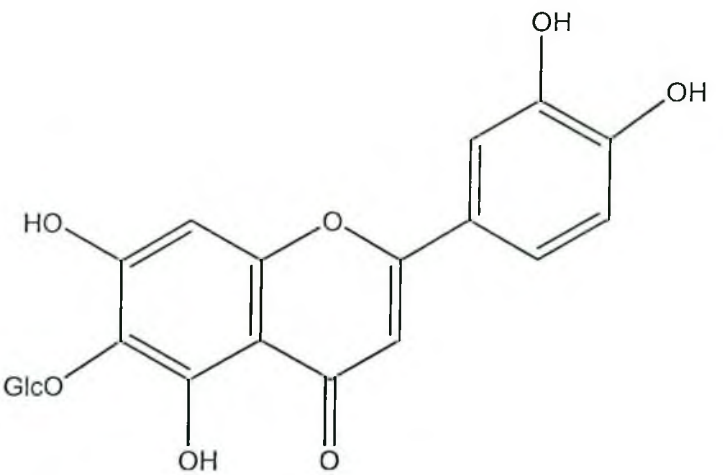
Flavonoids were isolated from endophytes. Tricin **(24)** and related flavone glycosides **(25-27),** toxic to mosquito larvae, have been isolated from endophyte-infected blue grass (*Poa ampla*) (Ju et al, 1998).



Tricin R=H **(24)**

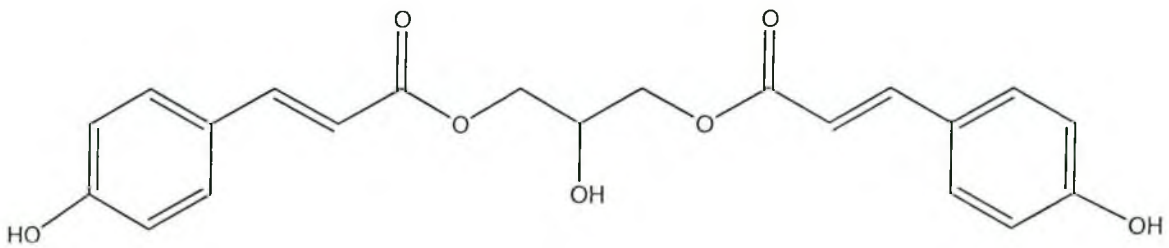
R= Glc **(25)**

R= Rha-Glc **(26)**



27

Phenylpropanoids and lignans were isolated from endophytes. The compounds **(28-29)** were characterized from stromata of *Epichloe typhina* on *Phleum pratense* (Koshino et al, 1988). Interestingly, coniferin **(30)** and syringin **(31),** two monolignol glucosides produced by the host plant, were ascertained to be specifically recognized by the endophytic *Xylariaceae* species as chemical signals during the establishment of fungus-plant interactions (Chapela et al, 1991).



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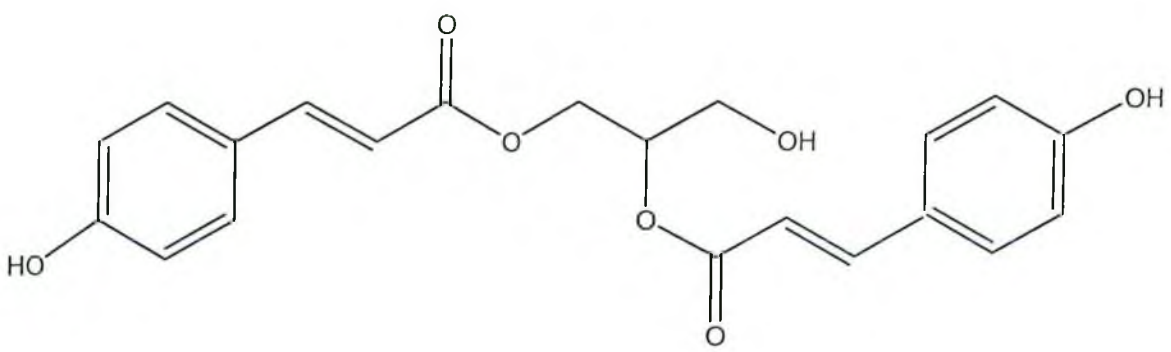

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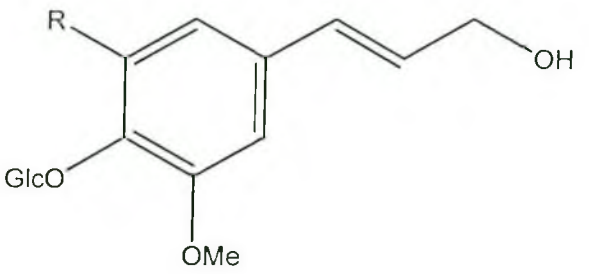
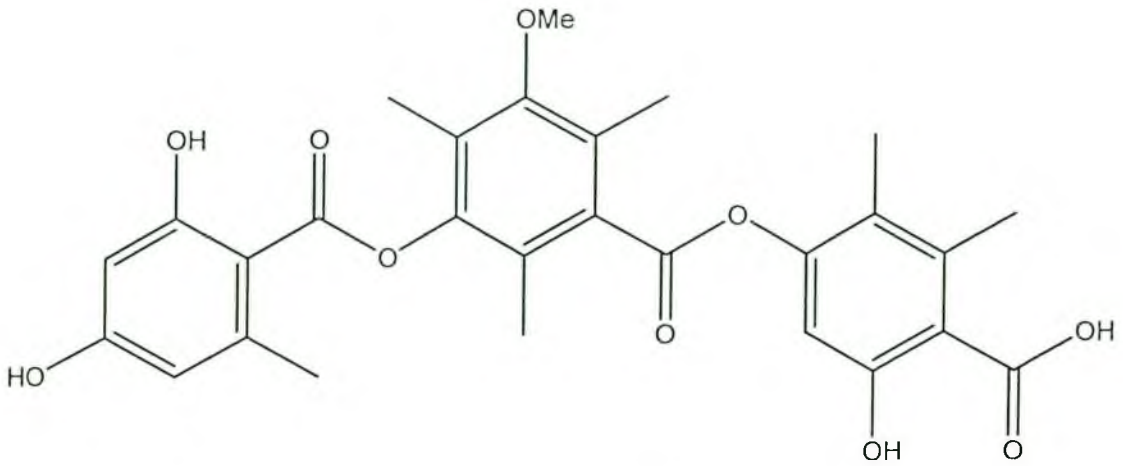


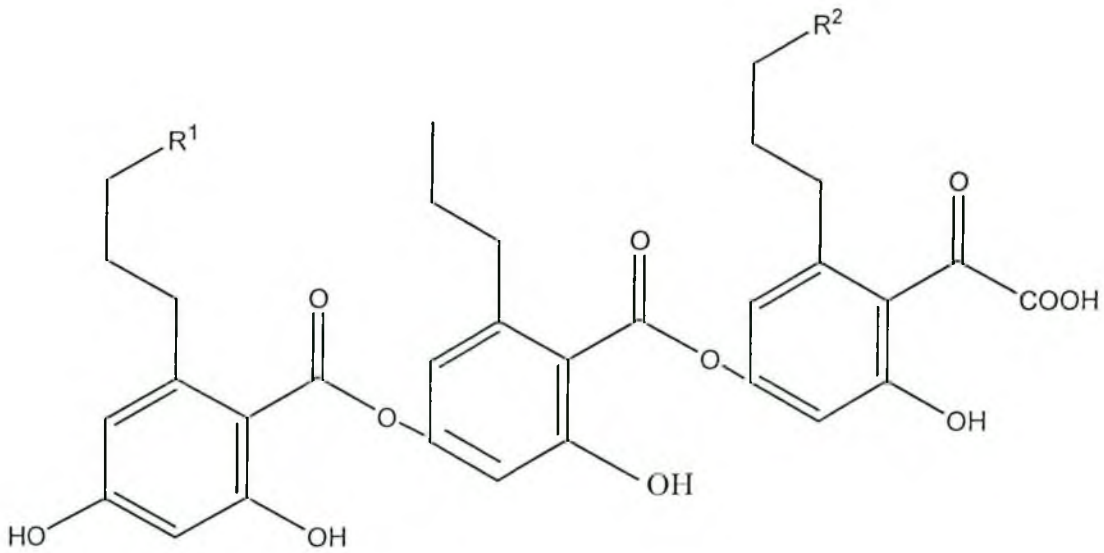
Coniferin R= H (**30**)

Syringin R= OMe (**31**)

Phenol and phenolic acids, frequently detected in cultures of endophytes, often have pronounced biological activities. 2-Hydroxy-6-methylbenzoic acid was isolated from endophytic *Phoma* sp. and shown to be antibacterial (Yang et al, 1994). From *Colletotrichum gloeosporioides,* an endophytic fungus of *Artemisia mongolica*, an antimicrobial tridepside colletotric acid (**32**) was characterized (Zou et al, 2000). Furthermore, two isomeric novel tridepsides cytonic acids A **(33)** and B **(34)** were reported as human cytomegalovirus (an ubiquitous opportunistic pathogen) protease inhibitors from the culture of the endophytic fungus *Cytonaema* sp. isolated from *Quercus* sp (Guo et al, 2000).



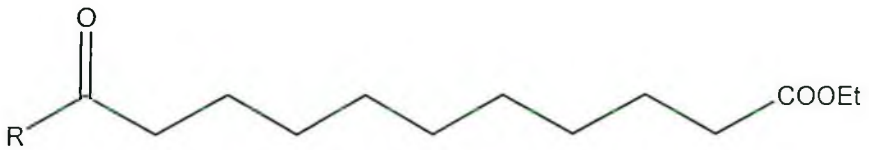
Colletotric acid (**32**)



Cytonic acid A R^1= Et, R^2 = H (**33**)

Cytonic acid B R^1= H, R^2 = Et (**34**)

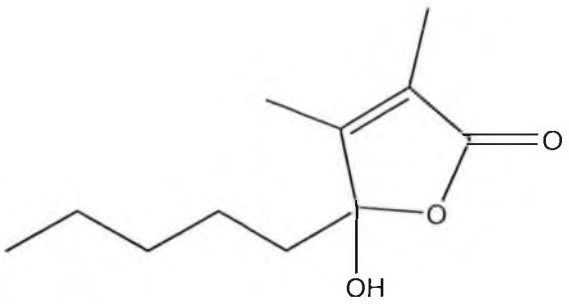
Aliphatic compounds were also isolated from endophytic fungus. Four antifungal aliphatic compounds (**35-38**) were characterized from stromata of *E. typhina* on *P. pretense* (Koshino et al, 1989). From an endophyte of the eastern larch, a novel ester metabolite was isolated as antibacterial agent against *Vibrio salmonicida, Pseudomonas aeruginosa* and *Staphylococcus aureus* (Findlay et al, 1997). Phomodiol (**39**) and phomopsolide B, metabolites of endophytic *Phomopsis* spp. present in the genus *Salix* and non-willow plants (Horn et al, 1996).



35 R= H

36 R= H, 2, 3-epoxide

37 R= O



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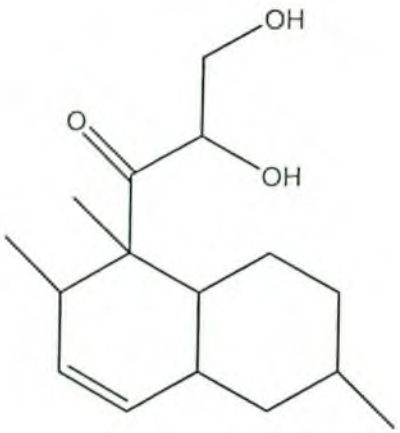
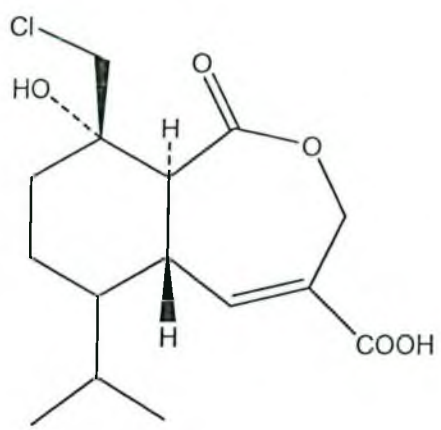


Phomodiol (**39**)

Chlorinated metabolites were also isolated from endophytic fungi. Three chlorinated metabolites from endophytic fungi were reported including an insect-toxic heptelidic acid chlorohydrin **(40),** and the two antimicrobial and algicidal compounds (-)-mycorrhizin A (**41**) and (+)-cryptosporiopsin (**42**). They were also isolated from cultures of balsam fir needle endophyte *Phyllosticta* sp. strain 76 (Calhoun et al, 1992) tree endophytes *Pezicula* sp. and *P. livida* strain 1156 (Schulz et al, 1995), respectively.



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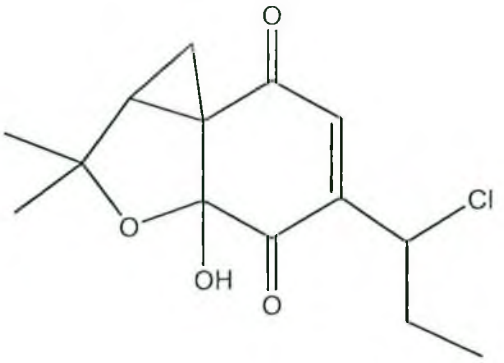

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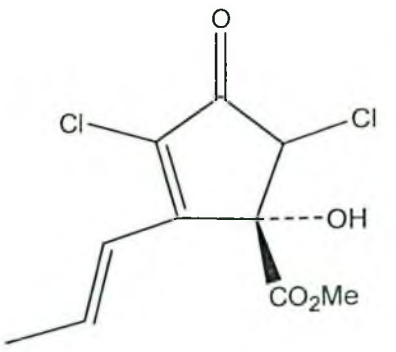
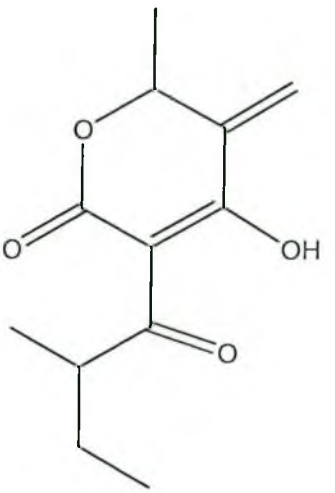


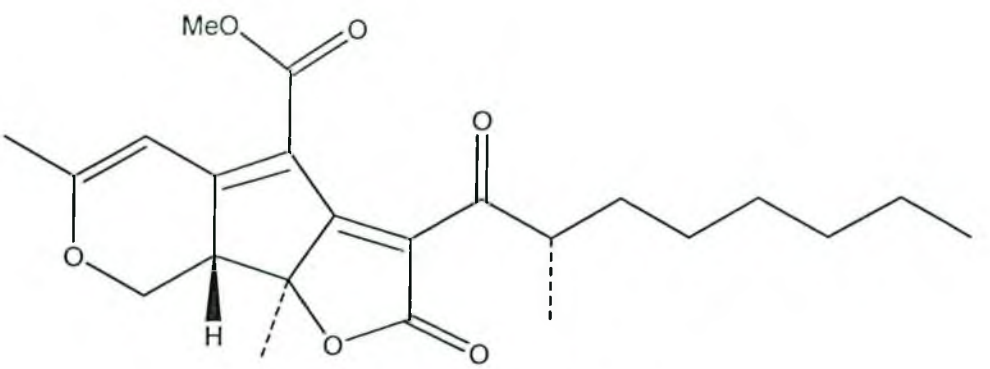
42

An antifungal pentaketide **(43)** was characterized from a *Fusarium* sp., an endophytic fungus living in the interior part of *Selaginella pallescens* stem (Brady and J. Clardy, 2000).

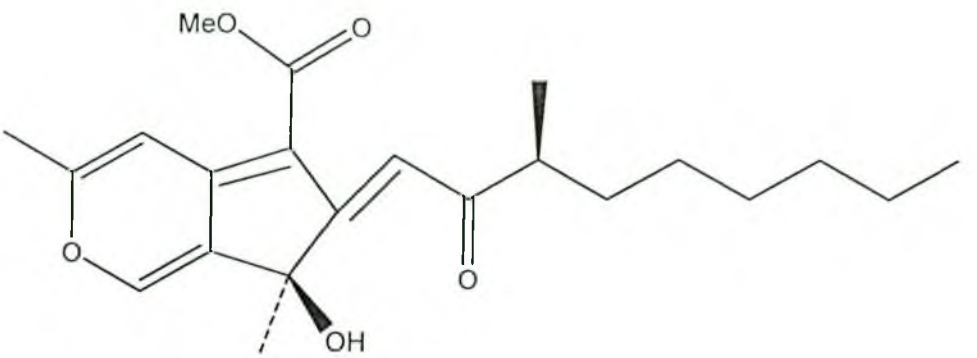


43

Two novel antitumor metabolites sequoiatones A **(44)** and B **(45)** were isolated from an endophytic fungus *Aspergillus parasiticus* of redwood (Stierle et al 1999).

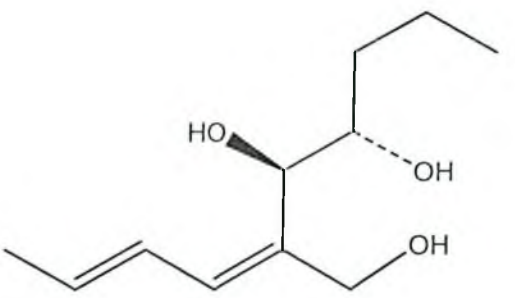


sequoiatone A **(44)**



sequoiatone B (**45**)

Three monoterpenes, C-methylated acetogenins (**46-48),** were produced by *Pestalotiopsis* spp., endophytic fungi of *Taxus brevfoli* (Pulici et al, 1997).



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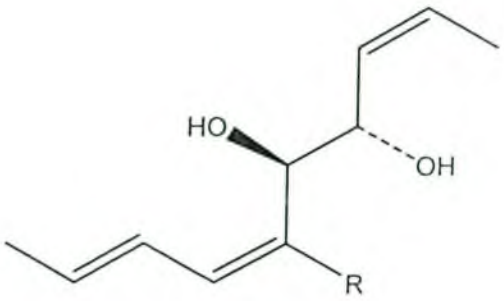
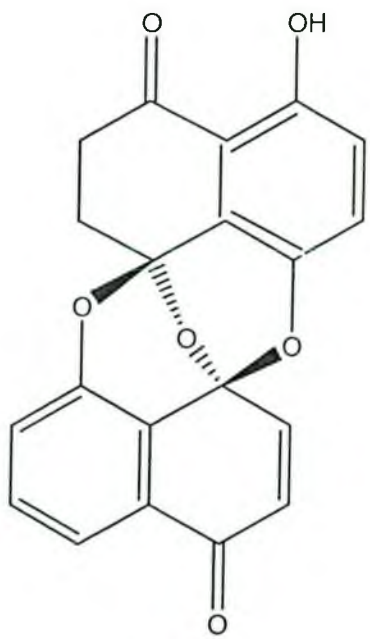


47 R=CH_2OH

48 R=CHO

Naphthoquinone spiroketals: preussomerin EG_1 (**49**), preussomerin EG_2 (**50**), preussomerin EG_3 (**51**) and deoxypreussomerin B (**52**) were isolated from endophytic fungi *Edenia gomezpompae*, derived from the leaves of *Callicarpa acuminate* (Macías-Rubalcava et al, 2008).



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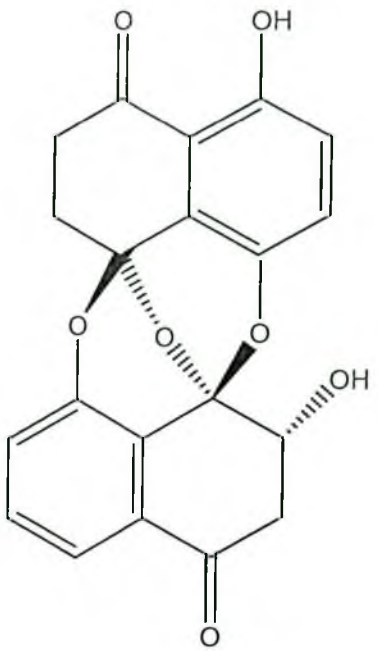

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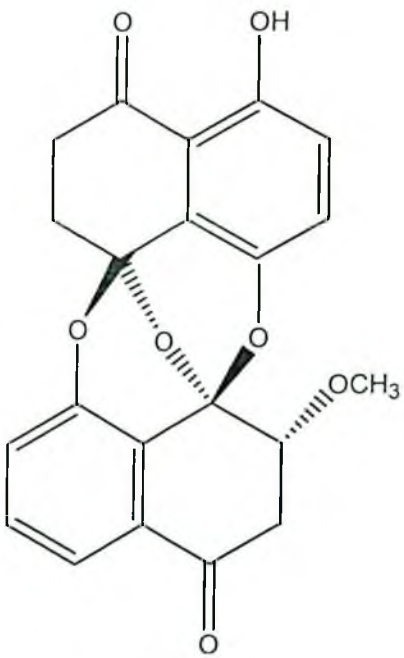

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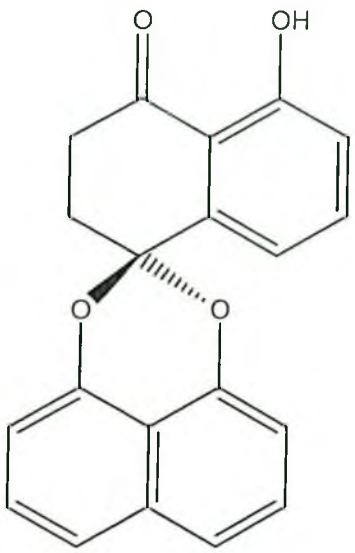
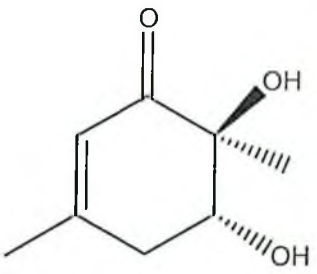


52

Four polyketides, leptosphaerone C (**53**), penicillenone (**54**), arugosin I (**55**) and 9-demethyl FR-901235 (**56**), as well as five known compounds, bacillosporin A, bacillosporin C, sequoiamonascin D, sequoiatone A, and sequoiatone B were isolated from the *Penicillium* sp. JP-1, an endophytic fungus isolated from *Aegiceras corniculatum* (Lin et al, 2008).



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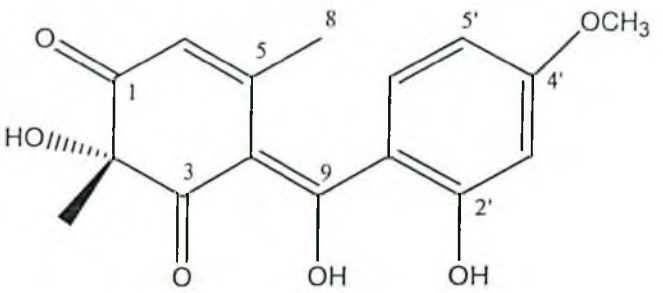

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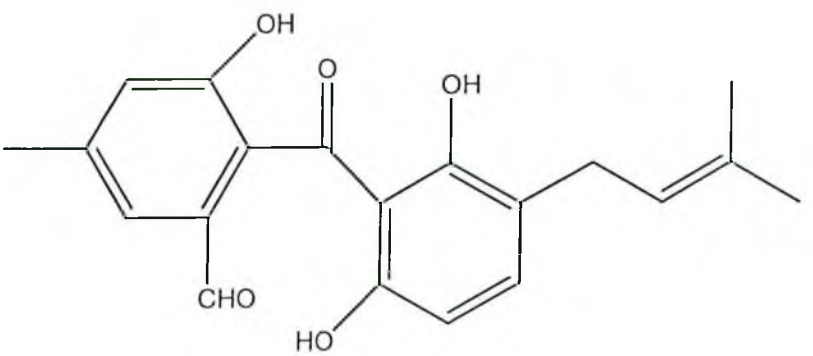

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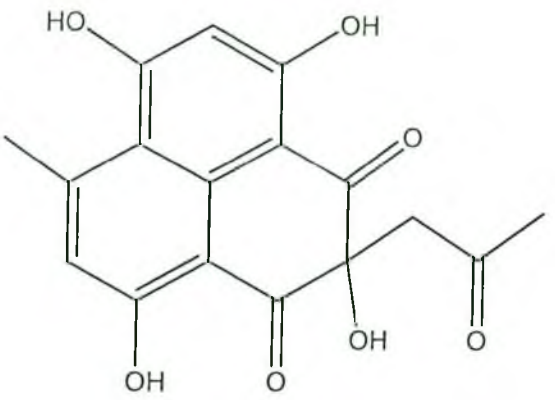
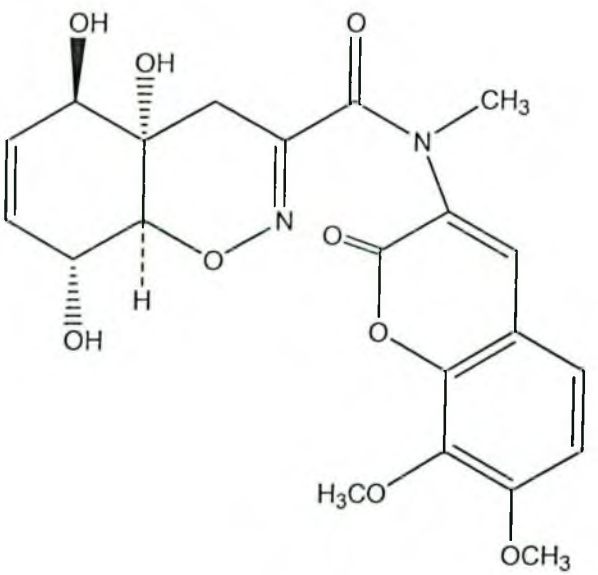


56

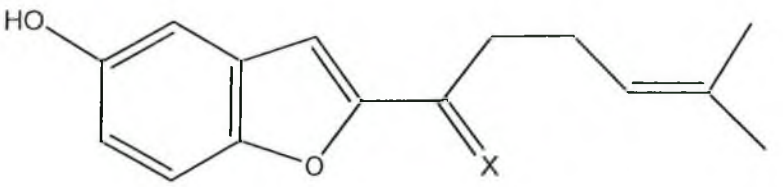
A modified dipeptide trichodermamide C **(57)** was isolated from the endophytic fungus Eupenicillium sp. (Rohan et al, 2008).



57

From the endophytic fungus *Phomopsis* sp. PSU-D15, three metabolites named as phomoenamide, phomonitroester and deacetylphomoxanthone B, were isolated together with three known compounds, dicerandrol A, (1*S*,2*S*,4*S*)-*p*-menthane-1,2,4-triol and uridine (Rukachaisirikul et al, 2008).

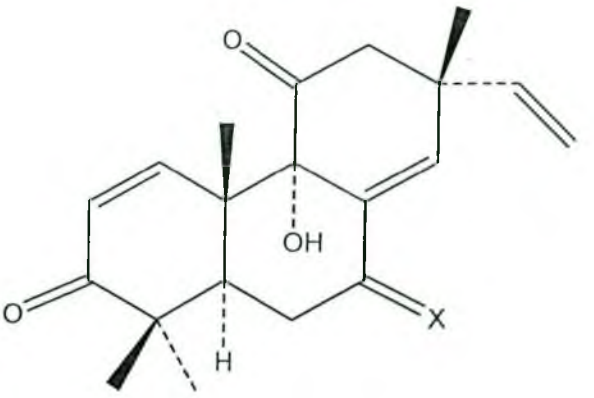
Two benzofuran-carrying normonoterpene derivatives **58** and **59**, toxic to spruce bud worm larvae and/or cells, have been characterized from a culture of an unidentified endophytic fungus obtained from wintergreen *Gaultheria procumbens* (Findlay et al, 1997).



58 X=O

59 X=OH, H

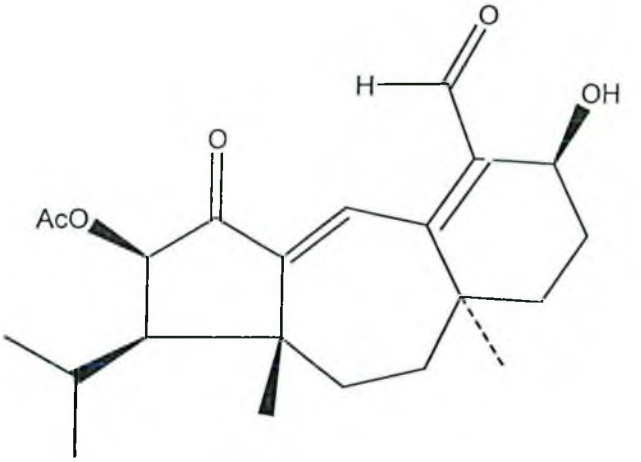
Two insect toxins **(60)** and **(61)** of a pimarane diterpene framework were isolated from the broth of an unidentified endophyte from a needle of the balsam fir *Abies balsamea* (Findlay et al, 1995).



60 X=O

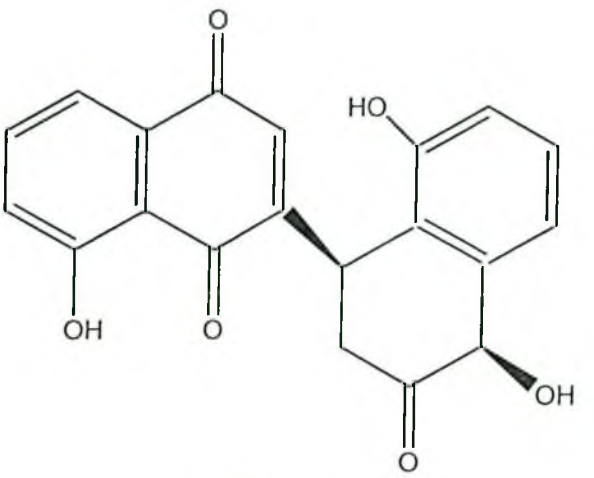
61 X=H

Guanacastepene **(62),** a novel ditrerpenoid produced by an unidentified fungus from the branch of *Daphnopsis Americana* growing in Guanacaste, Costa Rica, was shown to be antibacterial against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* (Singh et al, 2000).



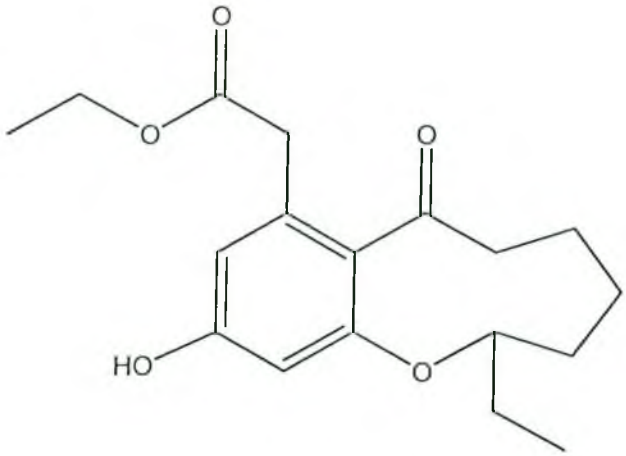
Guanacastepene (**62**)

From cultures of an unidentified endophyte obtained from an eastern larch (*Larix laricina*) needle, 8,1',5'-trihydroxy-3',4'-dihydro-1'*H*-[2,4'] binaphthalenyl-1,4,2'-trione **(63)** was characterized as a toxin to spruce budworm larvae (Findlay et al, 1997).



63

Three metabolites named phomopsin A (**64**), B (**65**) and C (**66**), together with cytosporone B (**67**) were isolated from the mangrove endophytic fungus, *Phomopsis* sp. ZSU-H76 obtained from the South China Sea (Huang, et al, 2008).



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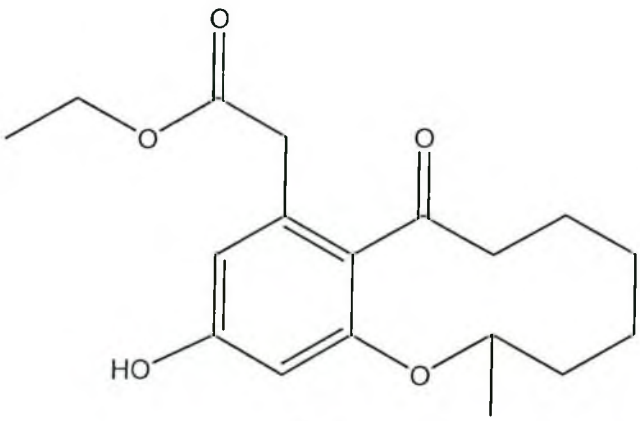

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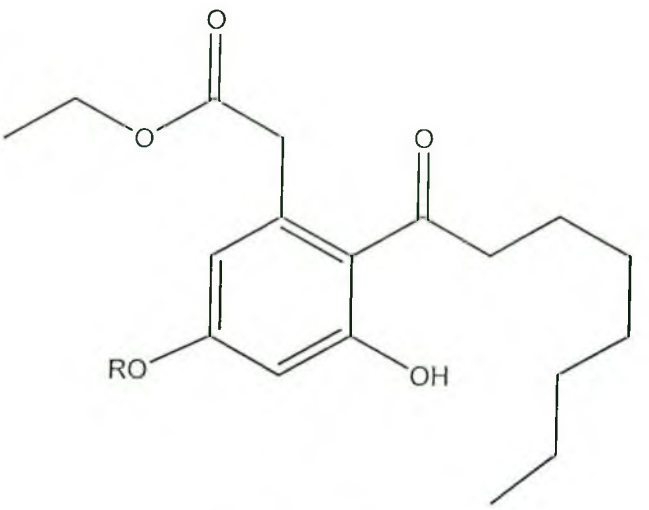


66 R= CH3

67 R=H

1.5 Selected medicinal plants: *Terminalia chebula* Retz and *Aquilaria malaccensis* Lamk

1.5.1 *Terminalia chebula* Retz

Terminalia chebula Retz is one of the important medicinal plants of Bangladesh. It is a tree belonging to the family Combretaceae. The plant naturally grows in Dhaka, Mymensingh, Tangail and Chittagong districts of Bangladesh and locally known as 'Haritaki'. It also grows in India, Nepal, Sri Lanka and Myanmar. It is generally used in triphala, a traditional Ayurvedic herbal formulation consisting of the dried fruits of three medicinal plants. This fruit is used to treat fever, caugh, asthma, jaundice, colic, hiccup, enlarged spleen and liver disorders. Externally they are used to treat eye discharges and as a local application to chronic ulcers and wounds

as a gargle in stomatitis (Ghani, 2003). *T. chebula* was selected for isolation of endophytic fungi.

1.5.2 *Aquilaria malaccensis* Lamk

The *Aquilaria malaccensis* Lamk is one of the medicinal plants of Bangladesh. The plant naturally grows in natural forest of Sylhet district. When the plant is attacked by exocyctic fungus the changes occurs in the middle lamella of timber. The exocyctic fungus secrets aromatic compounds which are used as agor oil. The oil has medicinal value as well. The wood after steam distillation is used in agor candle. Powdered wood is used as a perfume. Decoction of wood is used as a febrifuge, alterative, tonic, carminative, stomachic, laxative, diuretic and aphrodisiac. It is also useful in leucoderma and other skin diseases, hiccup, bronchitis, asthma, chronic diarrhea and diseases of the ear. It is specifically used as an aromatic, stimulant and nerve tonic (Ghani, 2003). This plant was also selected for isolation of endophytic fungi.

1.6 Objective of the work

There are more than 300,000 species of higher plants and 1 million species of endophytic fungi existing on this planet, and only a small portion has been explored phytochemically. It is believed that endophytic fungi/plants can provide potential bioactive compounds for the development of new 'leads'. Literature review showed that endophytic fungi are a poorly investigated group of microorganisms that represent an abundant and reliable source of bioactive compounds. Therefore, the objectives of the present work are to

i) isolate and culture of endophytic fungi from *Terminalia chebula* Retz and *Aquilaria malaccensis* Lamk and extract them;

ii) separate, purify and isolation of active compound (s) from extracts;

iii) identify and elucidate structures of isolated compounds by different spectroscopic techniques including one dimensional and two dimensional NMR;

iv) perform anti-bacterial activity assays of the extracts;

v) assess general toxicity (using brine shrimp lethality assay) of extracts.

CHAPTER- TWO

EXPERIMENTAL

2. Materials and Method

2.1 General methods

Some of general techniques used for isolation and characterization of the compounds from endophytic fungi of *Terminalia chebula* Retz and *Aquilaria malaccensis* Lamk are given below.

2.1.1 Solvents and chemicals

Analytical or laboratory grade solvents and chemicals used in the experiments were procured from E Merck (Germany). Commercial grade of ethyl acetate, acetone, methanol, ethanol, chlorofrom and dicholomethane were used after distillation.

2.1.2 Nutrient agar

Agar was used for fungal growth.

2.1.3 Evaporation

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

2.1.4 Freeze-drying

All freeze-drying were carried out by HETOSICC 52 (Hetolab Equipment, Denmark) freeze-dryer. Aqueous extracts and fractions were first frozen in

round bottom flasks in an ethanol freezer (Hetofrig cd 5, Hetobirkero, Denmark) at -30^{0}C to -40^{0}C and finally the materials were subjected to freeze-drying operation.

2.1.5 Shaker

All shaking were carried out using Orbital shaker (Thermo Forma, Refrigerated) at a temperature of 28^{0}C and 150 rpm/min.

2.1.6 Temperature of the fungus room

A wooden frame shelf having glass door was used in the fungus room for keeping the petridishes with media and fungus. Temperature of the room was maintained at ~22-25^{0}C.

2.1.7 Laminar flow

Inoculation and media preparation, antibacterial experiments were performed under a Laminar air flow (Thermo Forma. Class 11 A1, Biological Safety Cabinet) hood.

2.1.8 Autoclave

Media was autoclaved using HIRAYAMA autoclave (Hirayama MFG. Corp.) before use and all used petri dishes containing waste fungi were also autoclaved before discard.

2.1.9 Sterilization

All petri dishes were sterilized using a sterilizer (EYELA natural-sterilizer NDO-600ND) at 182° C for one hour before use.

2.1.10 Preparation of Potato-Carrot Agar media

Potato-carrot agar media was used for fungal growth. A mixture of grated potato (200 g) and grated carrot (200 g) in 1 L of water was boiled for 30 minutes. The mixture was cooled, mashed and the extract was collected by squeezing through a pre-cleaned cloth filter. The volume of the extract was adjusted up to 1L by adding water and to prepare potato-carrot agar media nutrient agar (12 g/L) was added. The agar media was autoclaved at 120° C at 1 atmosphere pressure for 30 minutes.

2.1.11 Preparation of media plate

Autoclaved potato-carrot agar media was poured into petridishes (90 mm x 90 mm) for fungal culture. Nearly 60 petridishes were made with 1L of the potato-carrot agar media.

2.2 Chromatographic methods

2.2.1 Thin layer chromatography (TLC)

Precoated TLC plates (0.2 mm thin coating of silica gel on aluminium sheets) were used throughout the experiments.

2.2.1a Application of the samples

Capillary tubes were used for the application of samples on TLC plates. The plates were run in different solvent systems by the ascending method in glass tanks or jars.

2.2.1b Development of plates

Location of spots

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

i) examination under a UV light with two different wavelengths, 254 and 350 nm.

ii) the plates were exposed to iodine vapor for a few minutes.

iii) the plates were sprayed with 1% ceric sulfate-sulfuric acid reagent followed by heating in an oven at 120^0C for 15 minutes.

2.2.2 Column Chromatography

2.2.2a Column

Glass column of different dimensions were used for separation of the compounds.

2.2.2b Stationary phase

Column grade silica gel (G-60; 230-400 mesh, particle size 0.04-0.063 mm, Ar.7734, ASTM, Merck) was used as stationary phase.

2.2.2c Mobile phase

For the fractionation of extract, the mobile phases were used on the basis of increasing polarity from Hexane to Methanol.

2.2.2d Preparation of column

The column was packed with silica gel slurry using hexane as the column equilibrating solvent. Three column volumes of mobile phase was passed through the column before applying the sample. The sample was applied on the top of the column bed in solid form.

2.3 Nuclear magnetic resonance (NMR) spectroscopy

The NMR spectra were recorded on a Bruker 400 MHz spectrometer at Bangladesh Council of Science and Industrial Research (BCSIR), laboratories, Dhaka on payment. The samples were dissolved either in $CDCl_3$ or CD_3OD. TMS was used as reference. In some case δ-value of solvent peak was taken as reference signal.

2.4 Infrared (IR) spectroscopy

A Shimadzu IR-470 spectrometer was used to obtain IR spectra and KBr pellete was used.

2.5 Gas chromatography mass spectroscopy (GC-MS)

It was done in Chemistry Department of Dhaka University.

2.6 HPLC (High Performance Liquid Chromatography)

2.6.1 Solvent Filtration

Methanol and Milli-Q water (deionized and hydrocarbon free) were filtered by disposable filter device (Polysulfone membrane and 0.45 μm pore size), which were used as mobile phase for HPLC.

2.6.2 High Performance Liquid Chromatography Determination

A Shimadzu SCL-10 *VP*, High Performance Liquid Chromatography having UV/visible detector was used for separation and isolation of compounds. Separation was performed on Reversed Phase C-18 column (Nova pack). The wavelength of detector was fixed at 252 nm. Samples were injected manually through a Rheodyne injector. Detector was connected to the computer for data processing. The working condition of HPLC was binary gradient; flow rate was 0.7 mL/min and injection volume was 100μL.

2.7 Studies of endophytic fungus of *Terminalia chebula* Retz

2.7.1 Collection of plant materials

The medicinal plant, *Terminalia chebula* Retz (locally known as Haritaki) was collected from a local nursery of Dhaka city.

Figure 2: Leaves of *Terminalia chebula* Retz

2.7.2 Surface sterilization of *Terminalia chebula* Retz

Leaves, stems and roots of the plant *Terminalia chebula* Retz were separated and cut into small pieces. Each plant part was surface sterilized with 70% ethanol, 3% sodium hypochloride and sterile water. Different parts of plants were cleaned and washed with water. The cleaned plant parts were kept successively into each of the solution for 3 minutes. The effectiveness of surface sterilization was checked by making an imprint of the treated portion on agar media.

2.7.3 Inoculation

The surface sterilized plant materials were inoculated on autoclaved potato-carrot agar media on a sterilized petridish (90 mm in diameter). Five pieces of leaves were inoculated on each petridish and 10 of such petridishes were used for leaves. Similarly 20 petridishes were used for stems and roots.

2.7.4 Isolation of fungi from *Terminalia chebula* Retz

After 21 days of inoculation it was found that leaves, stems and roots gave five, seven and nine fungi, respectively. By dereplication of common fungi, five strains (IR-1, IR-2, IR-4, IR-6 and IR-7) were found to have optimum growth for further cultivation. All of them were isolated from the roots.

2.7.5 Subculture of fungi

The five fungi IR-1, IR-2, IR-4, IR-6 and IR-7 from *T. chebula* was subcultured on semisolid potato-carrot agar media in small scale (10 petridishes for each fungus) for 21 days. Each of the cultured fungi was extracted with ethyl acetate and the TLC pattern of each fungal extract was observed. The TLC pattern of the strain IR-4, IR-6 and IR-7 were found to contain interesting compounds. These fungi were cultivated on large scale for chemical and biological investigation since they have minimum growth time and the extract showed well-separated spots on TLC.

2.7.6 Large scale growth of strain IR-6

The IR-6 strain was inoculated in 450 petridishes for large scale production and kept in a wooden frame shelf having glass door in the fungus room. Temperature of the room was maintained at ~22-25^{0}C. It took about 21 days for growing the fungi.

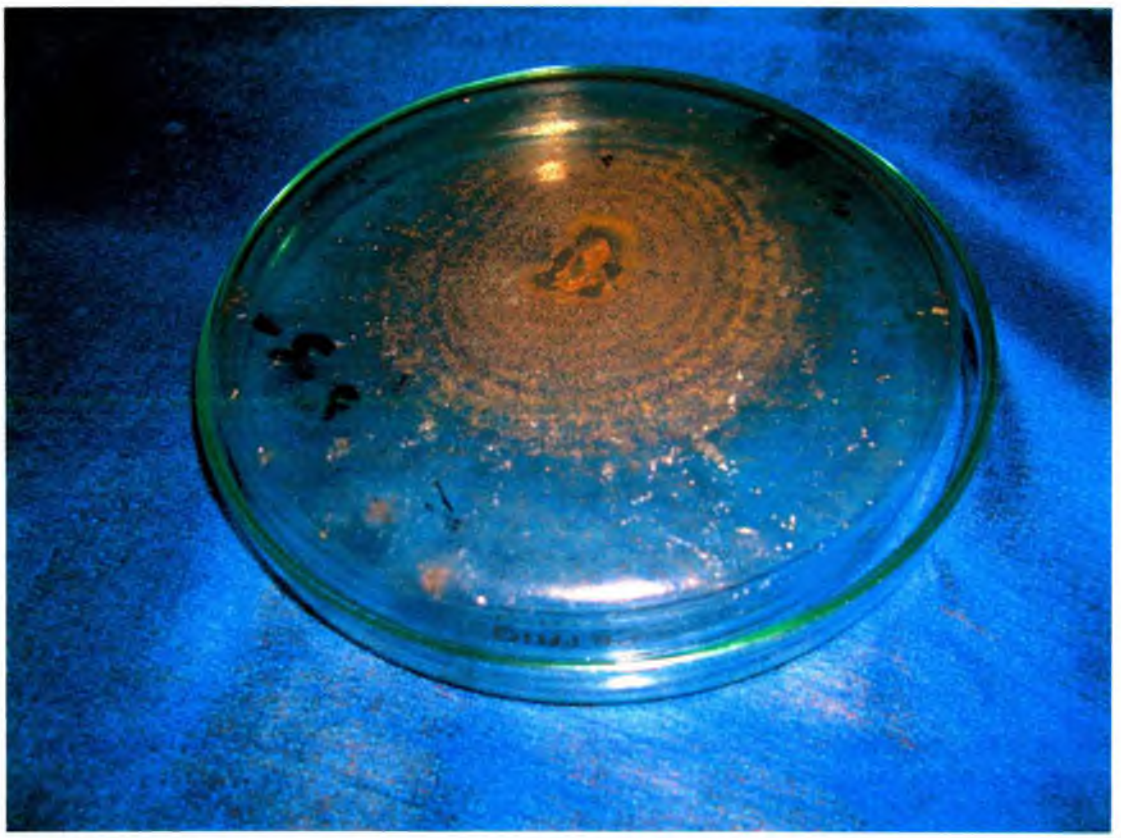


Figure 3: The fungus IR-6 from *T. chebula*

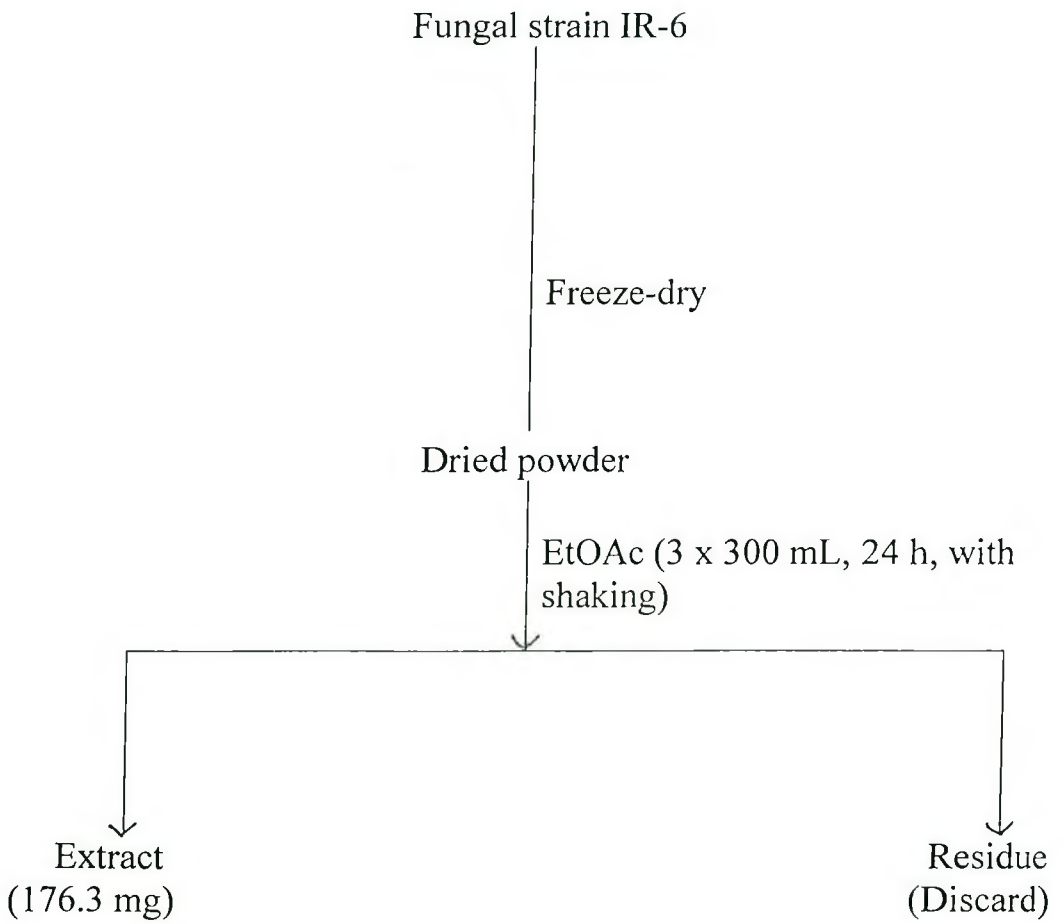
2.7.7 Extraction of the strain IR-6

The endophytic fungus IR-6 from *T. chebula* Retz was collected in a round bottom flask. The content was then subjected to freeze-drying. When the sample was completely dried, it was powdered in a mortar. The powdered

sample was extracted with ethyl acetate (3 x 1500 mL for 24 h, at room temperature). The extract was taken by decantation and the procedure was repeated for two more times and to get ethyl acetate extract (176.3 mg). A small part of the extract (~20 mg) was saved for antibacterial studies. Rest of the extract was used to isolate pure compounds.

Extraction of the fungal strain IR-6

Scheme 1



2.7.8 Isolation of compounds from the endophytic fungus, IR-6

2.7.8.1 Fractionation of ethyl acetate extract

The extract (156.3 mg) was dissolved in chloroform and silica gel (310 mg) was added to it. After evaporation of solvent the dried sample became ready for fractionation by column chromatography. A column was packed with silica gel using hexane as column equilibrating solvent. A three-column volume of mobile phase was passed through the column before applying the sample. After application of the sample, solvents of increasing polarities using hexane, dichloromethane and methanol were passed through the column. The fractions were collected in 10 mL test tubes. The eluted samples, collected in 109 test tubes were combined on the basis of their R_f values in TLC and finally eleven fractions were obtained. Amount of each sample and their TLC patterns are given in Table-2.

Table-2: TLC pattern of various fractions (from IR-6)

Fraction No.	Number of test tubes	TLC pattern	Amount (mg)
SR-1	9-12	Single spot	2.0
SR-2	13-16	Single spot	3.4
SR-3	19-23	Three different spots	12.4
SR-4	26-31	Single spot	6.8
SR-5	32-39	Three spots	7.1
SR-6	40-51	Three spots	7.0
SR-7	52-67	One spot with tailing	7.5
SR-8	68-78	Long tailing	5.0
SR-9	97-98	Mixture of several spots	4.0
SR-10	101-103	Two spots	2.1
SR-11	106-109	Single spot	4.6

2.7.8.2 Characterization of the compound SR-1 by spectroscopic methods

i) Physical properties:

It was off-white and gummy, soluble in the mixture of hexane & dichloromethane and hexane & ethyl acetate.

ii) Infrared (IR) spectroscopy

The IR spectrum of the compound SR-1 (KBr pellet) had absorbances at 3400, 2900, 2850, 1720, 1450, and 1250 cm^{-1}.

iii) ^1H NMR (400 MHz) spectroscopy

The ^1H NMR spectrum of the compound SR-1 was taken in $CDCl_3$ and TMS was used as reference. The spectral data and their assignment are presented in Table-3.

Table-3: ^1H NMR (chemical shift, multiplicity, coupling constant, *J* in Hz) spectral data of the compound SR-1

^1H NMR δ in ppm	Splitting pattern and coupling constant, *J* in Hz	Integration	Types of proton
5.33	multiplet	1H	olefinic
5.32	multiplet	1H	olefinic
3.65	singlet	3H	oxymethyl
2.75	multiplet	2H	methylene
2.28	multiplet	2H	methylene
1.98	multiplet	2H	methylene
1.67	multiplet	2H	methylene
1.24	multiplet	14H	methylene
0.85	triplet, *J*=7.2	3H	methyl

2.7.8.3 Characterization of the compound SR-2 by spectroscopic methods

i) Physical properties:

The compound SR-2 was light yellow coloured and gummy. It was soluble in the mixture of hexane & dichloromethane and hexane & ethyl acetate.

ii) Infrared (IR) spectroscopy

The IR spectrum of the compound showed absorbance at 3450, 2900, 1730, and 1450 cm^{-1}.

Table-4: ^{1}H NMR (chemical shift, multiplicity, coupling constant, *J* in Hz) spectral data of the compound SR-2

^{1}H NMR δ in ppm	Splitting pattern and coupling constant, *J* in Hz	Types of proton
5.36	multiplet	olefinic
5.33	multiplet	olefinic
4.12	multiplet	oxygenated methine
3.66	singlet	oxymethyl
2.76	multiplet	methylene
2.29	multiplet	methylene
2.03	multiplet	methylene
1.67	multiplet	methylene
1.60	multiplet	methylene
1.54	multiplet	methylene
1.41	multiplet	methylene
1.27	multiplet	methylene
0.88	triplet, 7.2	methyl

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

The ^{1}H NMR spectrum of the compound SR-2 was taken in $CDCl_3$ and TMS was used as reference signal. The spectral data and their assignments are presented in Table-4.

iv) ^{13}C NMR spectroscopy

The ^{13}C NMR spectrum of SR-2 were recorded using $CDCl_3$ as solvent. Dept-135 was also recorded. The values are given in Table-5.

Table-5: ^{13}C NMR data of the compound SR-2

Chemical shift, δ in ppm	Types of carbon
130.2	-CH
128.0	-CH
61.2	-CHOH
52.0	-CH
34.1	$-CH_2$
32.0	$-CH_2$
31.5	$-CH_2$
29.7	$-CH_2$
29.6	$-CH_2$
29.3	$-CH_2$
29.1	$-CH_2$
27.2	$-CH_2$
25.6	$-CH_2$
25.0	$-CH_2$
22.6	$-CH_2$
14.1	$-CH_3$

2.7.8.4 Characterization of the compound SR-4 by spectroscopic methods

i) Physical properties

The compound SR-4 was deep yellow coloured and gummy in nature. It was soluble in the mixture of hexane/dichloromethane and hexane/ethyl acetate.

ii) Infrared (IR) Spectroscopy

The IR spectrum of the compound showed absorbance at 2900, 1730 and 1450 cm^{-1}.

iii) ^1H NMR (400 MHz) spectroscopy

The ^1H NMR spectra of SR-4 was taken using $CDCl_3$ as solvent and TMS was used as reference. The spectral data and their assignment are presented in Table-6.

Table-6: ^1H NMR (chemical shift, multiplicity, coupling constant, *J* in Hz) spectral data of the compound SR-4

^1H-NMR δ in ppm	Splitting pattern and coupling constant, *J* in Hz	Types of proton
5.33	broad singlet	olefinic
5.25	broad singlet	olefinic
4.20	multiplet	oxymethyl
2.30	multiplet	methylene
2.00	broad singlet	methylene
1.59	multiplet	methylene
1.24	broad singlet	methylene
0.86	triplet, 7.2	methyl

iii) ^{13}C NMR spectroscopy

The ^{13}C NMR spectrum of SR-4 were recorded using $CDCl_3$ as solvent. The Dept-135 was also recorded. The values are given in Table-7.

Table-7: ^{13}C NMR data of the compound SR-4

Chemical shift δ in ppm	Types of carbon
173.3	>C<
130.0	=CH
129.7	=CH
62.1	$-CH_2$
34.1	$-CH_2$
32.0	$-CH_2$
29.7	$-CH_2$
29.7	$-CH_2$
29.6	$-CH_2$
29.4	$-CH_2$
29.3	$-CH_2$
29.2	$-CH_2$
29.1	$-CH_2$
27.2	$-CH_2$
24.9	$-CH_2$
22.7	$-CH_2$
14.1	$-CH_3$

2.7.8.5 Purification of SR-6

SR-6 (7.0 mg) was refractionated by a small silica gel column using hexane, dichloromethane and acetone and three sub-fractions (SBR-1, 2.0 mg; SBR-2, 1.5 mg and SBR-3, 1.2 mg) were obtained.

Table-8: TLC pattern of three fractions (from fraction SR-6)

Fraction no.	TLC pattern	Amount (mg)
SBR-1	Single spot	2.0
SBR-2	Single spot	1.5
SBR-3	Single spot with tailing	1.2

2.7.8.6 Characterization of the compound SBR-1 by spectroscopic methods

^{1}H NMR (400 MHz) spectroscopy

The ^{1}H NMR spectra of SBR-1 was taken using $CDCl_3$ as solvent and TMS was used as reference. The spectral data and their assignment are presented in Table-9.

Table-9: ^{1}H NMR (chemical shift, multiplicity, coupling constant, *J* in Hz) spectral data of the compound SBR-1

^{1}H-NMR δ in ppm	Splitting pattern and coupling constant, *J* in Hz	Types of proton
5.35	doublet	olefinic
5.12	doublet	olefinic
3.65	triplet	oxygenated methine
2.03	multiplet	methine
1.55	singlet	methylene
1.24	singlet	methylene
0.87	triplet	methyl

2.7.8.7 Characterization of the compound SR-11 by spectroscopic methods

i) Physical properties

The compound SR-11 was light yellow coloured and solid in nature. It was soluble in dichloromethane and methanol.

ii) Infrared (IR) spectroscopy

The IR spectrum of the compound showed absorbance at 3350, 2920, 1715, 1450, 1260 and 740 cm^{-1}.

iii) ^1H NMR (400 MHz) spectroscopy of SR-11

The ^1H NMR spectra of SR-11 was taken using $CDCl_3$ as solvent and solvent peak was taken as internal standard. The spectral data and their assignment are presented in Table-10.

Table-10: ^{1}H NMR (chemical shift, multiplicity, coupling constant, *J* in Hz) spectral data of the compound SR-11

No. of proton	^{1}H NMR δ in ppm	Types of proton
2	5.80, *d* (*J*=16.0)	olefinic
3	7.35, *dd* (*J*=16.0, 8)	olefinic
4	4.22, *m*	oxygenated
5	2.40, *s*	methylene
7	4.80, *m*	oxygenated
8	2.26, *m*	methylene
10	5.50, *m*	olefinic
11	5.22, *m*	olefinic
12	2.12, *m*	methylene
14	3.99, *m*	oxygenated
15	1.90, *m*	methylene
16	1.88, *m*	methylene
19	1.21, *s*	methyl
20	1.21, *s*	methyl

iii) ^{13}C NMR spectroscopy of the compound SR-11

The ^{13}C NMR spectrum of SR-11 were recorded using $CDCl_3$ as solvent. The Dept-135 was also recorded. The spectral data and their assignment are presented in Table-11.

Table-11: ^{13}C NMR data of the compound SR-11

No. of carbon	Chemical shift value δ in ppm	Types of carbon
1	176.0	O=C<
6	166.8	>C=O
2	117.1	=CH
3	152.5	=CH
4*	75.6	-C-OH
5	26.6	$-CH_2$
7	72.0	-CH-O-
8	31.8	$-CH_2$
9	51.8	-CH
10	130.2	=CH
11	136.7	=CH
12	29.6	$-CH_2$
14	72.1	>C-O-
15	34.0	$-CH_2$
16	41.0	$-CH_2$
17	44.3	-CH
18	43.0	-CH
19/20	20.7	$-CH_3$

* interchange

2.8 Studies of endophytic fungus of *Aquilaria malaccensis* Lamk

2.8.1 Collection of plant materials

Agor wood is a resinous wood that sometimes occurs in trees belonging to the *Aquilaria* genus, *Thymelaeceae* family. The young plant *Aquilaria malaccensis* (locally known as Agor) was collected from Sylhet. The plant was used to isolate endophytic fungi.

Figure 4 : Different parts of *Aquilaria malaccensis* Lamk

2.8.2 Surface sterilization of plant material

Leaves, stems and roots of the plant *A. malaccensis* were separated and cut into small pieces. Each plant part was surface sterilized with 70% ethanol,

3% sodium hypo chloride and sterile water. Different parts of plants were cleaned and washed with water. The cleaned plant parts were kept successively into each of the solution for 3 minutes. The effectiveness of surface sterilization was checked by making an imprint of the treated portion on agar media.

2.8.3 Inoculation

The surface sterilized plant materials were inoculated on autoclaved potato-carrot agar media on a sterile petridish (90 mm in diameter). Five pieces of leaves were inoculated on each petridish and a total of 10 petridishes were used for leaves. Similarly 20 petridishes were used for stems and roots.

2.8.4 Isolation of fungi from *A. malaccensis*

After 21 days of inoculation it was found that leaves, stems and roots gave two, four and three fungi, respectively. They were labeled as strain AL-1, AL-2, AS-1, AS-2, AS-3, AS-4, AR-1, AR-2 and AR-4. The effectiveness of surface sterilization was checked by making an imprint of the treated portion on a media plate (Schulz *et al.*1998). If the surface sterilization was successful no fungal colonies developed on the imprint medium.

2.8.5 Subculture of fungi

The fungal strain AL-2 from leaf of *A. malaccensis* was sub-cultured on semisolid potato-carrot agar media in a small scale (10 petridishes) for 21 days. The TLC pattern of AL-2 found to contain several interesting compounds. The AL-2 was cultivated on large scale for chemical and

biological investigation since it has minimum growth time and the extract showed well-separated spots on TLC.

2.8.6 Large scale growth of the strain AL-2

The selected AL-2 fungal strain was inoculated in 400 media plates (90 mm in size) each time and it was repeated for four times. It took about 21 days for growing the fungi.

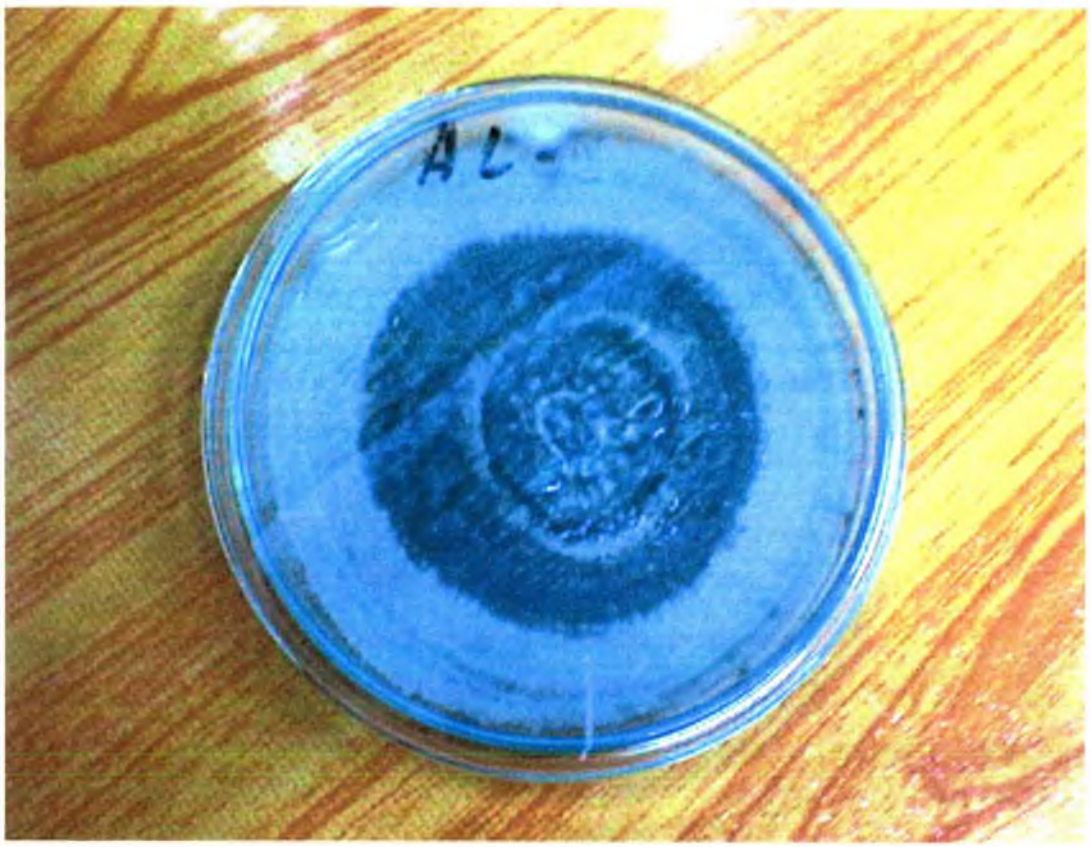


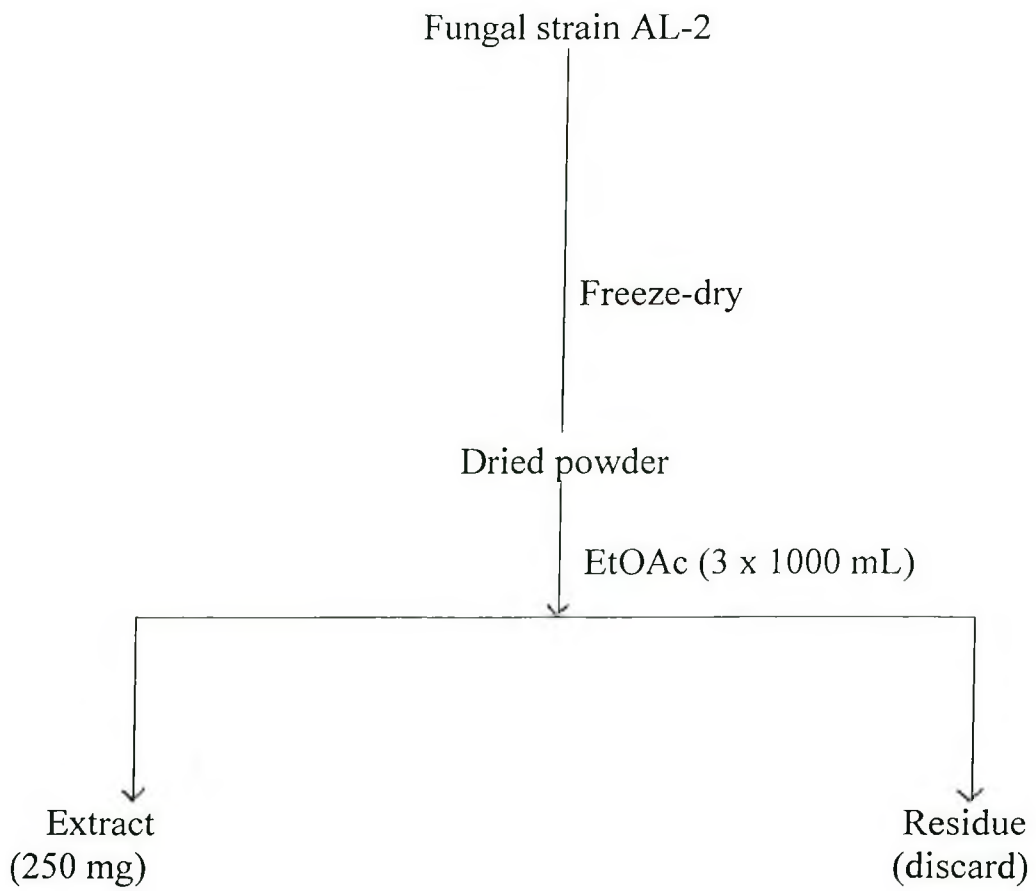
Figure 5: The fungus from *A. malaccensis*

2.8.7 Extraction of the strain AL-2

The endophytic fungus AL-2 from *A. malaccensis* was collected into a round bottom flask. The content was then subjected to freeze-drying. When the sample was completely dried, it was powdered in a mortar. The powdered sample was extracted with ethyl acetate for 24 hours, at room temperature. The extract was taken by decantation and this procedure was repeated for two more times. The ethyl acetate extract was evaporated to dryness and 250 mg was obtained.

Extraction of the fungal strain AL-2

Scheme 2



2.8.8 Isolation of compounds from endophytic fungus AL-2

2.8.8.1 Fractionation of ethyl acetate extract

The extract (200 mg) was dissolved in chloroform and silica gel (400 mg) was added to it. After evaporation of solvent the dried sample became ready for fractionation by column chromatography. A column was packed with silica gel using hexane as column equilibrating solvent. A three column volume of mobile phase was passed through the column before applying the sample. After application of the sample, solvents of increasing polarities from 100% hexane to 50% MeOH with dichloromethane were used for elution. The fractions were collected in 10 mL test tubes. The eluted samples collected in 235 test tubes were combined on the basis of their R_f values on TLC and finally sixteen fractions were obtained. Amount of each sample and their TLC patterns are given in Table-12.

Table- 12: Fractions of Ethyl acetate extract of AL-2

Fraction No.	Number of test Tubes	TLC pattern	Amount (mg)
S-1	11-20	Long tail	2.0
S-2	19-32	Long tail	19.7
S-3	34-44	Tailing	3.5
S-4	47-56	Single spot	2.5
S-5	57-65	Two spots with tail	6.8
S-6	66-85	Three spots	15.6
S-7	89-113	Two spots	12.0
S-8	115-133	Three spots	7.0
S-9	139-152	Three spots with tail	13.8
S-10	153-157	Long tailing	1.3
S-11	158-163	Two spots	7.2
S-12	164-175	Single spot	11.3
S-13	175-207	Three spots with tail	24.8
S-14	218-220	Long tail	2.8
S-15	221-224	Three spots	1.8
S-16	231-235	Three spot with tail	20

2.8.8.2 Characterization of the compound S-4 by spectroscopic methods

i) Physical properties

The compound S-4 was yellow coloured and gummy. It was soluble in dichloromethane, ethyl acetate and partially in methanol.

ii) ^{1}H NMR spectroscopy

The ^{1}H NMR spectra of S-4 was taken using $CDCl_3$ as solvent and TMS was used as reference. The spectral data and their assignment are presented in Table-13.

Table-13: ^{1}H NMR (chemical shift, multiplicity) spectral data of the compound S-4

No.	^{1}H NMR δ in ppm	Splitting pattern and coupling constant, *J* in Hz	Types of proton
H-5	7.82	doublet, 7.1	aromatic
H-6	7.67	doublet, 7.1	aromatic
H-8	7.50	singlet	aromatic
H-4	7.10	singlet	aromatic
H-2	6.97	singlet	aromatic
-OCH_3	3.94	singlet	oxymethyl
-O-H (1)	12.11	broad singlet	chelating proton

2.8.8.3 Characterization of the compound S-12 by spectroscopic methods

i) Physical properties

The compound S-12 was light yellow and solid. It was soluble in dichloromethane, ethyl acetate and partially in methanol.

ii) Infra red (IR) spectroscopy

The IR spectrum of the compound showed absorbance at 2900 and 1760 cm^{-1}.

iii) ^{1}H NMR spectroscopy

The ^{1}H NMR and ^{13}C NMR spectrum of the compound S-12 were taken in $CDCl_3$ and TMS was used as reference. The spectral data and their assignment are presented in Table-14.

Table-14: ^{1}H NMR (chemical shift, multiplicity and coupling constant, *J* in Hz) spectral data of the compound S-12

^{1}H NMR δ in ppm	Splitting pattern and coupling constant, *J* in Hz	Types of proton
5.29	multiplet	olefinic
2.25	multiplet	olefinic
1.97	multiplet	methylene
1.56	broad singlet	methylene
1.20	-	methylene
0.85	triplet, 7.6	methyl

iv) ^{13}C NMR spectroscopy of the compound S-12

The ^{13}C NMR spectrum of S-12 were recorded using $CDCl_3$ as solvent. The Dept-45 was also recorded. The values are given in Table-15.

Table-15: ^{13}C-NMR data of the compound S-12

^{13}C-NMR	Types of carbon
179.1	>C<
132.0	H-C=
129.0	H-C=
34.0	$-CH_2$
32.0	$-CH_2$
29.7	$-CH_2$
29.5	$-CH_2$
29.4	$-CH_2$
29.1	$-CH_2$
24.7	$-CH_2$
22.7	$-CH_2$
14.1	$-CH_3$

2.8.8.4 Purification of fraction S-16 by High Performance Liquid Chromatography (HPLC)

The fraction S-16 (20 mg) was dissolved in methanol and purified by High Performance Liquid Chromatography (HPLC).

HPLC Conditions are as follows:

HPLC Shimadzu Class 10 *VP*
UV-Visible detector; wavelength: 254 nm
Column: Discovery C-18 (25 cm x 4.6 mm, 5 μm)
Mobile phase: Methanol : Water :: 40: 60
Flow rate: 0.7 mL/min
Injection volume: 100 μL

Isolated fractions and their amount are given in Table-16.

Table-16: Retention time of the fraction S-16

Retention time (min)	Fraction No.	Amount (mg)
18.00	S-16C	2.0
19.30	S-16D	2.0

2.8.8.5 Characterization of the compound S-16C by spectroscopic methods

i) Physical properties

The compound S-16C was light yellow and solid. It was soluble in dichloromethane with a few drop of methanol.

ii) Infra red (IR) spectroscopy

The IR spectrum of the compound had absorbances at 2900 and 1650 cm^{-1}.

iii) ^1H NMR spectroscopy

The ^1H NMR spectrum of the compound S-16C was taken in $CDCl_3$ & CD_3OD and solvent peak was taken as internal standard. The spectral data and their assignment are presented in Table-17.

Table-17: ^1H NMR (chemical shift, multiplicity, coupling constant, *J* in Hz) spectral data of the compound S-16C

^1H NMR δ in ppm	Multiplicity and Coupling constant, *J* in Hz	Types of proton
7.45	doublet, 8.4	aromatic
6.57	doublet, 8.4	aromatic
4.75	doublet, 6.8	-O-CH_2
4.62	doublet, 6.9	-O-CH_2
3.70	singlet	oxymethyl
1.25	multiplet	aliphatic
0.98	triplet, 7.2	methyl

2.8.8.6 Characterization of the compound S-16D by spectroscopic methods

i) Physical properties

The compound S-16D was off-white and solid. It was soluble in dichloromethane.

ii) Infra red (IR) spectroscopy

The IR spectrum of the compound had absorbance at 1640 and 1490 cm^{-1}.

iii) ^{1}H NMR spectroscopy

The ^{1}H NMR spectrum of the compound S-16D was taken in $CDCl_3$ and solvent peak was taken as internal standard. The spectral data and their assignment are presented in Table-18.

Table-18: ^{1}H NMR (chemical shift, multiplicity, coupling constant, *J* in Hz) spectral data of the compound S-16D

No.	^{1}H NMR δ in ppm	Multiplicity and coupling constant, *J* in Hz	Types of proton
5	8.42	broad singlet	aromatic
3	7.59	doublet, 7.7	aromatic
2′	7.30	doublet, 7.4	aromatic
4	7.10	multiplet	aromatic
3′	6.97	doublet of doublet, 7.7	aromatic
5	6.60	singlet	aromatic
6	3.79	singlet	-O-CH_3
4′	3.64	singlet	-O-CH_3

2.9 Biological studies of the ethyl acetate extract of endophytic fungi

2.9.1 Antibacterial activity

The ethyl acetate extract of the fungal strain IR-6, isolated from *Terminalia chebula* and the strain AL-2, isolated from *Aquilaria malaccensis* were used to access the antibacterial activity. The specific concentration of the extract of IR-6 and AL-2 were 120 and 200 μg/μL respectively. Sterile filter paper disc of 6 mm in diameter were loaded with 200/120 μg/disc using micropipette and were dried under laminar airflow hood. Standard antibiotic, *Kanamycin* (30 μg/disc) was used as a positive control. The loaded discs were placed in petridishes (90 mm in diameter) containing sterile nutrient agar medium seeded with test microorganisms using sterile transfer loop for antibacterial screening. The plates were kept at 4°C for 12 hours. Then the plates were kept in an incubator at 37°C for 12-18 hours. The antibacterial activities of the test agent was determined by measuring the diameter of the zone of inhibition in mm (Choudhary, et al, 1999).

The antibacterial activity of ethyl acetate extract of IR-6 was performed against two gram-positive bacteria i.e. *Bacillus cereus, Staphylococcus aureus* and three gram-negative bacteria i.e. *Escherichia coli* 0157, *Shigella boydii, Pseudomonas aeruginosa.*

The antibacterial activity of ethyl acetate extract of AL-2 was performed against two gram-positive bacteria i.e. *Bacillus cereus* and *Staphylococcus aureus* and five gram-negative bacteria i.e. *Escherichia coli* 0157,

Salmonella typhi, Shigella boydii, Pseudomonas aeruginosa and *Vibrio cholerae.* The results are given in Table -19.

Table-19: Zone of inhibition of the fungal extract against bacteria

Name of the organism	Zone of inhibition (mm)	
	Standard *Kanamycin*	EtOAc Extract
	30 µg/disc	200 µg/disc
Gram-positive bacteria		
Bacillus cereus	36	12
Staphylococcus aureus	40	10
Gram-negative bacteria		
Escherichia coli 0157	37	0
Shigella boydii	39	0
Pseudomonas aeruginosa	42	0
Salmonella typhi	40	0
Vibrio cholerae	35	9

--inactive

Zone of inhibition: 7-9 mm= insignificant, 10-12 mm= mild activity, 13-15 mm=moderate activity, above 15 mm=significant.

2.9.2 Brine shrimp lethality Assay

Brine shrimp lethality assay (Meyer et al, 1982) ethyl acetate extract of the fungus IR-6 and AL-2 was performed by the following standard protocol.

Test animal

Brimp shrimp (*Artemia salina*) was used as the test animal for the investigation of cytotoxicity.

Hatching and maintenance of brine shrimp

Brine shrimp prefer a temperature between 28-30° C and salinity of 30-35 ppm, a pH of 8-9, and strong aeration under a continuous light regime. After these conditions were fulfilled, about one teaspoon of brine shrimp eggs were added into the systems (38% brine solution) and approximately after 48 hour hatching the phototropic nauplii were collected with a pipette from the lighted side and concentrated in a small beaker and applied for testing.

Preparation of test sample

Ethyl acetate extract (10 mg) was dissolved in 4 mL ethyl acetate solvent. The solution was then taken in vials as 1.0 mL in each three vials, 200 μL in each three and 40 μL in each three vials. The solvent of these vials was evaporated in a vacuum dryer. Brine solution (8 mL) was added in each vial and a few drops of yeast solution were added in each vial. Now ten shrimps were transferred in each vial using a micropipette. For control, one vial with 8 mL brine solution and 10 shrimps were taken with a few drops of yeast solution.

Counting of shrimps

The vials containing different amounts of test samples were observed and the number of survived nauplii in each test tube was counted first time after 24 h and 2nd time after 48 h. From this, the percentage of lethality of brine shrimp nauplii was counted at each vial for the sample. An approximate linear correlation was observed when logarithm of amount (in mg) was plotted against percentage of mortality and the values of LD_{50} were calculated manually. Although there was no mortality in the control group, the test samples shown different mortality rate at different amounts of sample, which was found to increase with increasing amount of the sample. The LD_{50} values of the extract compounds are shown in Table-20 and 21.

Table-20: Brine shrimp lethality assay of ethyl acetate extract obtained from the fungus IR-6

Sample	Amount of dose in mg	Number of shrimps	Vials no.	After 24 hours		After 48 hours	
				Alive	Dead	Alive	Dead
EtOAc Extract	2.5	10	1 2 3	1 2 0	9 8 10	0 0 0	10 10 10
	0.5	10	1 2 3	9 7 8	1 3 2	5 3 5	5 7 5
	0.1	10	1 2 3	10 9 9	0 1 1	8 8 7	2 2 3

Table-21: Brine shrimp lethality assay of ethyl acetate extract obtained from the fungus AL-2

Sample	Amount of dose in mg	Number of shrimps	Vials no.	After 24 hours		After 48 hours	
				Alive	Dead	Alive	Dead
EtOAc Extract	2.5	10	1	8	2	6	4
			2	6	4	5	5
			3	8	2	6	4
	0.5	10	1	9	1	8	2
			2	10	0	9	1
			3	9	1	8	2
	0.1	10	1	10	0	10	0
			2	10	0	10	0
			3	10	0	10	0

CHAPTER- THREE

RESULTS AND DISCUSSION

3.1 Results and Discussion

Natural products derived from plants, endophytic fungi and marine fungi have been used for the treatment of various diseases for many years. An endophyte is a bacterial or fungal microorganism, which spends the whole or part of its life cycle colonizing inter and/or intra-cellularly inside the healthy tissues of the host plants. The word endophyte means "in the plant" and refers to all organisms that grow within plants. Fungal endophytes are fungi that live within their host plants without causing any noticeable symptoms of disease to the plant. Different types of secondary metabolites have been isolated from different endophytic fungi. Recently, study on endophytic fungi from medicinal plants has received much attention to the scientist because endophytic fungi are believed to be an excellent potential source of biologically active compounds. Two medicinal plants, *Terminalia chebula* Retz and *Aquilaria malaccensis* Lamk were selected to separate endophytic fungi from them, and to isolate secondary metabolites from selective fungal strains.

Terminalia chebula Retz is one of the important medicinal plants of Bangladesh. The plant naturally grows in Dhaka, Mymensingh, Tangail and Chittagong districts. It is generally used in triphala, a traditional Ayurvedic herbal formulation consisting of the dried fruits of three medicinal plants (Ghani, 2003).

The *Aquilaria malaccensis* Lamk is one of the medicinal plants of Bangladesh. The plant naturally grows in natural forest of Sylhet district. When the plant is attacked by exocyctic fungus the changes occurs in the

middle lamella of timber. The exocyctic fungus secrets aromatic compounds which are used as agor oil. The oil has medicinal value as well. The wood after steam distillation is used in agor candle. Powdered wood is used as a perfume. It is specifically used as an aromatic, stimulant and nerve tonic.

3.2 Studies of endophytic fungus of *Terminalia chebula* Retz

The leaves, stems and roots of the plant *Terminalia chebula* Retz were separated and cut into small pieces. Each plant part was surface sterilized with 70% ethanol, 3% sodium hypochloride and sterile water. Different parts of plants were cleaned and washed with water. The surface sterilized plant materials were inoculated on autoclaved potato-carrot agar media on a sterilized petridish (90 mm in diameter). After 21 days of inoculation it was found that leaves, stems and roots gave five, seven and nine fungi, respectively. By dereplication of common fungi, five strains from roots (IR-1, IR-2, IR-4, IR-6 and IR-7) were found to have optimum growth for further cultivation. The five fungi IR-1, IR-2, IR-4, IR-6 and IR-7 from *T. chebula* was subcultured on semisolid potato-carrot agar media in small scale (10 petridishes for each fungus) for 21 days. Each of the cultured fungi was extracted with ethyl acetate and the TLC pattern of each fungal extract was observed. The TLC pattern of the strain IR-4, IR-6 and IR-7 were found to contain interesting compounds. These fungi were cultivated on large scale for chemical and biological investigation since they have minimum growth time and the extract showed well-separated spots on TLC. Only the IR-6 strain was inoculated in 450 petridishes for 21 days for large scale production.

The endophytic fungus IR-6 from *T. chebula* Retz was collected in a round bottom flask. The content was then subjected to freeze-drying. When the sample was completely dried, it was powdered in a mortar. The powdered sample was extracted with ethyl acetate (3 x 1500 mL for 24 h, at room temperature). The extract was taken by decantation and the procedure was repeated for two more times and to get ethyl acetate extract (176.3 mg). A small part of the extract (~20 mg) was saved for antibacterial studies.

3.2.1 Isolation of compounds from the strain IR-6

The ethyl acetate extract (156.3 mg) was fractionated by silica gel column using hexane, dichloromethane and methanol as eluting solvents. The fractions were collected in 10 mL test tubes. The eluted samples, collected in 109 test tubes were combined on the basis of their R_f values in TLC and finally eleven fractions (SR-1, SR-2, SR-3, SR-4, SR-5, SR-6, SR-7, SR-8, SR-9, SR-10 and SR-11) were obtained (**Table-2**). Among them four fractions SR-1 (2.0 mg), SR-2 (3.4 mg), SR-4 (6.8 mg) and SR-11 (4.6 mg) showed single spot in TLC and collected as pure compounds. The fraction SR6 (7.0 mg) was refractionated by a small silica gel column using hexane, dichloromethane and acetone and three sub-fractions (SBR-1, 2.0 mg; SBR-2, 1.5 mg and SBR-3, 1.2 mg) were obtained.

3.2.1.1 Characterization of the compound SR-1 by spectroscopic methods

The TLC of compound SR1 in different solvent media was found to be a single compound and it was characterized by spectroscopic methods (IR, ^{1}H, and ^{13}C NMR).

Physical properties

It was off-white and gummy, soluble in the mixture of hexane & dichloromethane and hexane & ethyl acetate.

Infra red (IR) spectroscopy

In the IR spectrum of the compound SR-1, absorbance at 3400 cm^{-1} indicated the presence of hydroxyl group. The absorbances at 2900 cm^{-1} and 2850 cm^{-1} indicated the aliphatic CH stretching. Absorbance at 1720 cm^{-1} indicated the presence of carbonyl functional group. Absorbance at 1450 cm^{-1} indicated the aromatic double bond (C=C). Absorbance at 1250 cm^{-1} indicated the presence of oxymethyl of carbonyl group.

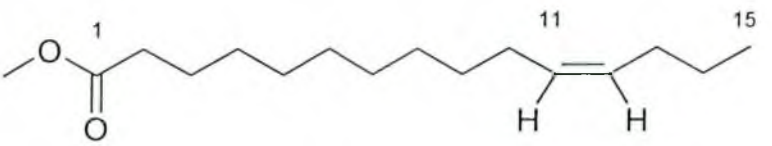
^{1}H NMR (400 MHz) spectroscopy

^{1}H NMR spectrum of the compound was recorded in $CDCl_3$ and TMS was used as reference. The spectrum showed a number of large signals in the

aliphatic region, signals in the olefinic double bond region. In addition to that a number of very small signals appeared in the aldehydic region.

The ^{1}H NMR spectrum showed signals at 5.33 (*m*) and 5.32 ppm (*m*) for the presence of two olefinic protons. Two signals at 3.65 ppm (3H, *s*) and a triplet at 0.85 ppm (3H, *t*) in the ^{1}H NMR spectrum indicated the presence of oxygeneted methyl and a methyl group attached to methylene group respectively. Two multiplets at δ_H = 2.28 and δ_H = 2.75 ppm indicated the presence of two methylene groups. A large signal at 1.24 ppm was assigned for the presence of seven methylene groups. From the analysis of spectral data of the compound SR-1 it was revealed that the signals were due to the presence of pentadec-11-enoic acid methyl ester. Pentadec-11-enoic acid ester was earlier reported to be present in herbal medicine (Nahar, 2007).

The structure of the compound is given below:



Pentadec-11-enoic acid methyl ester

3.2.1.2 Characterization of the compound SR-2 by spectroscopic methods

Physical properties

The compound SR-2 was light yellow coloured and gummy. It was soluble in the mixture of hexane & dichloromethane and hexane & ethyl acetate.

Infra red (IR) spectroscopy

In the IR spectrum of the compound SR-2 the absorbance at 3450 cm^{-1} was for hydroxyl group. The absorbance at 2900 cm^{-1} was accounted for saturated hydrocarbon. Absorbance at 1730 cm^{-1} was due to carbonyl functional group. The absorbances at 1450 cm^{-1} was accounted for unsaturated hydrocarbon.

^{1}H NMR (400 MHz) spectroscopy

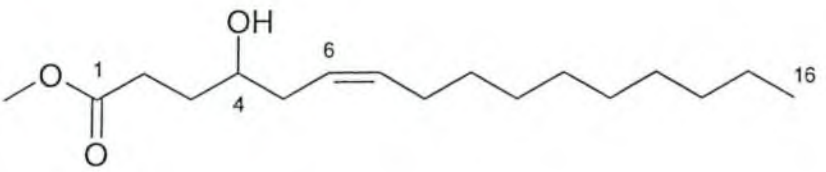
The ^{1}H NMR spectrum of the compound was recorded in $CDCl_3$ and TMS was used as reference signal. In the ^{1}H NMR spectrum, the signal at 2.29 ppm indicated the oxymethyl group. The ^{1}H NMR spectrum of SR-2 showed two signals at 5.36 (*m*) and 5.33 ppm (*m*) were due to two olefinic protons. The ^{1}H NMR also had signals at 4.12 (*m*), 3.66 (*s*) and 0.88 ppm (*t*) were due to the presence of oxygenated methine, oxymethyl and methyl group respectively. Several other signals at 2.76, 2.29, 2.03, 1.67, 1.60, 1.54, 1.41, 1.27 were due to methylene groups.

^{13}C NMR (400 MHz) spectroscopy

In the ^{13}C NMR spectrum several signals between 22.6–34.1 ppm were accounted for methylenes and one signal at 14.1 ppm, for methyl were observed. Chemical shift at 130.2 and 128.0 ppm were due to olefinic carbons. The signals at 61.2 and 52.0 ppm were due to oxygenated methine carbon and oxygenated methyl carbon.

Combining all the spectral data of SR-2, it was found to be a methyl ester of long chain fatty acid having one double bond at 6,7-position and one hydroxyl group at the four position of the molecule. So the compound SR-2 is 4-hydroxy-hexadec-6-enoic acid methyl ester. The fatty acid esters are the common compounds that are produced by endophytic fungi (Strobel, 2008).

The structure of the compound SR-2 is given below:



3.2.1.3 Characterization of the compound SR-4 by spectroscopic method

Physical properties

The compound SR-4 was deep yellow coloured and gummy in nature. It was soluble in the mixture of hexane & dichloromethane and hexane & ethyl acetate.

Infra red (IR) spectroscopy

In the IR spectrum of the compound SR-4, absorbance at 2900 cm^{-1} was for aliphatic stretching. The absorbance at 1730 cm^{-1} was well characteristic of carbonyl functional group and absorbance at 1450 cm^{-1} was for aliphatic stretching.

^{1}H NMR (400 MHz) spectroscopy

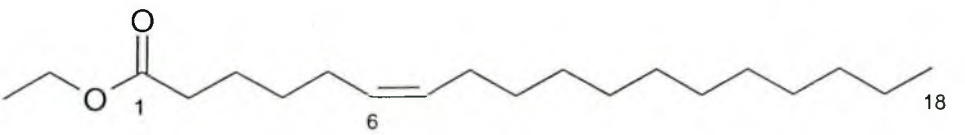
^{1}H NMR spectrum of the compound was recorded in $CDCl_3$ and TMS was used as reference. In the ^{1}H NMR spectrum the two unresolve broad signals at 5.33 and 5.25 ppm were due to the presence of two olefinic protons. A distorted triplet at 0.86 ppm (6-H) was due to two methyl groups. A broad signal at 1.24 ppm was due to severel methylene protons. An unresolve broad signal at 1.59 ppm was due to methylene proton present in the molecule. Another broad signal at 2.00 ppm was also accounted for methylene proton attached to carbonyl functional group. A multiplet at 2.30 ppm was due to methylene proton attached to double bond.

^{13}C NMR (400 MHz) spectroscopy

In the ^{13}C NMR spectrum signal at 173.3 ppm was due to ester carbonyl functional group while signals at 130.0 ppm and 129.7 ppm were due to the olefinic carbons. In the DEPT spectrum downward signal at δ_C = 62.1 ppm was due to oxygenated methylene group. In the up field region, downward signals at 22.7 to 34.1 ppm were due to several methylene carbons.

Combining all the spectral data of SR-4 it was found to be octadec-6-enoic acid ethyl ester. The fatty acids are the common compounds that are produced by endophytic fungi (Strobel, 2008).

The structure of the compound is given below:

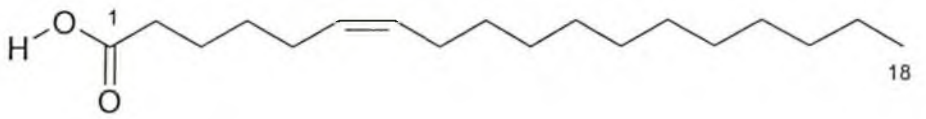


Octadec-6-enoic acid ethyl ester

3.2.1.4 Characterization of the compound SBR-1 by spectroscopic method

^{1}H NMR (400 Hz) spectroscopy

In the ^{1}H NMR spectrum the signal at 1.55 ppm was due to methylene proton. A signal at 1.24 ppm was accounted for several methylene groups in the molecule. A triplet at 0.87 ppm was due to methyl proton. Signal at 2.03 ppm was due to methine proton and signal at 3.65 ppm was due to the presence of oxygenated methine proton. Two doublets at 5.35 and 5.12 ppm were accounted for olefinic double bond. SBR-1 was octadeca-6-enoic acid.



The ^{1}H NMR spectra data of SBR-2 and SBR-3 looked very similar to SBR-1. Due to small in amount ^{13}C NMR spectrum of SBR-2 (1.5 mg) and SBR-3 (1.2 mg) were not recorded and it was not possible to determine their structures.

3.2.1.5 Characterization of the compound SR-11 by spectroscopic methods

Physical properties

The compound SR-11 was light yellow colored and solid in nature. It was soluble in dichloromethane and methanol.

Infra red (IR) spectroscopy

The IR spectrum of the compound SR-11 gave absorbance at 3350 cm^{-1} which was characteristic of hydroxyl group. Absorbance at 2920 cm^{-1} indicated the aliphatic CH stretching. The absorbance at 1715 cm^{-1} was for carbonyl functional group. Absorbance at 1450 cm^{-1} indicated the aromatic double bond (C=C). The absorbance 1260 cm^{-1} indicated the presence of oxymethyl of carbonyl group.

^{1}H NMR (400 MHz) spectroscopy

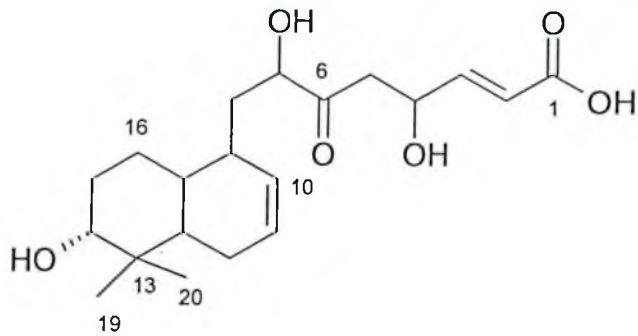
The ^{1}H NMR spectrum of the compound was recorded in $CDCl_3$ and solvent peak was used as reference. In the ^{1}H NMR spectrum signal at 1.21 ppm (6H, *s*) was due to methyl proton. One doublet of doublet of olefinic proton at 7.35 ppm (J=16 Hz, 8 Hz). Another doublet at 5.80 ppm (J=16 Hz) having large coupling constant (J=16 Hz) due to exocyclic double bond present in the molecule. Two multiplets at 5.50 and 5.22 ppm were indicated the presence of two olefinic protons in the ring system. The chemical shifts at 3.99 ppm (*m*), 4.22 ppm (*m*), 4.80 ppm (*m*) were indicated the presence of three methyne protons attached to three oxygenated carbon. An unresolved

signal at 2.40 ppm, multiplets at 2.26, 2.12, 1.90, 1.88 ppm indicated the presence of five methylene groups were present in the molecule SR-11.

^{13}C NMR spectroscopy

The ^{13}C NMR and DEPT spectrum gave eighteen signals for twenty carbons. The most down field signal at 176.0 ppm was due to carboxylic carbon. The signal at 168.0 ppm was due to carbonyl carbon. The lowfield signal at 152.5, 136.7, 130.2 and 117.1 ppm were due to the presence of four olefinic carbons. Chemical shifts at 75.6 and 72.1, and 72.0 ppm were due to the presence of oxygenated methyne protons in the compound. Three aliphatic methyne protons appear that 51.8, 44.3 and 43.0 ppm. Five methyl signals appear at 41.0, 34.0, 31.8, 29.6 and 26.6 ppm. Signal at 20.7 ppm was accounted for two methyl carbon attached to a quaternary carbon.

In the ^{1}H-^{1}H COSY spectrum strong correlation was observed between chemical shifts at 5.22 ppm with 5.50 ppm and chemical shifts at 5.80 ppm correlate with 7.35 ppm. Combining all the spectral data IR, ^{1}H- and ^{13}C NMR including DEPT, ^{1}H-^{1}H COSY, HMBC, the structure of the compound SR-11 was elucidated as the following structure.



To the best of our knowledge, the SR-11 is a new compound and is given name as terminatol by us.

3.3 Studies of endophytic fungus of *Aquilaria malaccensis* Lamk

Following the same procedure as described for *Terminalia chebula* Retz nine fungi were isolated from leaves, steams and roots of the plant *A. malaccensis* and lebelled as AL-1, AL-2, AS-1, AS-2, AS-3, AS-4, AR-1, AR-2 and AR-4. One fungal strain AL-2 was subcultured and was extracted with ethyl acetate. The TLC pattern of the extract of the AL-2 strain showed the presence of several interesting compounds. It was then fermented on large scale for chemical and biological investigation. AL-2 fungal strain was subcultured in 400 petridishes and the fungas was extracted with ethyl acetate for 24 hours, at room temperature. The ethyl acetate extract was then evaporated and 250 mg extract was obtained from 400 plates of fungus.

3.3.1 Isolation of compounds from the strain AL-2

The ethyl acetate extract of AL-2 (200 mg) was fractionated with silica gel column and sixteen fractions were obtained (**Table -13**). Among them two fractions (S-4; 3.4 mg and S-12; 11.3 mg) gave single spot in TLC and collected as pure compounds. The fraction S-16 (20 mg) gave three spots with tailing in TLC (6% methanol in dichloromethane). The TLC pattern indicated that the fraction might contain polar compounds. It was purified by HPLC in reversed phase C-18 column. HPLC profile of the sample showed two major peaks having retention time 18.00 and 19.30 min. Two major

fractions were collected and evaporated, and lebeled as S-16C (2.0 mg) and S-16D (2.0 mg).

3.3.1.1 Characterization of the compound S-4

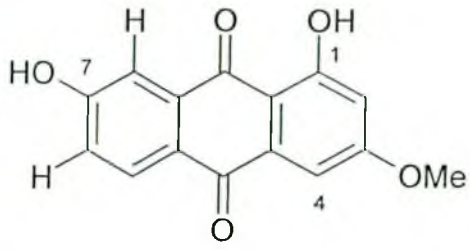
Physical properties

The compound S-4 was yellow coloured and gummy. It was soluble in dichloromethane, ethyl acetate and partially in methanol.

^{1}H NMR (400 MHz) spectroscopy

The ^{1}H NMR spectrum of the compound S-4 was recorded in $CDCl_3$ and TMS was used as reference. The ^{1}H NMR spectrum of the compound S-4 gave several signals in the aromatic region. One sharp signal at 3.94 ppm was assigned for oxygenated methyl proton. Two doublets at 7.82 (J=7.1 Hz) and 7.67 ppm (J= 7.1 Hz) indicated two aromatic protons in *o*-position coupling each other. Other three singlets in the aromatic region 7.50, 7.10, 6.97 ppm indicated that they do not have any neighbouring hydrogen. The ^{1}H NMR signals with the reported compounds of plant and fungal origin from the literature, it is evident that chemical shift at 12.11 ppm was due to the chelation of H-bonding of carbonyl group and carbonyl-oxygen and hydroxyl proton of aromatic system (Aly et al, 2008). Literature survey showed that the chemical shift of S-4 fitted is similar to macrosporin isolated from an endophytic fungus of *Ampelomyces* sp. isolated from the medicinal plant *Urospermum picroides*. Therefore structure of S-4 was confirmed as 1,7-dihydroxy-3-methoxyanthraquinone.

The structure of the compound is given below:



1, 7-dihydroxy-3-methoxyanthraquinone

3.3.1.2 Characterization of the compound S-12

Physical properties

The compound S-12 was light yellow and solid. It was soluble in dichloromethane, ethyl acetate and partially in methanol.

Infra red (IR) spectroscopy

In the IR spectrum, absorbance at 2900 cm^{-1} indicated the presence of aliphatic CH stretching. Absorbance at 1760 cm^{-1} was well characteristic of carbonyl functional group.

^{1}H NMR (400 MHz) spectroscopy

The ^{1}H NMR spectrum of the compound was recorded in $CDCl_3$ and TMS was used as reference. In the ^{1}H NMR spectrum there are several signals at

upfield region. Large signal at 1.20 ppm was accounted for five methylene groups in the compound. The signal at 2.25 ppm was due to one methylene group attached to carbonyl group. Other two broad signals at 1.97 and 1.56 ppm were accounted for two methylene groups attached to two olefinic carbons. Triplet at 0.85 ppm having *J* value 7.6 Hz was due to a methyl group attached to methylene group ($-CH_2$). Multiplet at 5.29 ppm was accounted for two olefinic protons.

^{13}C NMR spectroscopy

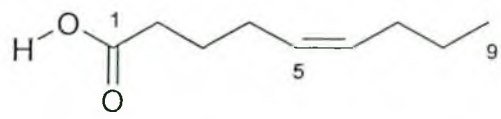
In the ^{13}C NMR spectrum the most downfield signal at 179.1 ppm was due to carboxylic carbon. Two small signals at 132.0 and 129.0 ppm were due to two olefinic carbons. The most upfield signals at 14.1 was due to terminal methyl group present in S-12. Other two signal at 22.7 and 24.7 ppm were due to two methylene carbons.

Signal at 32.0 and 29.7 were due to two methylene carbon closed to olefinic carbon. Other three signals at 29.5, 29.4 and 29.0 were accounted for three methylene groups in the compound. Signal at 34.0 was due to a carbon attached to carbonyl carbons.

Chemical shifts at 0.85 ppm was due to a methyl proton attached to methylene group correlate with 1.20 ppm which was due to methylene groups. Chemical shifts at 1.20 ppm correlate with 1.56 ppm which was due to methylene proton attached to olefinic carbons.

Combining the physical properties, absorbances in IR spectrum, signals of ^{1}H-, and ^{13}C-NMR including DEPT and ^{1}H-^{1}H COSY, S-12 was found to be

nona-5-enoic acid. The fatty acids are the common compounds that are produced by endophytic fungi (Strobel, 2008).



3.3.1.3 Characterization of the compound S-16C

Physical properties

The compound S-16C was light yellow and solid. It was soluble in dichloromethane with a few drop of methanol.

Infra red (IR) spectroscopy

In IR spectrum, the absorbance at 2900 cm^{-1} indicated the aliphatic CH stretching. Absorbance at 1650 indicated the presence of unsaturated double bond (C=C).

^{1}H NMR (400 MHz) spectroscopy

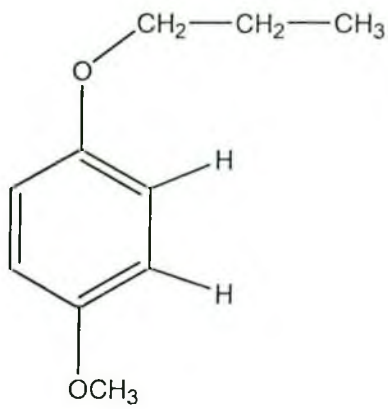
The ^{1}H NMR spectra of S-16C was taken using mixture of $CDCl_3$ & CD_3OD and solvent peak was taken as internal standard.

In ^{1}H NMR spectrum two most downfield signal at 7.45 (2H, *J*=8.4 Hz) and 6.57 ppm (2H, *J*=8.4 Hz) showed the presence of *p*-disubstituted benzene ring in S-16C. Two sets of doublet at 4.75 ppm (1H, *J*=6.8 Hz) and 4.62 ppm (1H, *J*=6.9 Hz) were accounted for
two protons of oxygenated methylene group. A sharp singlet (3H) at 3.70 ppm for a oxymethyl group. Triplet at 3.69 was due to two protons attached of oxygenated methylene group which was overlapped with the peak at 3.70 ppm. Chemical shift at 0.98 ppm was due to methyl proton and 1.25 ppm was due to methylene proton (multiplet).

The doublet at 6.57 and 7.45 ppm showed strong crossed peak in ^{1}H-^{1}H COSY spectrum. Strong correlation of chemical shift at 0.98 ppm (triplet) with 1.25 ppm (multiplet).

Combining IR, ^{1}H-, ^{1}H-^{1}H COSY spectrum S-16C was determined as 4-methoxy propyl benzyl ether. Although it is a common compound isolated from endophytic fungi but it is uncommon for the plant.

The structure of the compound is given below:



4-Methoxy propyl benzyl ether

3.3.1.4 Characterization of the compound S-16D

Physical properties

The compound S-16D was off-white and solid. It was soluble in dichloromethane.

Infra red (IR) spectroscopy

In the IR spectrum , absorbance at 1640 cm^{-1} indicated for carbonyl carbon and 1490 cm^{-1} was for aromatic ring.

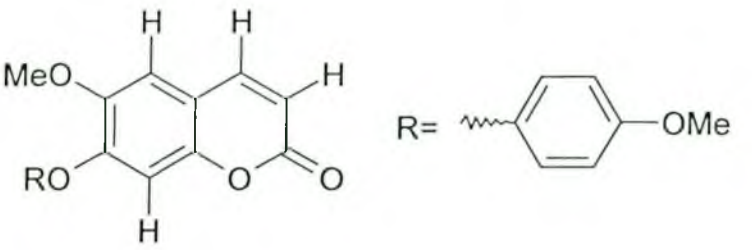
^{1}H NMR (400 MHz) spectroscopy

^{1}H NMR spectrum of the compound was recorded in $CDCl_3$ and solvent peak was taken as internal standard. In the ^{1}H NMR spectrum, the compound S-16D has several downfield signals between 6.0 to 8.5 ppm. Two doublets at 7.30 ppm (J=7.4 Hz) and 6.97 ppm (J= 7.7 Hz) were due to *p*-substituted aromatic proton. Two sharp singlets at 3.79 (3H), 3.64 (3H) were due to two oxymethyl groups attached to aromatic moiety. The sharp singlet (1H) at 8.42 ppm was assigned due to one aromatic proton which had no neighbouring hydrogen. A multiplet at 7.10 ppm and another doublet at 7.57 ppm (J=7.7 Hz) were due to two aromatic protons. A broad signal at 6.60 ppm was due to an aromatic proton.

The multiplet at 7.10 and doublet at 7.57 ppm showed crossed peak in ^{1}H-^{1}H COSY spectrum. Chemical shifts at 6.97 ppm correlated with the chemical shift at 7.30 ppm.

The extensive literature survey was carried out to elucidate the structure of the compound. Combining spectroscopic data (IR, NMR spectrum and ^{1}H-^{1}H COSY) and comparing NMR spectral data of the reported compound the structure of the compound S-16D was elucidate as substituted scopoletin.

The structure of the compound is given below:



substituted scopoletin

3.4 Biological studies of the ethyl acetate extract of endophytic fungi

3.4.1 Antibacterial activity

The ethyl acetate extract of the fungal strain IR-6, isolated from *Terminalia chebula* and the strain AL-2, isolated from *Aquilaria malaccensis* were used to access the antibacterial activity. The specific concentration of the extract of IR-6 and AL-2 were 120 and 200 µg/µL respectively. Sterile filter paper disc of 6 mm in diameter were loaded with 200/120 µg/disc using micropipette and were dried under laminar airflow hood. Standard antibiotic, *Kanamycin* (30 µg/disc) was used as a positive control. The antibacterial activities of the test agent was determined by measuring the diameter of the zone of inhibition in mm.

The antibacterial activity of ethyl acetate extract of IR-6 was performed against two gram-positive bacteria i.e. *Bacillus cereus, Staphylococcus aureus* and three gram-negative bacteria i.e. *Escherichia coli* 0157, *Shigella boydii, Pseudomonas aeruginosa.* The antibacterial activity of ethyl acetate extract of AL-2 was performed against two gram-positive bacteria i.e. *Bacillus cereus* and *Staphylococcus aureus* and five gram-negative bacteria i.e. *Escherichia coli* 0157, *Salmonella typhi, Shigella boydii, Pseudomonas aeruginosa* and *Vibrio cholerae.*

The ethyl acetate extract of the strain IR-6 did not show any activity. The extract of the strain AL-2 showed mild activity against two gram-positive bacteria i.e. *Bacillus cereus* and *Staphylococcus aureus.*

3.4.2 Brine shrimp lethality Assay

The ethyl acetate extract of the fungal strain IR-6, isolated from *Terminalia chebula* and the strain AL-2, isolated from *Aquilaria malaccensis* were tested for general toxicty against brine shrimp lethality assay as described in Section. Although both extracts showed low activities against brine shrime assay, IR-6 is more toxic than AL-2.

REFERENCES

References

Ainsworth, G.C.,"Introduction and keys to higher *texa.* In *the Fungi*: An Advanced Treatise", 1973, IV**B**, 1-7.

Aly, A. H., Edrada-Ebel, R.A., Wray, V., Müller, W.E.G., Kozytska, S., Hentschel, U., proksch, P., Ebel, R., Bioactive metabolites from the endophytic fungus *Ampelomyces* sp. isolated from the medicinal plant *Urospermum picroides*; Phytochemistry 2008, **69**, 1716-1725.

Anke, T. and Erkel, G., In *The Mycota.* 2002, Vol.**X**. *Industrial Application*, 93-108.

Arvigo, R., Balick, M., *Rainforest Remedies,* Lotus Press, Twin Lakes 1993.

Baker, D., Mocek, U., Garr, C., *Biodiversity: New Leads for Pharmaceutical and Agrochemical Industries* (Eds.: Wrigley, S.K., Hayes, M.A., Thomas, R., Chrystal, E.J.T., Nicholson, N.), The Royal Society of Chemistry, Cambridge, UK 2000, pp. 66-72.

Bhat, S.V., Bajwa, B.S., Dornauer, H., de Souza, N. J., Fehlhaber, H.W., *Tetrahedron Lett.* 1977, 1669-72.

Boursnell, J. G., The Symbiotic seed-borne fungus in the *Cistaceae, Annls Bot.*, 1950, **14,** 217-243.

Brady, S.F. and Clardy, J., *J. Nat. Prod.*, 2000, **63**, 1447.

Bugni, T.S., Ireland, C.M., Marine-derived fungi: a chemically and biologically diverse group of microorganisms. Nat. Prod. Rep., 2004. **21**, 143-146.

Calhoun, L.A., Findlay, J.A., Miller, J.D. and Whitney, N.J., *Mycol. Res.,* 1992, **96**, 281.

Carrol, G., Ecology, 1988, **69**, 2-9.

Caruso, M., Colombo, A. L., Fedeli, L., Pavesi, A., Quaroni, S., Saracchi, M. and Ventrella, G., *Ann. Microbiol*, 2000, **50**, 3.

Chang, H.-M., But, P.P.-H., *Pharmacology and Applications of Chinese Materia Medica,* World Scientific Publishing, Singapore 1986, **1** & **2**.

Chapela, I. H., Petrini, O. and Hagmann, L., *Physiol. Mol. Plant Pathol.*, 1991, **39**, 289.

Chen, G.Y., Lin, B.R., Lin, Y.C., Xie, F.C., Lu, W., Fong, W.F., A new fungicide produced by a *Streptomyces* sp. GAAS7310. J. Antibiot. 2005, **58**, 519-522.

Chen, G.Y., Lin, Y.C., Wen, L., Vrijmoed, L.L.P., Jones, E.B.G., Two new metabolites of a marine endophytic fungus (No. 1893) from an estuarine mangrove on the South China Sea Coast. Tetrahedron 2003, **59**, 4907-4909.

Choudhary, A. R., M.I. and Thomson, W., Manual of Bioassay Techniques for Natural Product Chemists, Harwood Academic Publishers, Amsterdam, 1999, pp 18-19.

Clark, A.M., *Pharm. Res.* 1996, **13**, 1133-1144.

Claydon, N., Grove, J.F. and Pople, M., Elm bark beetle boring and feeding deterrents from *Phomopsis oblonga*, Phytochemistry 1985, **24**, 937-943.

Cragg, G. M., Newman, D. J., Snader, K. M., Natural Products in drug discovery and development. *Journal of Natural Products,* 1997, **60**, 52-60.

Dev, S., *Environ. Health Perspect.*1999, **107**, 783-789.

Dreyfuss, M.M. and Chapela, I.H (1994), In *The Discovery of Natural Products with Therapeutic Potential*, 49-80.

Farnsworth, N.R., Akerele, O., Bingel, A.S., Soejarto, D.D., Guo, Z., *Bull. WHO* 1985, **63**, 965-981.

Fellows, L.E., Fleet, G.W., *Alkaloidal glycosidase inhibitors from plants in Natural Products Isolation: Separation Methods for Antimicrobials, Antiviral and Enzyme inhibitors, Journal of Chromatography Library* (Eds.: Wagman, G.H., Cooper, R.), Elsevier, Amsterdam, 1989, **43**, 13.

Findlay, J. A., Buthelezi, S., Lavoie, R., Pena-Rodriguez, L. and Miller, J. D., *J. Nat. Prod.*, 1995, **58**, 1759.

Findlay, J. A., Buthelezi, S., Li, G., Seveck, M. and Miller, J. D., *J. Nat. Prod.*, 1997, **60**, 1214.

Findlay, J. A., Li, G. and Johnson, J. A., *Can. J. Chem.*, 1997, **75**, 716.

Findlay, J. A., Li, G., Penner, P.E. and Miller, J. D., *J. Nat. Prod.*, 1995, D.E., **58**, 197.

Ghani, A. Medicinal Plants of Bangladesh with Chemical Constituents and Uses, Second Edition, 2003, Asiatic Society of Bangladesh, pp. 103, 408.

Grabley, S., Thiericke, R., *Drug Discovery from Nature* (Eds.: Grabley, S., Thiericke, R.), Springer-Verlag, Berlin 1999, pp. 3-33.

Grifo, F., Newman, D., Fairfield, A.S., Bhattacharya, B., Grupenhoff, J.T., *The Origins of Prescription Drugs* (Eds.: Grifo, F., Rosenthal, J.), Island Press, Washington, D.C. 1997, p.131.

Guo, B., Dai, J.-R., Ng, S., Y. Huang, Y., Leong, C., Ong, W. and Karte, B. K., *J. Nat. Prod.*, 2000, **63**, 602.

Hamburger, M., Marston, A., Hostettmann, K., *Adv. Drug. Res.* 1991, **20**, 167-215.

Hawksworth, D.L., *Mycological Research*, 2001, **105**, 1422-1431.

Horn, W. S., Simmonds, M. S. J., Schwartz, R. E. and Blaney, W. M., *Mycologia*, 1996, **88**, 588.

Huang, Z., Cai, X., Shao, C., She, Z., Xia, X., Chen, Y., Yang, J., Zhou, S., Lin, Y.; Chemistry and weak antimicrobial activities of phomopsins produced by mangrove endophytic fungus *Phomopsis* sp. ZSU-H76 ; Phytochemistry 2008, **69**, issue 7, 1604-1608.

Ju, Y., Sacalis, J. N. and Still, C. C., *J. Agric. Food Chem.*, 1998, **46**, 3785.

Kapoor, L.D., *CRC Handbook of Ayurvedic Medicinal plants,* CRC press, Boca Raton 1990.

Kappor, L. D., CRC Handbook of Ayurvedic Medicinal Plants. CRC Press. Boca Raton, Florida, 1990, 416-417.

Kinghorn, A.D., *J. Nat. Prod.* 1987, **50**, 1009-1024.

Klaymann, D.L., *Science* 1985, **228**, 1049.

Koga, H., Hirai, Y., Kanda, K., Tsukiboshi, T. and Uematsu, T., Successive transmission of resistance to bluegrass webworm to perennial ryegrass and tall

fescue plants by artificial inoculation with *Acremonium* endophytes, *Japan Agricultural Reseaech Quaterly,* 1997, **31**, 109-115.

Koshino, H., Terada, S.-I., Yoshihara, T., Sakamura, S., Shimanuki, T., Sato, T. and Tajimi, A., *Phytochem.*, 1988, **27**, 1333.

Koshino, H., Yoshihara, T., Okuno, M., Sakamura, S., Tajimi, A. and Shimanuki, T., *Biosci. Biotech, Biochem.*, 1992, **56**, 1096.

Koshino, H., Yoshihara, T., Sakamura, S., Shimanuki, T., Sato, T. and Tajimi, A., *Agric. Biol. Chem.*, 1989, **53**, 2527.

Krohn, K., Michel, A., Beckman, K. and Root, N., *Abstracts of ASOMPS X,* 18-23 Nov., 2000, Dhaka, Bangladesh.

Kwon-Chang, K.J., and Bennett, J.E., *Medical Mycology*, 1992, **IX**, 102-105.

Latch, G.C.M. and Christensen, M.J., Artificial infections of grasses with endophytes, *Annals of Applied Biology,* 1985, **107**, 17-24.

Lee, J.C., Lobkovsky, E., Pliam, N.B., Strobel, G.A. and Clardy, J., *J. Org. Chem.,* 1995, **60**, 7076.

Leuchtmann, A. and Caly, K., Experimental infection of the host grasses and sedges with *Atkinsonella hypoxylon and Balansia Cyperi* (Balansiae, Clavicipitaceae), Mycologia, 1985, **80,** 192-199.

Li, J. Y., Sidhu, R. S., Ford, E. J., Long, D. M., Hess, W. M. and Strobel, G. A., *J. Ind. Microbiol. Biot.,* 1998, **20**, 259.

Li, J. Y., Strobel, G., Sidhu, R., Hess, W. M. and Ford, E. J., *Microbiology*, 1996, **142**, 2223.

Lin, Y.C., Wu, X. Y., Deng, Z.J., Wang, J., Zhou, S.N., Vrijmoed, L.L.P., Jones, E.B.G., The metabolites of the mangrove fungus *Verruculina enalia* no. 2606 from a salt lake in the Bahamas. Phytochemistry 2002, **59**, 469-471.

Lin, Y.C., Wu, X.Y., Feng, S., Jiang, G.C., Luo, J.H., Zhou, S.N., Vrijmoed, L.L.P., Jones, E.B.G., Krohn, K., Steingroever, K., Zsila, F., Five unique compounds: xyloketals from mangrove fungus *Xylaria* sp. from the South China Sea coast. J. Org. Chem. 2001, **66**, 6252-6256.

Lin, Y.C., Wu, X.Y., Feng, S., Jiang, G.C., Luo, J.H., Zhou, S.N., Vrijmoed, L.L.P., Jones, E.B.G., A novel *N*-cinnamoylcyclopeptide containing an allenic ether from the fungus *Xylaria* sp. (Strain #2508) from the South China Sea. Tetrahedron Lett. 2001a, **42**. 449-451.

Lin, Z., Zhu, T., Fang, Y., Gu, Q. Zhu, W., Polyketides from *Penicillium* sp. JP-1, an endophytic fungus associated with the mangrove plant *Aegiceras corniculatum*, Phytochemistry, 2008, **69**, 1273-1278.

Lu, H., Zou, W.X., Meng, J. C., Hu, J. and Tan, R. X., *Plant Sci.,* 2000, **151**, 67.

Macías-Rubalcava, M. L., Hernández-Bautista, B. E., Jiménez-Estrada, M., González, M. C., Glenn, A. E., Hanlin, R. T., Hernández-Ortega, S., Saucedo-García, A., Muria-González, J. M., and Anaya, A. L., Naphthoquinone spiroketal with allelochemical activity from the newly discoverd endophytic fungus *Edenia gomezpompae,* Phytochemistry, 2008, **69**, 1185-1196.

Meyer, B.N., N.R., Paum, J.E., Lacobsen, L.B., Nichols, D.E., Mc Laughlin, J.L., *Planta Medica*, 1982, **45**, 31-32.

Miles, C.O., diMena, M.E., Jacobs, S.W.L., Garthwaite, I., Lane, G.A., Pestidge, R.A., Marshal, S.L., Wilkinson, H.H, Schardl, C.L., Ball, O.J.P. and Latch, C.M., Endophytic fungi in indigenous australasian grassess associated with toxicity to livestock. *Applied and Environmental Microbiology* 1998, **64**, 601-606.

Nahar, N., Evalution of two herbal antidiabetic drug Jambadayrist and Handmade bori, M.Sc. Thesis, DU, 2007.

Newman, D.J., Cragg, G.M., Snader, K.M., *J.Nat.Prod.* 2003, **66**, 1022-1037.

Petrini, O., In *Microbial Ecology of Leaves*, Springer-Verlag, New York, 1991, 179.

Powell, R.G. and Petroski, R.J., *Nat. Toxins,* 1992, **1,** 163.

Pulici, M., Sugawara, F., Koshino, H., Okada, G., Esumi, Y., Uzawa, J. and Yoshida, S., *Phytochemistry,* 1997, **46**, 313.

Qiu, D. Y., Huang, M. J., Fang, X.H., Zhu, C. and Zhu, Z. Q., *Acta Mycol. Sinica,* 1994, **13**, 314 [in Chinese].

R. A. Davis, J. Longden, V. M. Avery and P. C. Healy; The Isolation, structure determination and cytotoxicity of the new fungal metabolite, trichodermamide C. Bioorganic & Medicinal Chemistry Letter 2008, **18**, 2836-2839.

Rukachaisirikul, V., Sommart, U., Phongpaichit, S., Sakayaroj, J., Kirtikara, K., Metabolites from the endophytic fungus *Phomopsis* sp. PSU-D15, Phytochemistry, 2008, **69**, 783-787.

Samuelsson, G., Drugs of Natural Origin, A Textbook of Pharmacognosy. 4th revised ed., Swedish Pharmaceutical Press, Stockholm, 1999.

Schardl, C.L. and Phillips, T.D., *Plant Dis.,* 1997, **81**, 430.

Schultes, R.E., Raffauf, R.F., *The Healing Forest,* Dioscorides Press, Portland 1990.

Schultz, B., Boyle, C., Draeger, S., Rommert, A. and Krohn, K., *Mycological Research,* 2002, **1069**, 996-1004.

Schultz, B., Rommert, A. –K., Dammann, U., Aust, H, -J., and Strack, D., The endophyte–host interaction: a balanced antagonism? *Mycol Res*., 1999, **105**, 1275-1283

Schulz, B., Sucker, J., Aust, H. J., Krohn, K., Ludewig, K., Jones, P.G. and Döring, D., *Mycol. Res*., 1995, **99**, 1007.

Singh, M. P., Janso, J. E., Luckman, S. W., Brady, S. F., Clardy, J., Greenstein, M. and Maiese, W. M., *J. Antibiot*., 2000, **53**, 256.

Stierle, A., Stierle, D. and Bugni, T., *J. Org. Chem.*, 1999, **64**, 5479.

Stierle, A., Strobel, G. and Stierle, D., *Science,* 1993, **260**, 214.

Stierle, A., Strobel, G., Stierle, D., Grothaus, P. and Bignami, G., *J. Nat. Prod.,* 1995, **58**, 1315.

Stone, J. K., Bacon, C.W. and White, J. F., An overview of endophytic microbes: endophytism defined. In: Bacon, C. W. and White, J.F. (eds), *Microbial Endophytes*, 2000, chap.1, pp. 3-29. Marcel Dekker: New York.

Strobel, G. A. et al., *Microbiology*, 1999, **145,** 1919-1926.

Strobel, G. A., Hess, W.M., Li, J. Y., Ford, E., Sears, J., Sidhu, R. S. and Summerell, B., *Aust. J. Bot.,* 1997, **45**, 1073.

Strobel, G. A., Rainforest Endophytes and Bioactive Products. *Crit. Rev. Biotechnol.*, 2002, **22**, 315-333.

Strobel, G. and Daisy, B., *Microbiol. Mol. Biol. Rev.*, 2003, **67**, 491-502.

Strobel, G., Daisy, B., Castillo, U., Harper, J., *J. Nat. Prod.* 2004, **67**, 257-268.

Strobel, G., Yang, X., Sears, J., Kramer, R., Sidhu, R. S. and Hess, W. M., *Microbiology,* 1996, **142**, 435.

Strobel, G.A.,Knighton, B., Kluck, K., Ren, Y., Livinghouse, T., Griffin, M., Spakowicz, D. and Sears, J., The production of myco-diesel hydrocarbons and their derivatives by the endophytic fungus *Gliocladium roseum* (NRRL 50072), Microbiology, 2008, **154**, 3319-3328.

Suffness, M., *Taxol: Science and Applications,* CRC Press, Boca Raton, Fl. 1995.

Sylvia, D. M. and Sinclair, W. A., Phenolic compounds and resistance to fungal pathogens induced in primary roots of Douglas-fir seedlings by the ectomycorrhizal fungus *Laccaria laccata. Phytopathology*, 1983, **73,** 390-397.

Tan, R.X., and Zou, W.X., "Endophytes: A reach source of functional metabolites" *Natural Product Chemistry,* 2001, **18**, 448-459.

Taxane Anticancer Agents, eds. Georg, G. I., Chen, T. T., Ojima, I. and Vyas, D. M., American Chemical Society, Washington, DC, 1994.

Wang, J., Li, G., Lu, H., Zhang. Z., Huang, Y. and Su, W., *FEMS Microbiol. Lett.,* 2000, **193**, 249.

Wani, M.C., Talor, H.L., Wall, M.E., Coggon, P., McPhail, A.T., *J. Am. Chem. Soc.* 1971, **93**, 2325-2327.

Webber, J., "A natural control of Dutch elm disease", Nature, London 1981, **292**, 449-451.

Yang, R.Y., Li, C.Y., Lin, Y.C., Peng, G.T., She, Z.G., Zhou, S.N., Lactones from a brown alga endophytic fungus (No. ZZF36) from the South China Sea and their antimicrobial activities. Bioorg. Med. Chem. Lett. 2006, **16**, 4205-4208.

Yang, X., Strobel, G., Stierle, A., Hess, W. M., Lee, J. and Clardy, J., *Plant Sci.*, 1994, **102**, 1.

Zou, W. X., Meng, J. C., Lu, H., Chen, G. X., Shi, G. X., Zhang, T. Y. and Tan, R. X., *J. Nat. Prod.*, 2000, **63**, 1529.

APPENDIX

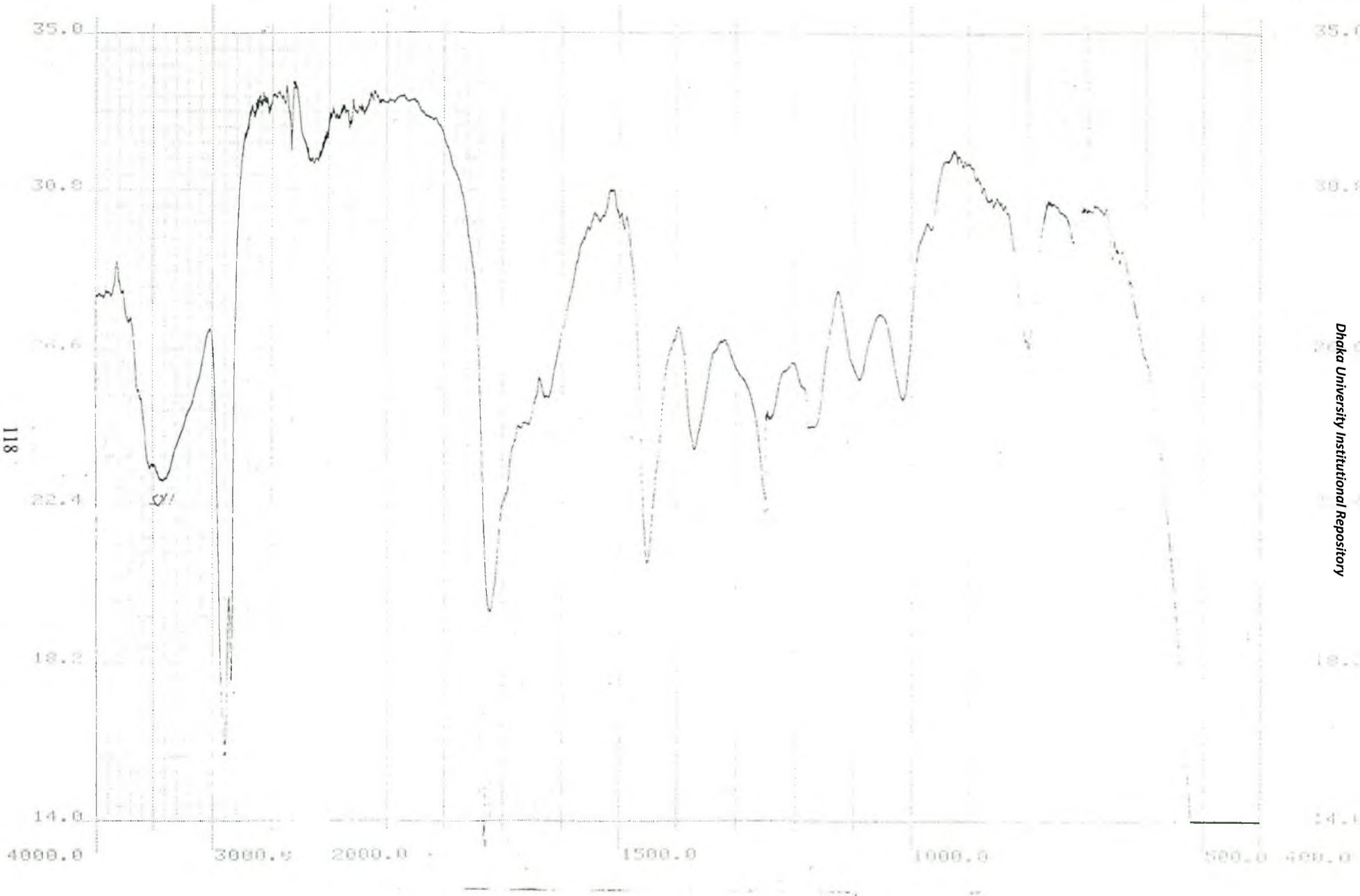


Figure-6: IR spectrum of compound SR-1

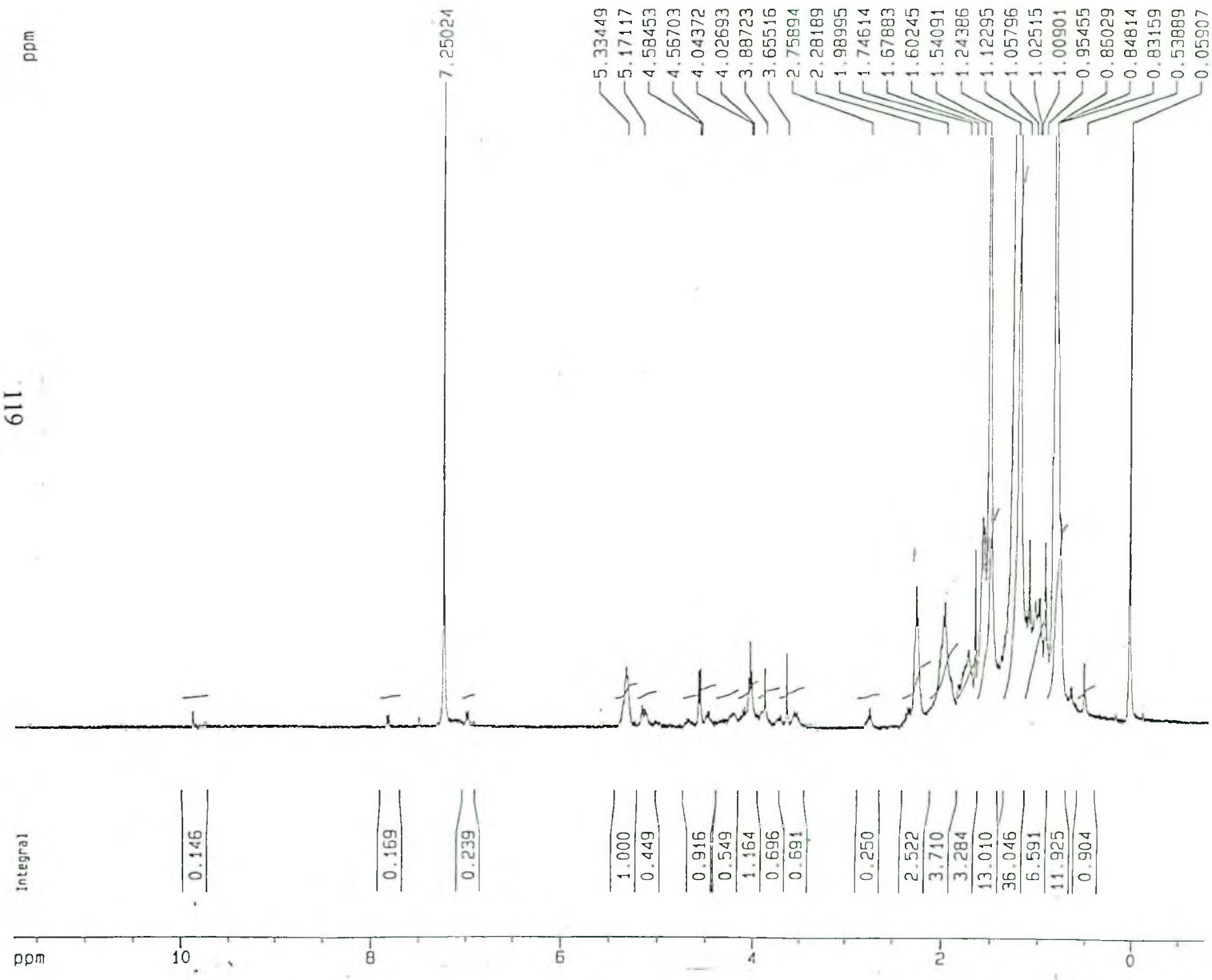


Figure-7: ^{1}H NMR spectrum of compound SR-1

Current Data Parameters	
NAME	A3048
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters	
Date_	20061010
Time	12.36
INSTRUM	dpx400
PROBHD	5 mm Multinuc
PULPROG	zg30
TD	32768
SOLVENT	Aceton
NS	128
DS	2
SWH	6410.256 Hz
FIDRES	0.195625 Hz
AQ	2.5559540 sec
RG	512
DW	78.000 usec
DE	6.00 usec
TE	310.0 K
D1	1.00000000 sec

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

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

1D NMR plot parameters	
CX	20.00 cm
F1P	11.734 ppm
F1	4695.18 Hz
F2P	-0.788 ppm
F2	-315.37 Hz
PPMCM	0.62610 ppm/cm
HZCM	250.52742 Hz/cm

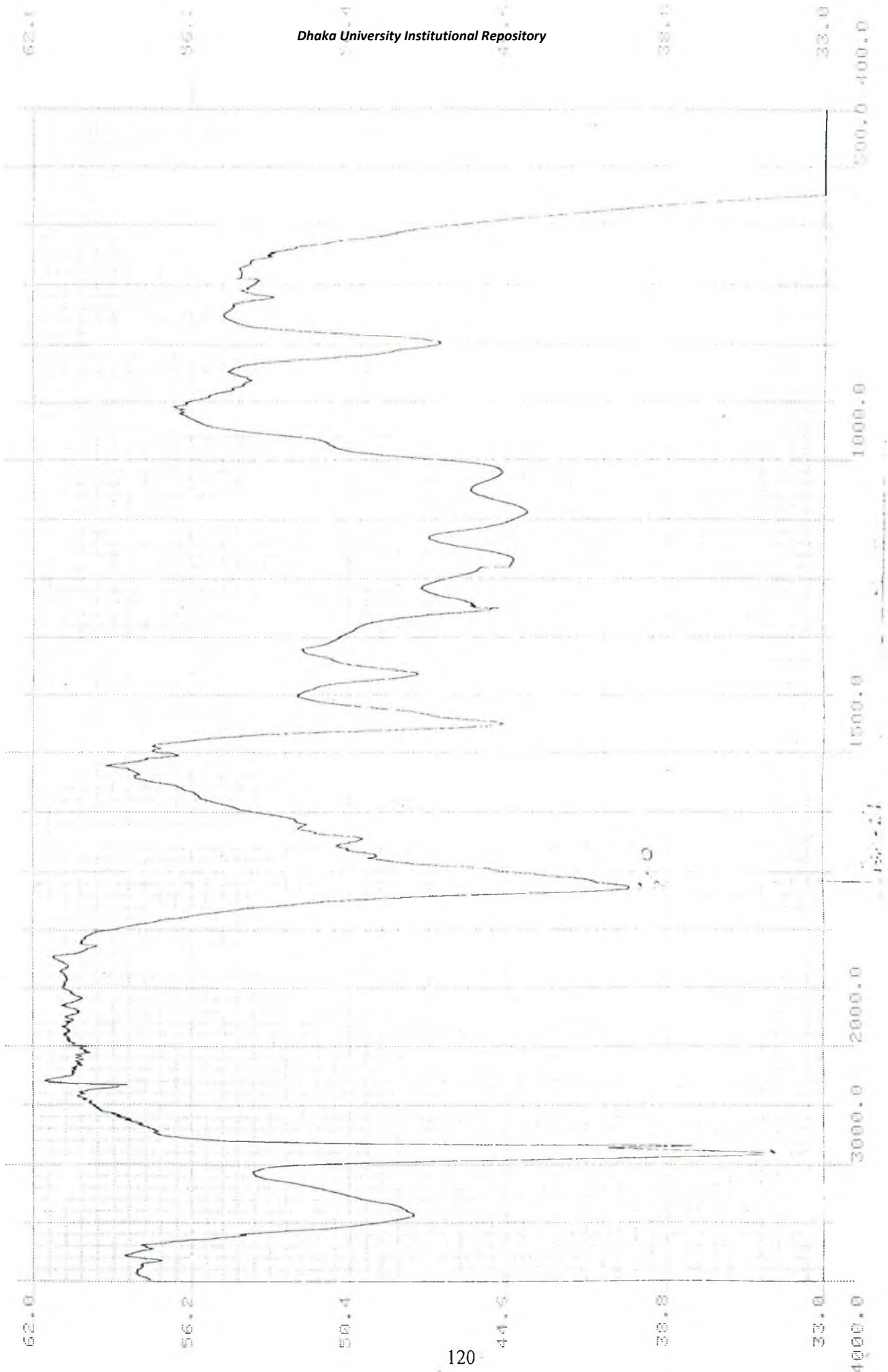


Figure-8: IR spectrum of compound SR-2

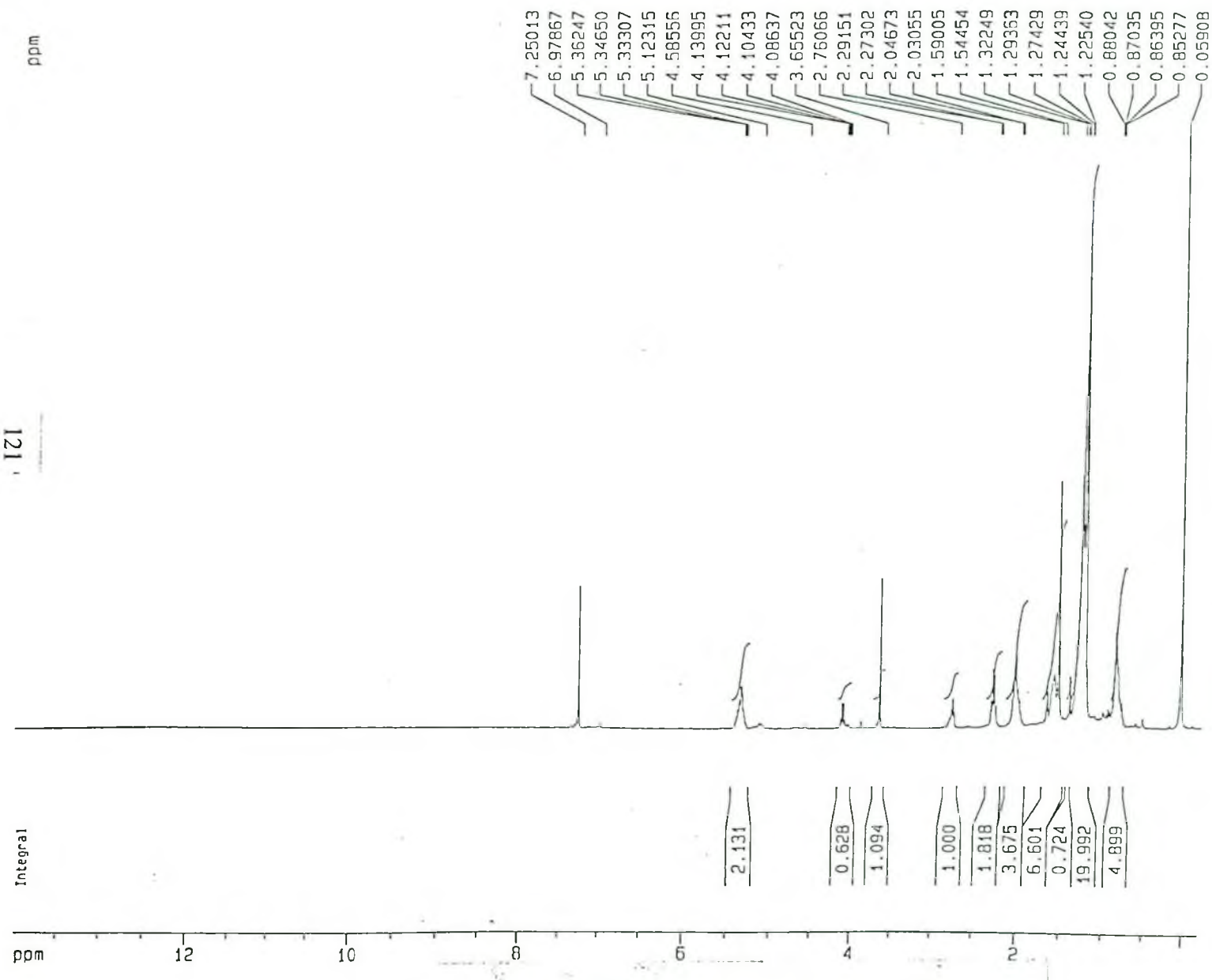


Figure-9: ^{1}H NMR spectrum of compound SR-2

Current Data Parameters	
NAME	A3049
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters	
Date_	20061010
Time	12.48
INSTRUM	dpx400
PROBHD	5 mm Multinuc
PULPROG	zg30
TD	32768
SOLVENT	Aceton
NS	128
DS	2
SWH	6410.256 Hz
FIDRES	0.195625 Hz
AQ	2.5559540 sec
RG	512
DW	78.000 usec
DE	6.00 usec
TE	310.0 K
D1	1.00000000 sec

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

F2 - Processing parameters	
SI	32768
SF	400.1400123 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.40

1D NMR plot parameters	
CX	20.00 cm
F1P	13.982 ppm
F1	5594.90 Hz
F2P	-0.213 ppm
F2	-85.07 Hz
PPMCM	0.70975 ppm/cm
HZCM	283.99869 Hz/cm

Fig- ^{1}H NMR spectroscopy of compound SR-2

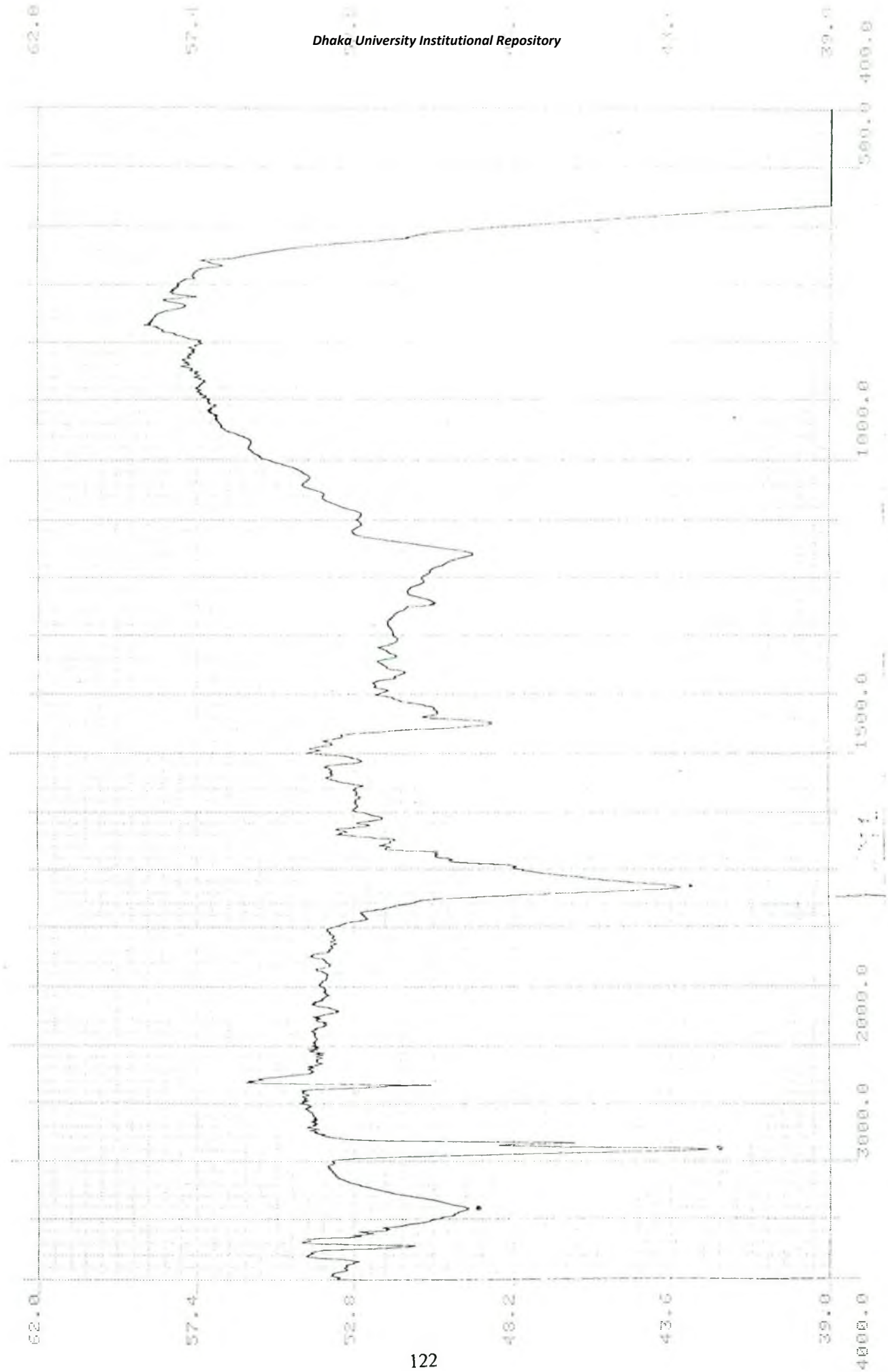
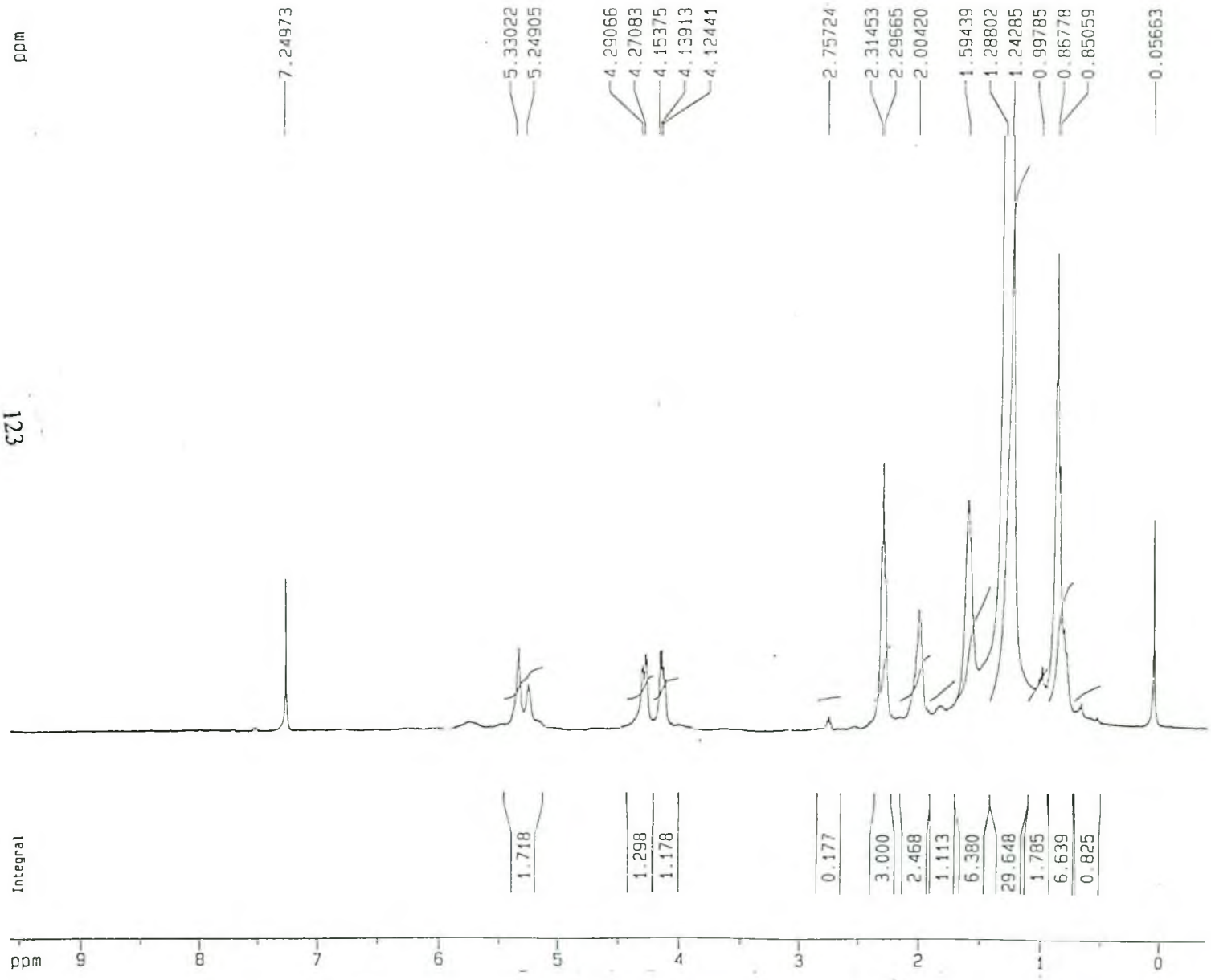


Figure-10: IR spectrum of compound SR-4



Current Data Parameters

NAME	A3475
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20070508
Time	17.29
INSTRUM	dpx400
PROBHD	5 mm Multinuc
PULPROG	zg30
TD	32768
SOLVENT	CDCl3
NS	128
DS	2
SWH	6410.256 Hz
FIDRES	0.195625 Hz
AQ	2.5559540 sec
RG	181
DW	78.000 usec
DE	6.00 usec
TE	310.0 K
D1	1.00000000 sec

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

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

F2 - Processing parameters

SI	32768
SF	400.1400124 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	9.571 ppm
F1	3829.86 Hz
F2P	-0.399 ppm
F2	-159.65 Hz
PPMCM	0.49851 ppm/cm
HZCM	199.47528 Hz/cm

Figure-11: ^{1}H NMR spectrum of compound SR-4

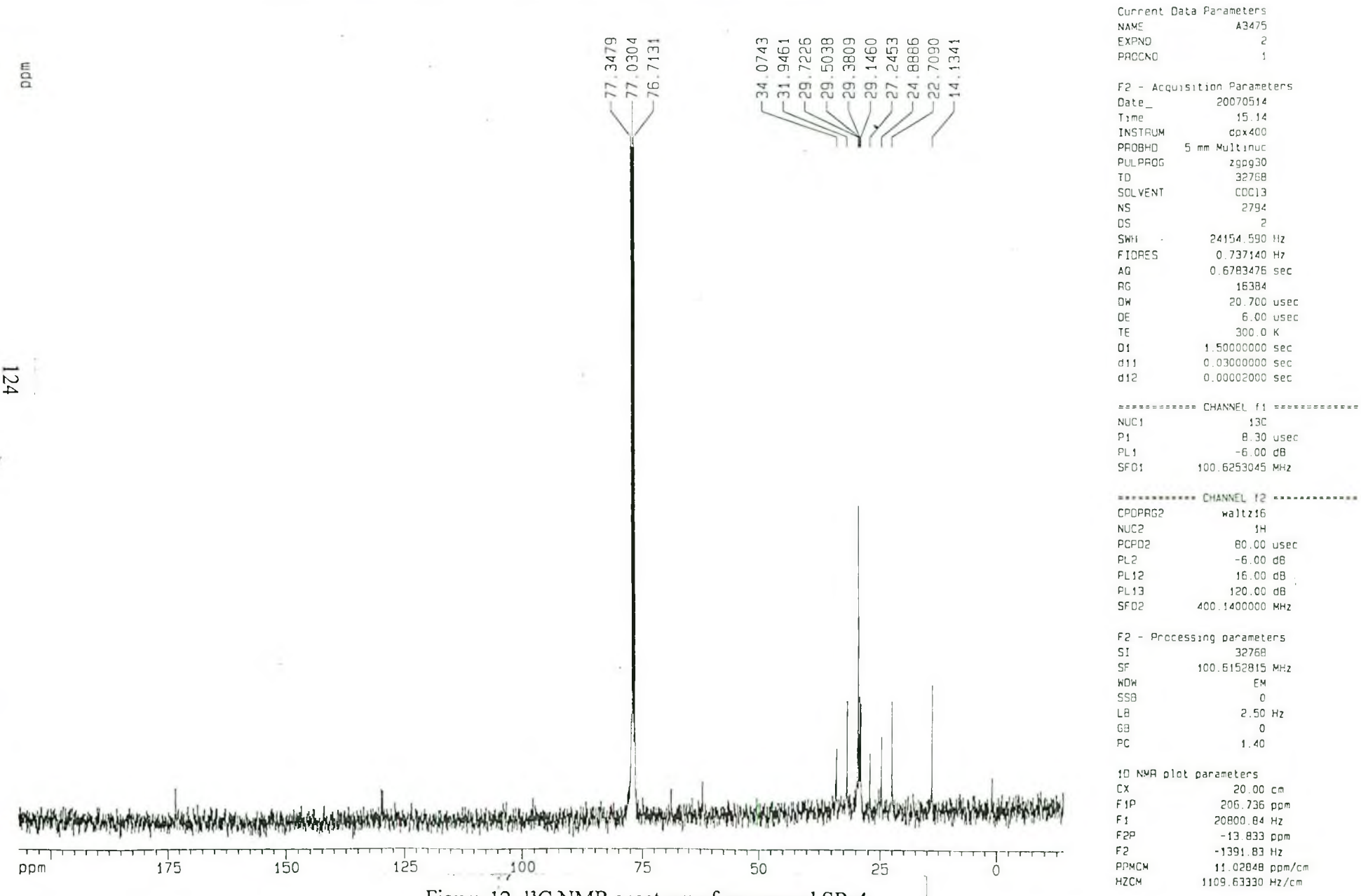
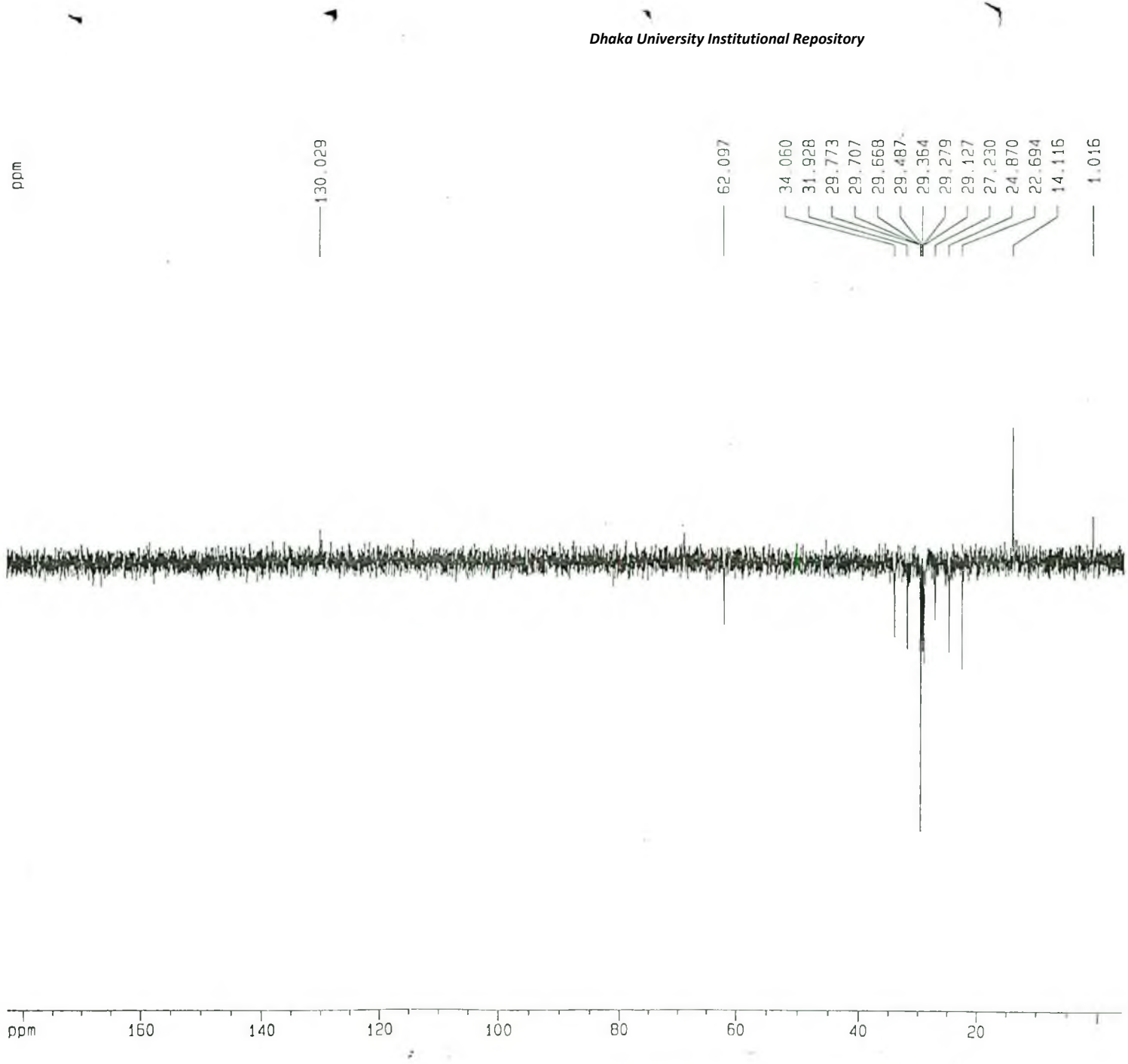
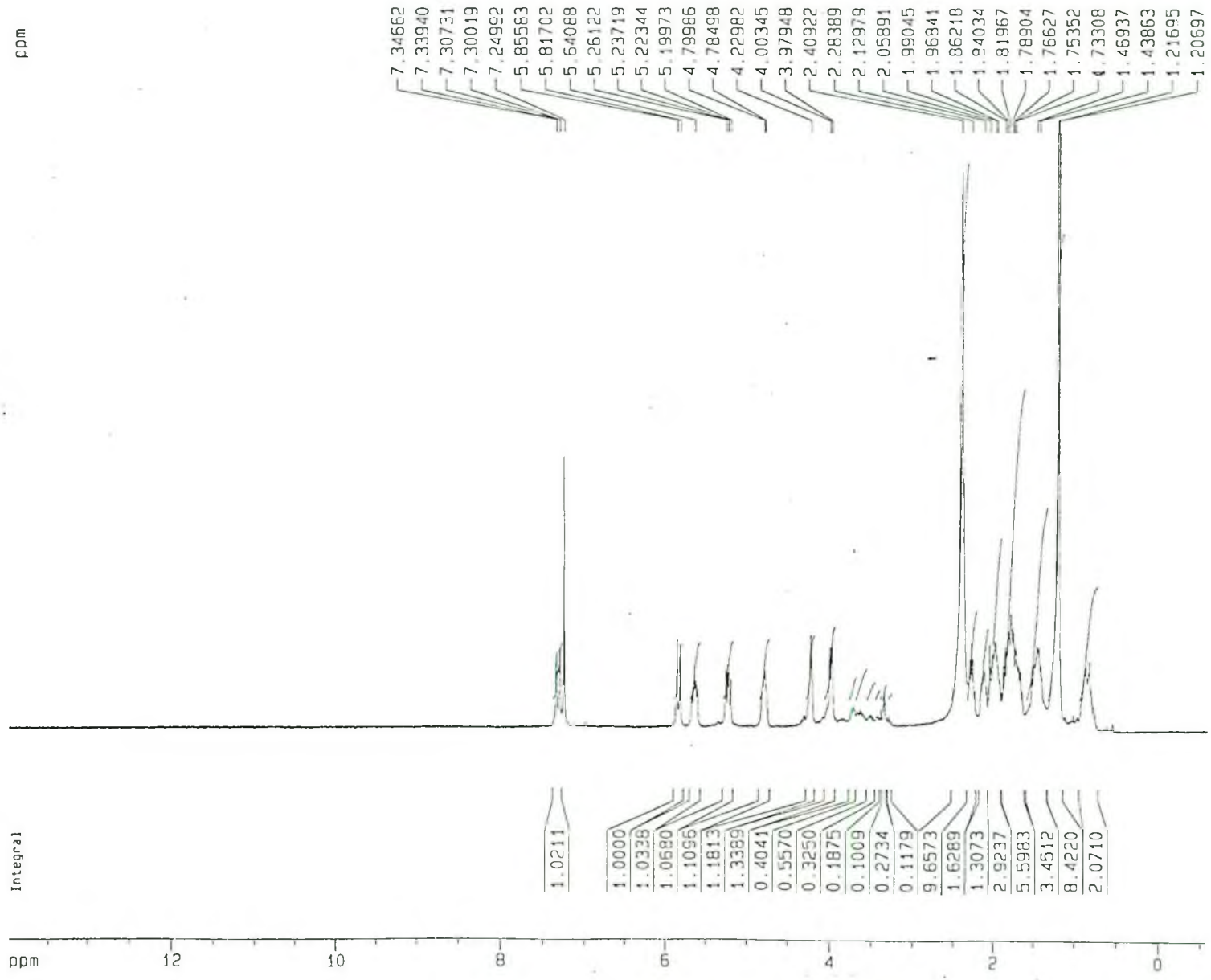


Figure-12: ^{13}C NMR spectrum of compound SR-4



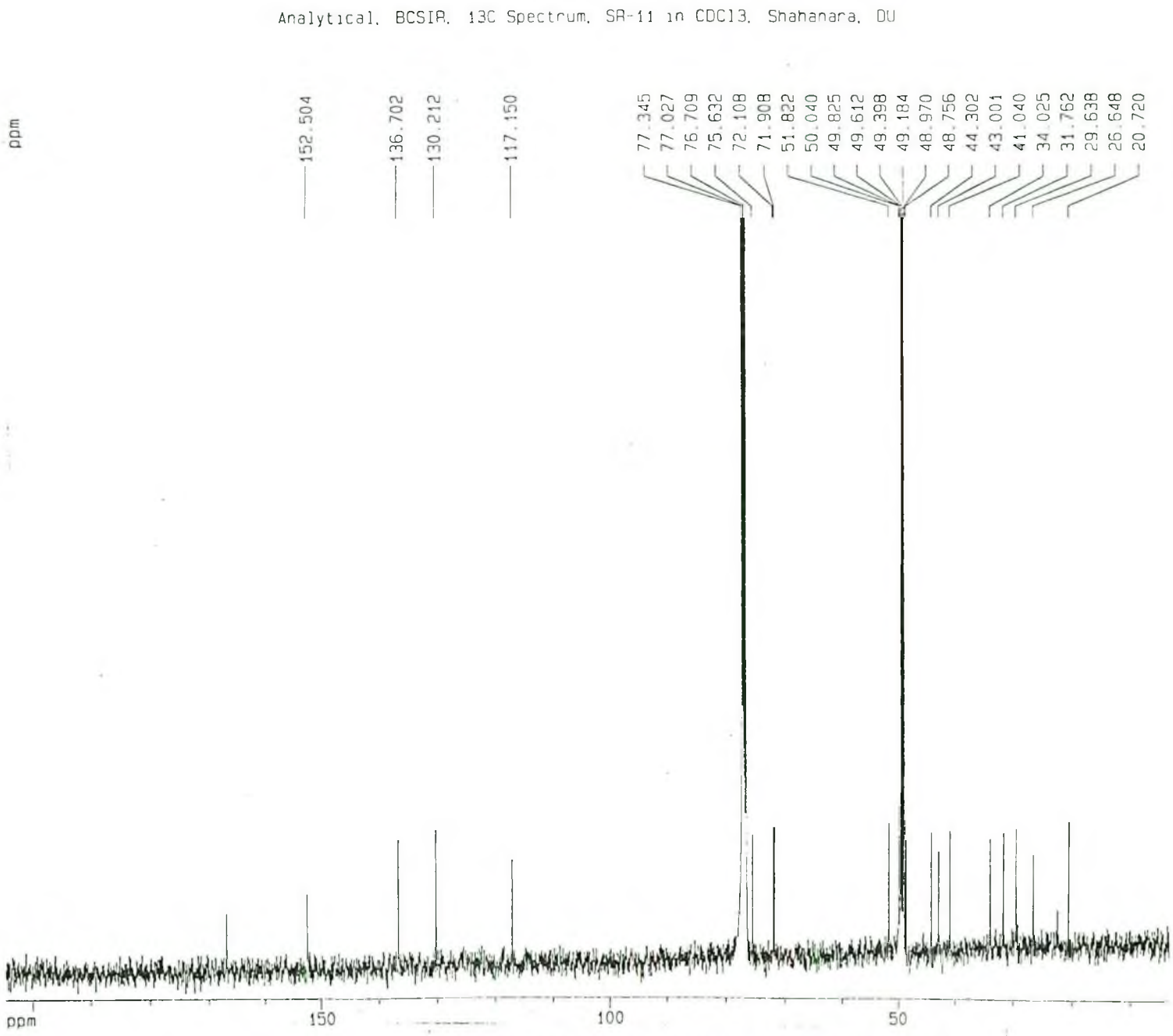

```
Current Data Parameters
NAME                  A3475
EXPNO                     3
PROCNO                    1

F2 - Acquisition Parameters
Date_              20070514
Time                  16.50
INSTRUM               dpx400
PROBHD     5 mm Multinuc
PULPROG             dept135
TD                    32768
SOLVENT               CDCl3
NS                      585
DS                        8
SWH               24154.590 Hz
FIDRES            0.737140 Hz
AQ                0.6783476 sec
RG                    13004
DW                20.700 usec
DE                 6.00 usec
TE                 300.0 K
CNST2           145.0000000
D1                4.00000000 sec
d2                0.00344828 sec
d12               0.00002000 sec
DELTA             0.00000764 sec

========== CHANNEL f1 ==========
NUC1                    13C
P1                 6.00 usec
p2                 12.00 usec
PL1                -6.00 dB
SFO1           100.6253045 MHz

========== CHANNEL f2 ==========
CPDPRG2            waltz16
NUC2                    1H
P3                 8.30 usec
p4                 16.60 usec
PCPD2              80.00 usec
PL2                -6.00 dB
PL12               16.00 dB
SFO2           400.1420007 MHz

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

1D NMR plot parameters
CX                  20.00 cm
F1P               182.662 ppm
F1                18378.60 Hz
F2P                -3.974 ppm
F2                -399.81 Hz
PPMCM              9.33179 ppm/cm
HZCM           938.92047 Hz/cm
```

Figure-13: DEPT NMR spectrum of compound SR-4




```
Current Data Parameters
NAME                  A3462
EXPNO                     2
PROCNO                    1

F2 - Acquisition Parameters
Date_              20070809
Time                 10.32
INSTRUM              dpx400
PROBHD     5 mm Multinuc
PULPROG              zg30
TD                  32768
SOLVENT             Aceton
NS                     128
DS                       2
SWH              6410.256 Hz
FIDRES           0.195625 Hz
AQ               2.5559540 sec
RG                     362
DW                78.000 usec
DE                 6.00 usec
TE                310.0 K
D1             1.00000000 sec

============ CHANNEL f1 ============
NUC1                   1H
P1                  8.30 usec
PL1                -6.00 dB
SF01         400.1428010 MHz

F2 - Processing parameters
SI                  32768
SF           400.1400128 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.40

1D NMR plot parameters
CX                 20.00 cm
F1P                13.981 ppm
F1               5594.32 Hz
F2P                -0.569 ppm
F2               -227.66 Hz
PPMCM            0.72749 ppm/cm
HZCM           291.09866 Hz/cm
```

Figure-14: ^{1}H NMR spectrum of compound SR-11



Current Data Parameters

NAME	A3462
EXPNO	3
PROCNO	1

F2 - Acquisition Parameters

Date_	20070809
Time	10.45
INSTRUM	cpx400
PROBHD	5 mm Multinuc
PULPROG	zgpg30
TD	32768
SOLVENT	CDCl3
NS	5474
DS	2
SWH	24154.590 Hz
FIDRES	0.737140 Hz
AQ	0.6783476 sec
RG	16384
DW	20.700 usec
DE	6.00 usec
TE	300.0 K
D1	1.50000000 sec
d11	0.03000000 sec
d12	0.00002000 sec

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

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

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

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

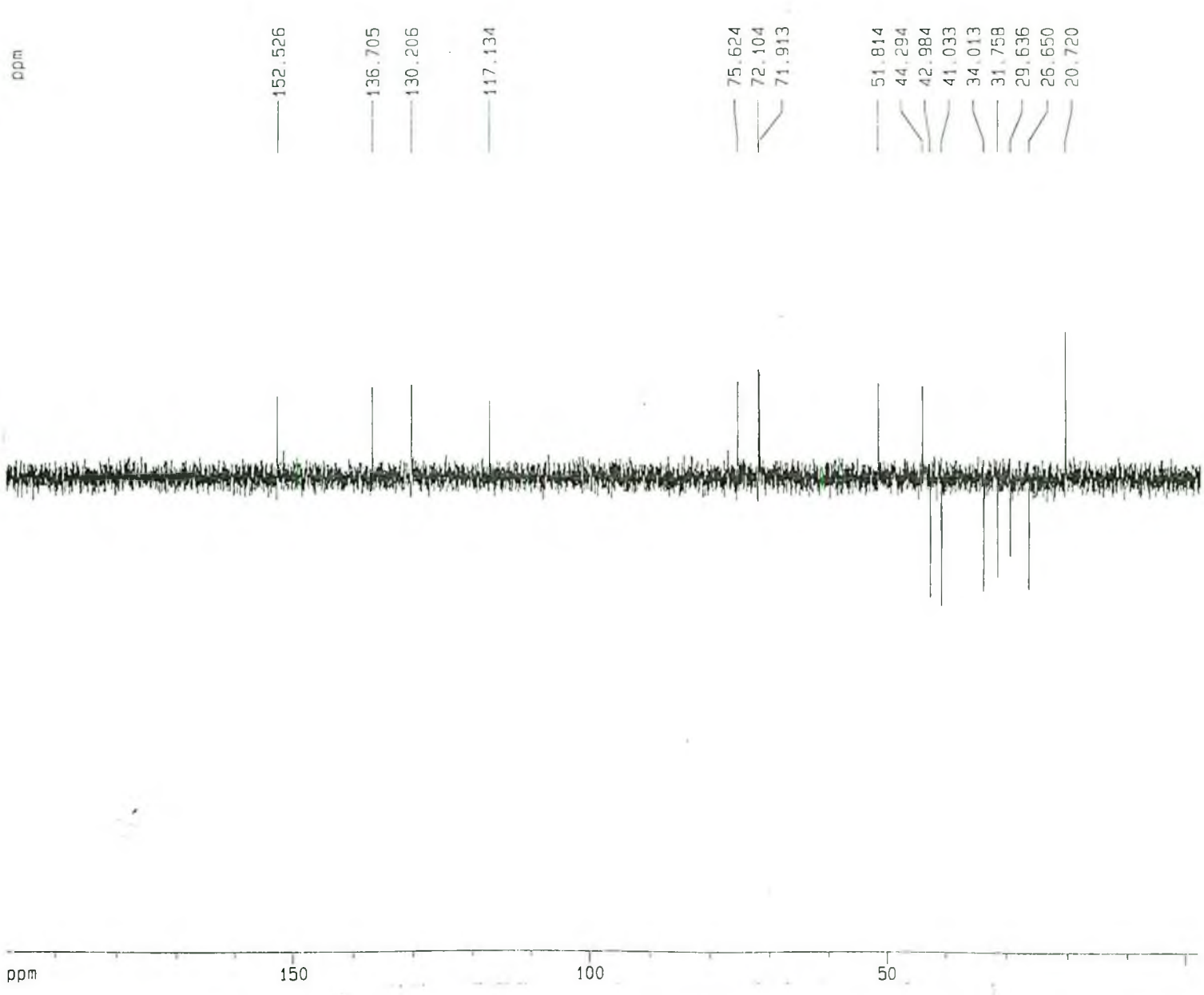
F2 - Processing parameters

SI	32768
SF	100.6152852 MHz
WDW	EM
SSB	0
LB	2.50 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	204.674 ppm
F1	20593.31 Hz
F2P	3.097 ppm
F2	311.61 Hz
PPMCM	10.07884 ppm/cm
HZCM	1014.08514 Hz/cm

Figure-15: ^{13}C NMR spectrum of compound SR-11




```
Current Data Parameters
NAME                  A3462
EXPNO                     4
PROCNO                    1

F2 - Acquisition Parameters
Date_              20070809
Time                  14.53
INSTRUM             dpx400
PROBHD     5 mm Multinuc
PULPROG           dept135
TD                  32768
SOLVENT             CDCl3
NS                    717
DS                      8
SWH             24154.590 Hz
FIDRES          0.737140 Hz
AQ              0.6783476 sec
RG                  13004
DW                20.700 usec
DE                 6.00 usec
TE                300.0 K
CNST2         145.0000000
D1              4.00000000 sec
d2              0.00344828 sec
d12             0.00002000 sec
DELTA           0.00000764 sec

============ CHANNEL f1 ============
NUC1                  13C
P1                 6.00 usec
p2                12.00 usec
PL1               -6.00 dB
SFO1          100.6253045 MHz

============ CHANNEL f2 ============
CPDPRG2          waltz16
NUC2                  1H
P3                 8.30 usec
p4                16.60 usec
PCPD2             80.00 usec
PL2               -6.00 dB
PL12              16.00 dB
SFO2          400.1420007 MHz

F2 - Processing parameters
SI                  32768
SF            100.6152852 MHz
WDW                   EM
SSB                    0
LB                 1.00 Hz
GB                     0
PC                 1.40

1D NMR plot parameters
CX                20.00 cm
F1P              199.090 ppm
F1              19930.85 Hz
F2P               -2.221 ppm
F2              -223.45 Hz
PPMCM            10.01553 ppm/cm
HZCM          1007.71527 Hz/cm
```

Figure-16: DEPT NMR spectrum of compound SR-11

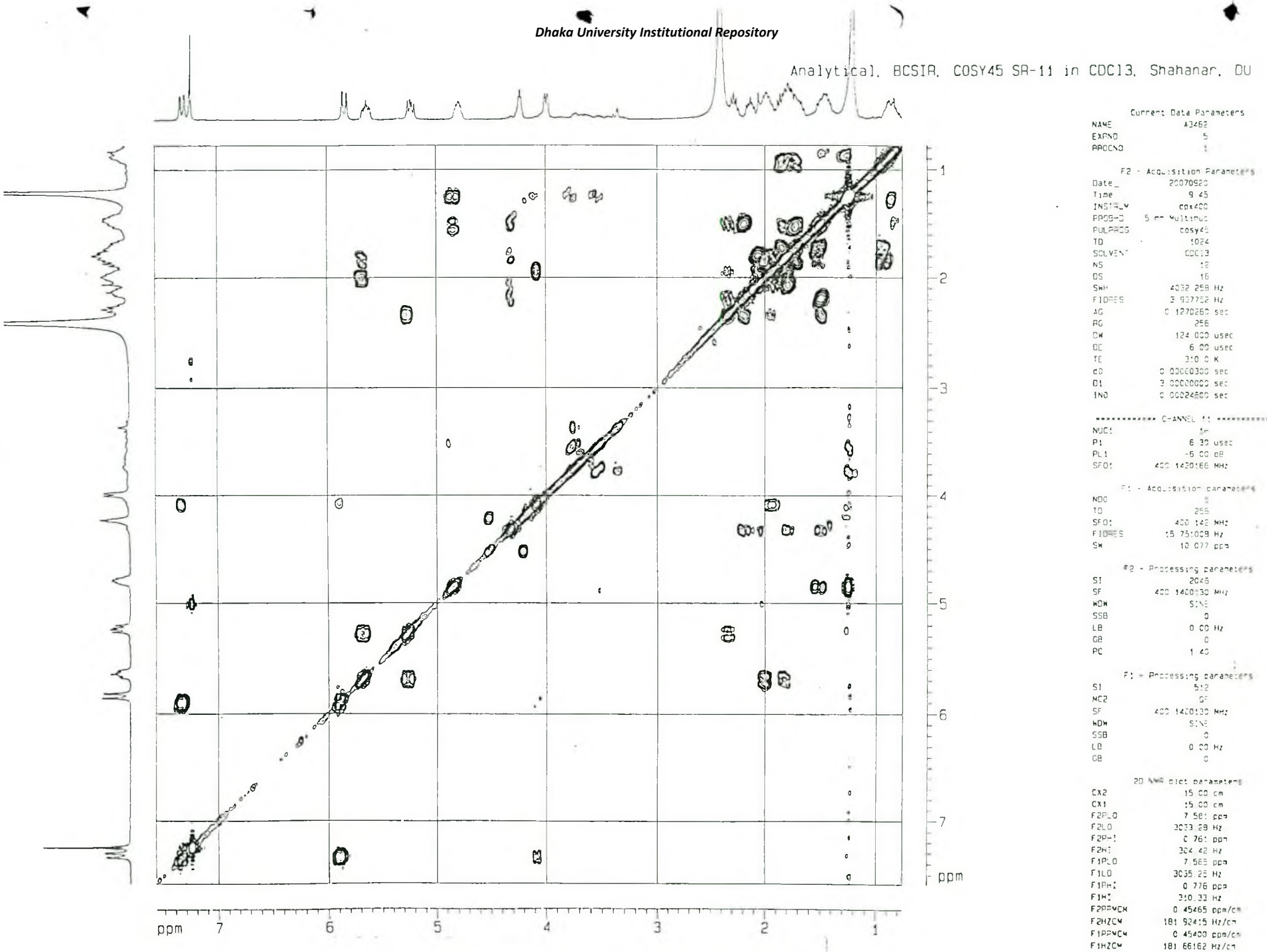


Figure-17: ^{1}H-^{1}H COSY spectrum of compound SR-11

Dr, Masih-U-Zama/Dr, Khalid/
1H/Cdcl3/S-4/

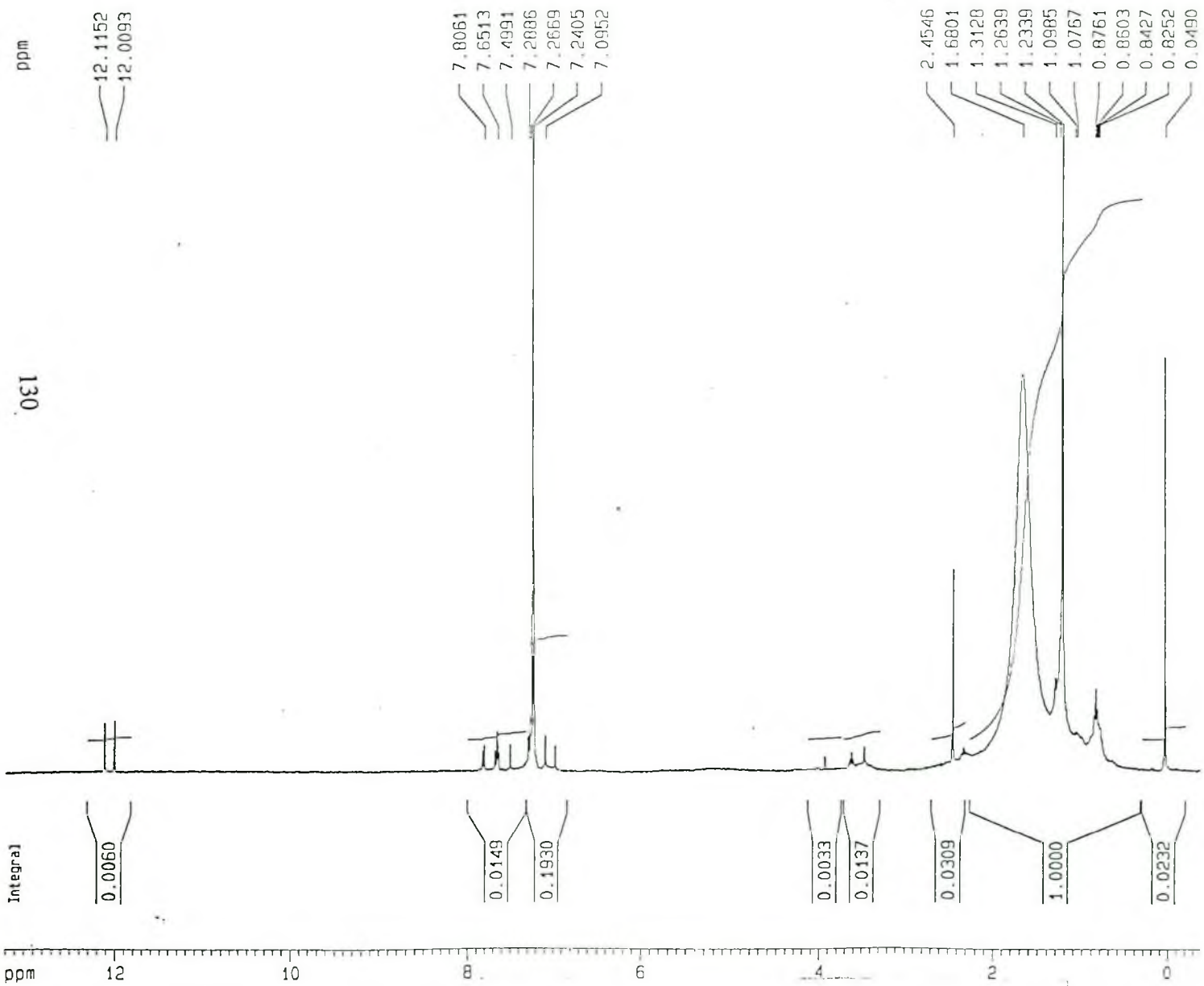


Figure-18: ^{1}H NMR spectrum of compound S-4

```
Current Data Parameters
NAME                  oct30
EXPNO                     1
PROCNO                    1

F2 - Acquisition Parameters
Date_              20071030
Time                  9.14
INSTRUM              spect
PROBHD    5 mm SEI 13C-1
PULPROG                zg
TD                  32768
SOLVENT              MeOD
NS                    128
DS                      0
SWH             7022.472 Hz
FIDRES          0.214309 Hz
AQ              2.3331316 sec
RG                 1149.4
DW                71.200 usec
DE                 7.00 usec
TE                300.0 K
D1             0.50000000 sec

======== CHANNEL f1 ========
NUC1                   1H
P1                  9.70 usec
PL1                 0.00 dB
SFO1         400.3330802 MHz

F2 - Processing parameters
SI                  16384
SF           400.3300159 MHz
WDW                    EM
SSB                     0
LB                  1.00 Hz
GB                      0
PC                   1.00

1D NMR plot parameters
CX                 20.00 cm
CY                  0.00 cm
F1P                13.208 ppm
F1                5287.48 Hz
F2P                -0.389 ppm
F2                -155.77 Hz
PPMCM           0.67985 ppm/cm
HZCM           272.16278 Hz/cm
```

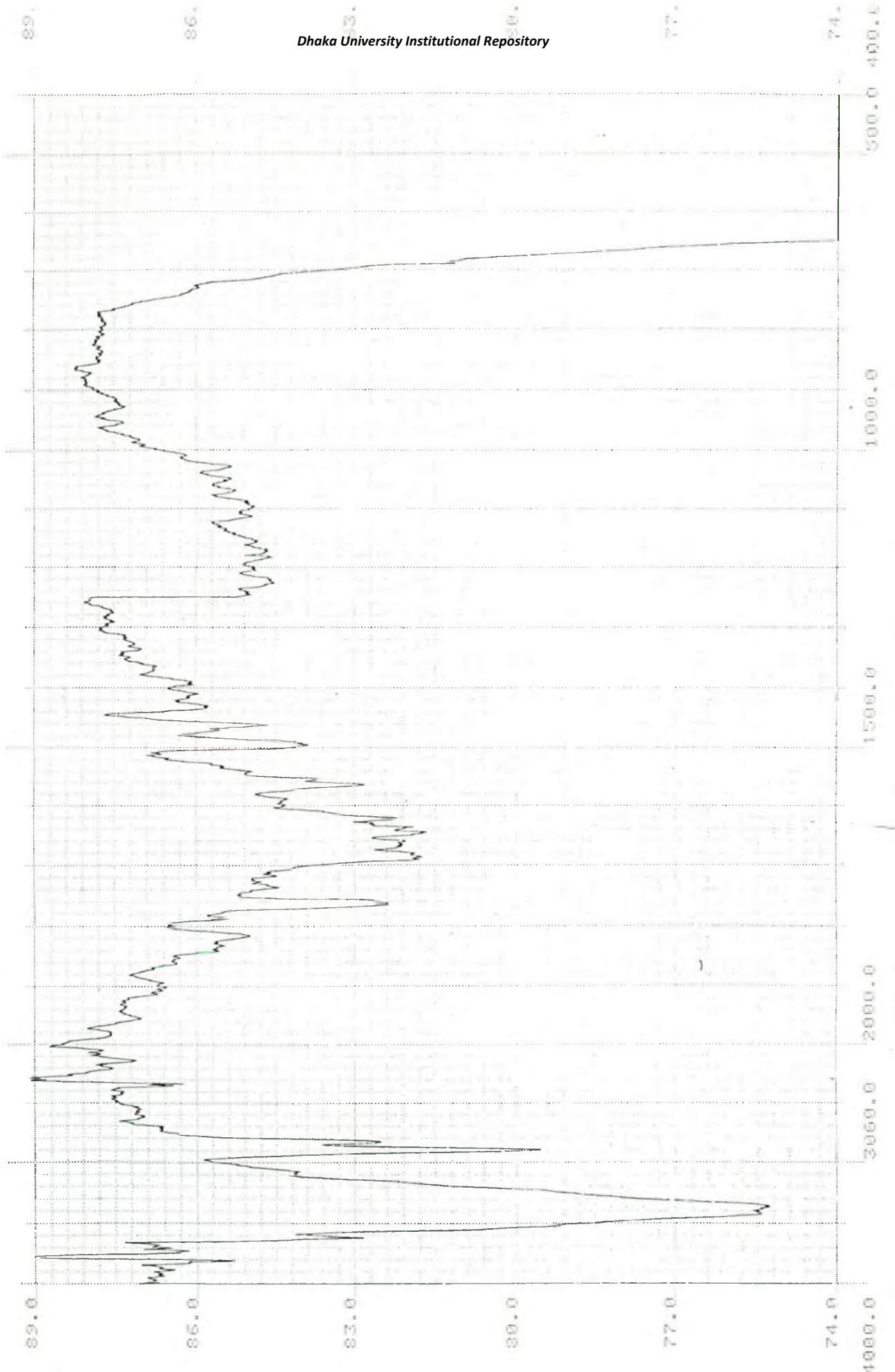
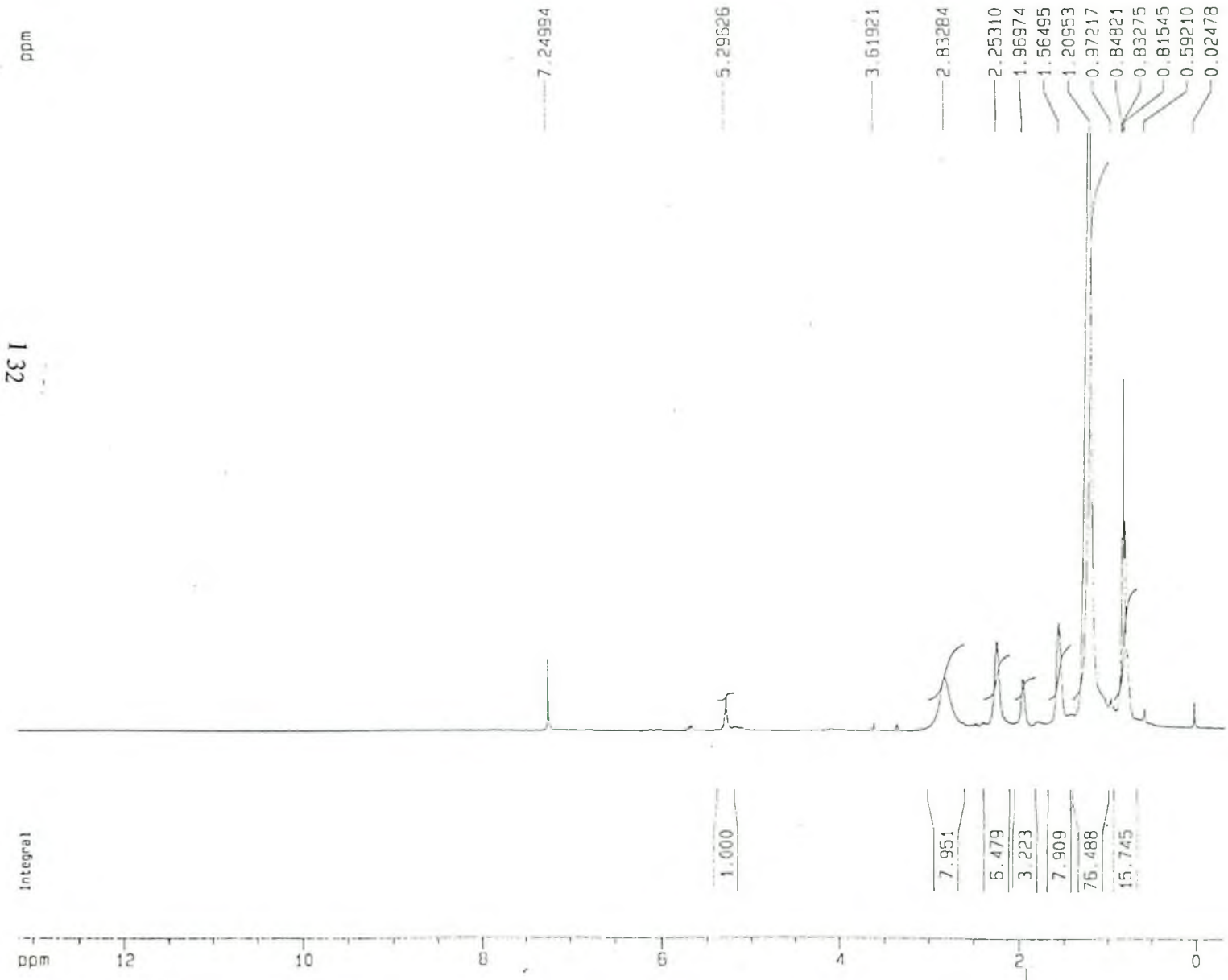


Figure-19: IR spectrum of compound S-12



Current Data Parameters

NAME	A3781
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20070822
Time	13.52
INSTRUM	dpx400
PROBHD	5 mm Multinuc
PULPROG	zg30
TD	32768
SOLVENT	CDCl3
NS	128
DS	2
SWH	6410.256 Hz
FIDRES	0.195625 Hz
AQ	2.5559540 sec
RG	128
DW	78.000 usec
DE	6.00 usec
TE	310.0 K
D1	1.00000000 sec

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

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

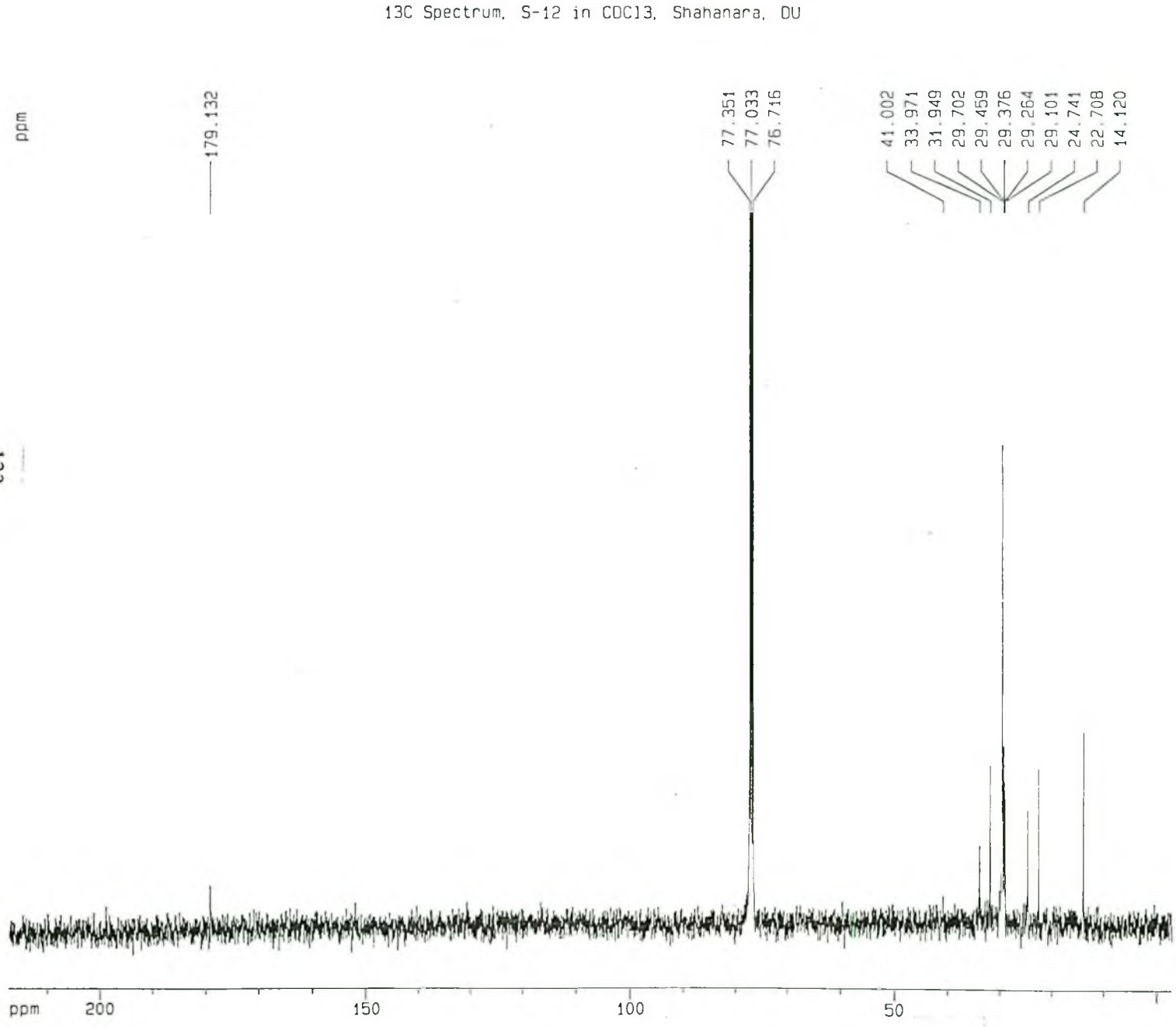
F2 - Processing parameters

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

1D NMR plot parameters

CX	20.00 cm
F1P	13.170 ppm
F1	5269.94 Hz
F2P	-0.315 ppm
F2	-126.03 Hz
PPMCM	0.67426 ppm/cm
HZCM	269.79577 Hz/cm

Figure-20: ^{1}H NMR spectrum of compound S-12



Current Data Parameters

NAME	A4328
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20080603
Time	11.22
INSTRUM	dpx400
PROBHD	5 mm Multinuc
PULPROG	zgpg30
TD	32768
SOLVENT	CDCl3
NS	1014
DS	2
SWH	24154.590 Hz
FIDRES	0.737140 Hz
AQ	0.6783476 sec
RG	16384
DW	20.700 usec
DE	6.00 usec
TE	300.0 K
D1	1.50000000 sec
d11	0.03000000 sec
d12	0.00002000 sec

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

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

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

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

F2 - Processing parameters

SI	32768
SF	100.6152801 MHz
WDW	EM
SSB	0
LB	2.50 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	217.115 ppm
F1	21845.08 Hz
F2P	-2.799 ppm
F2	-281.66 Hz
PPMCM	10.99571 ppm/cm
HZCM	1106.33667 Hz/cm

Figure-21: ^{13}C NMR spectrum of compound S-12

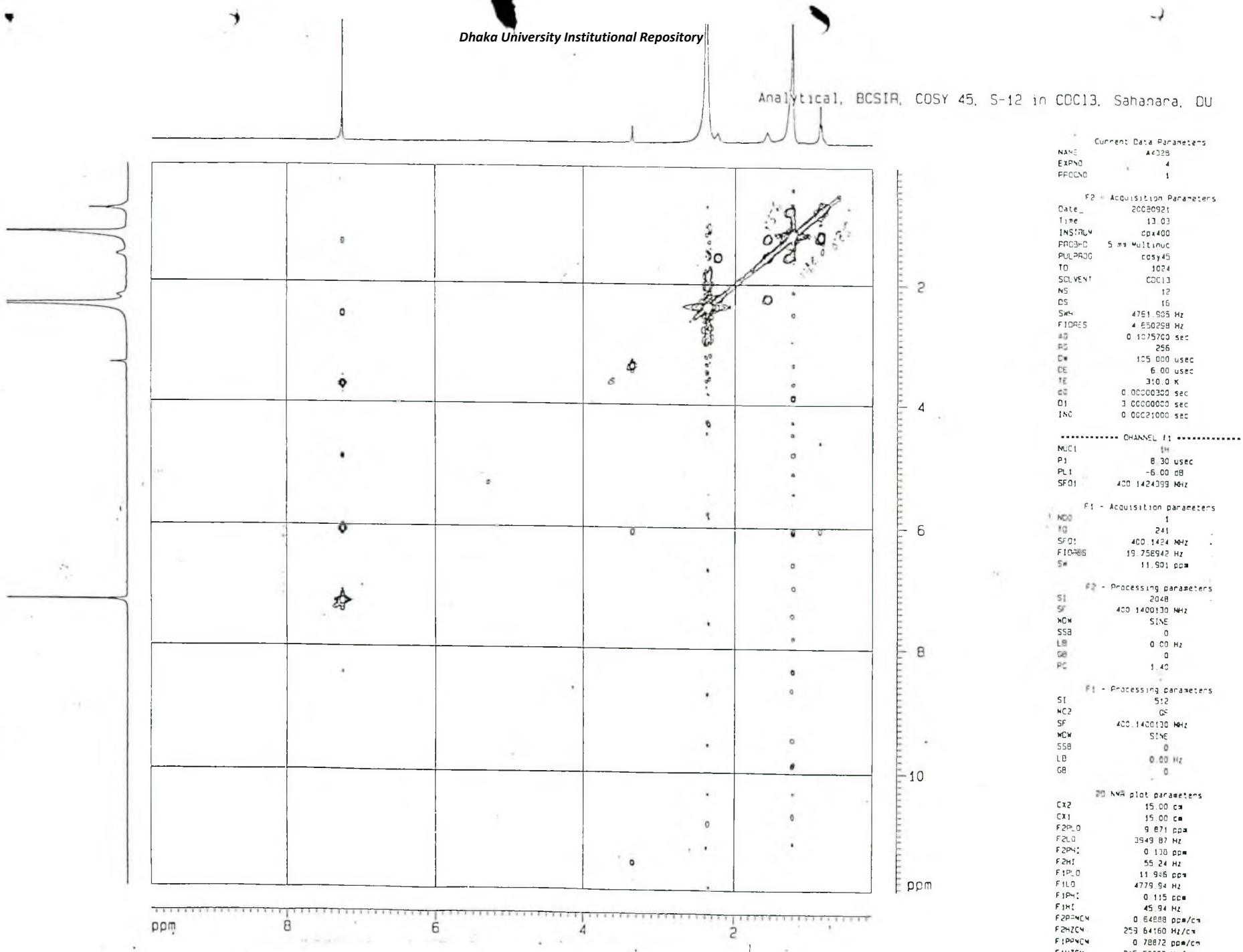


Figure-22: ^{1}H-^{1}H COSY spectrum of compound S-12

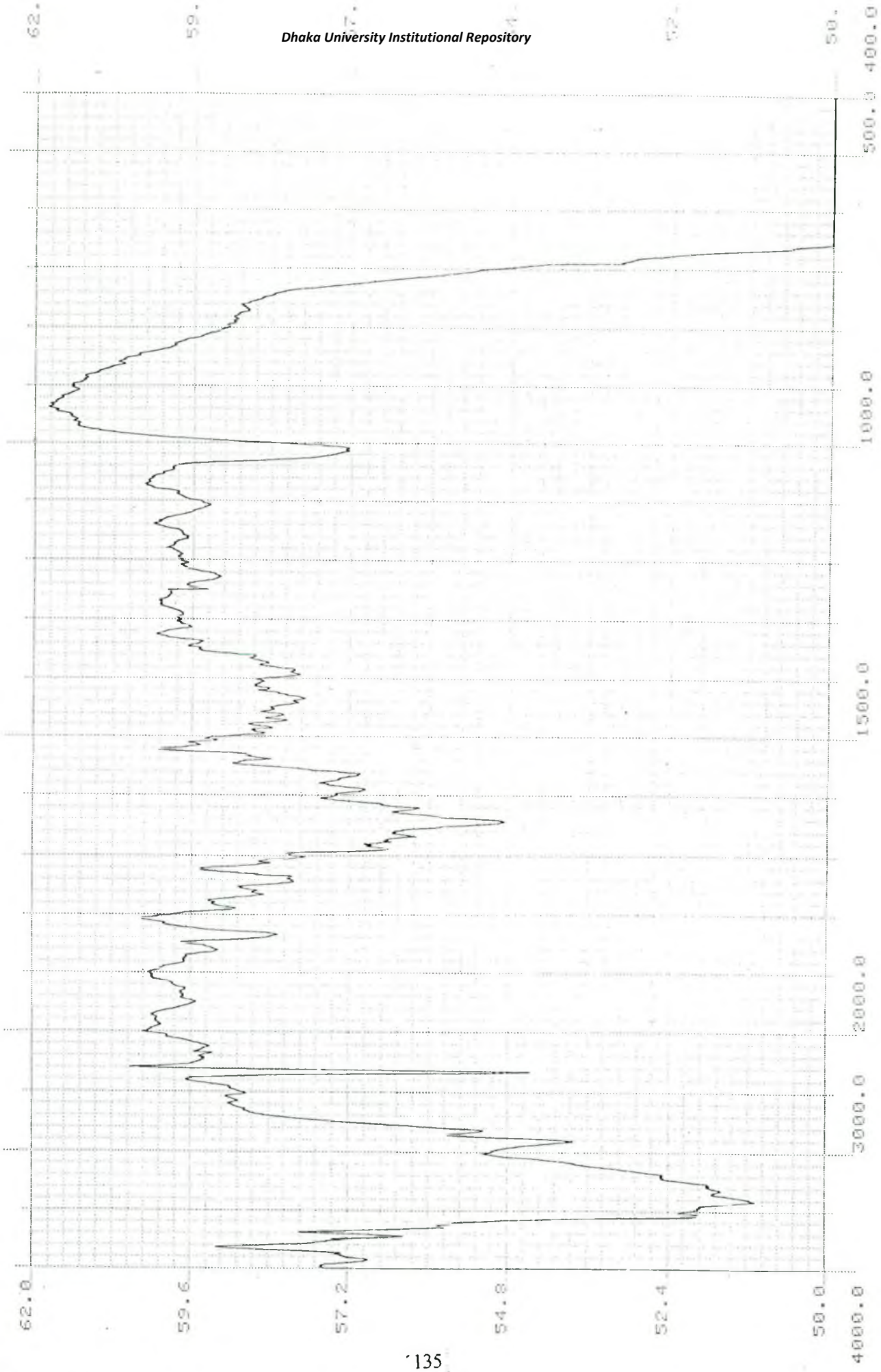
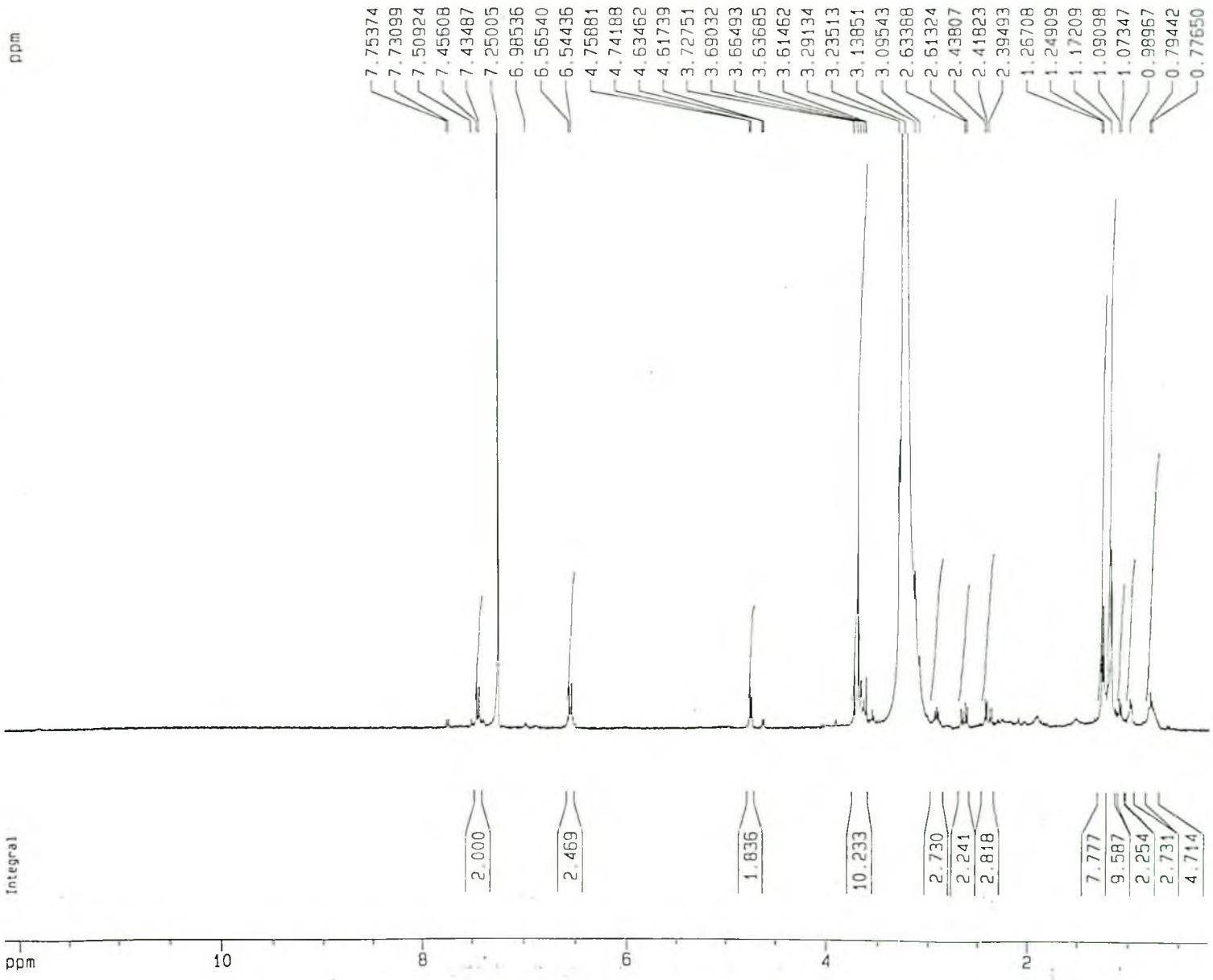


Figure-23: IR spectrum of compound S-16C



Current Data Parameters

NAME	A4227
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20080417
Time	16.32
INSTRUM	dpx400
PROBHD	5 mm Multinuc
PULPROG	zg30
TD	32768
SOLVENT	CDCl3
NS	128
DS	2
SWH	6410.256 Hz
FIDRES	0.195625 Hz
AQ	2.5559540 sec
RG	362
DW	78.000 usec
DE	6.00 usec
TE	310.0 K
D1	1.00000000 sec

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

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

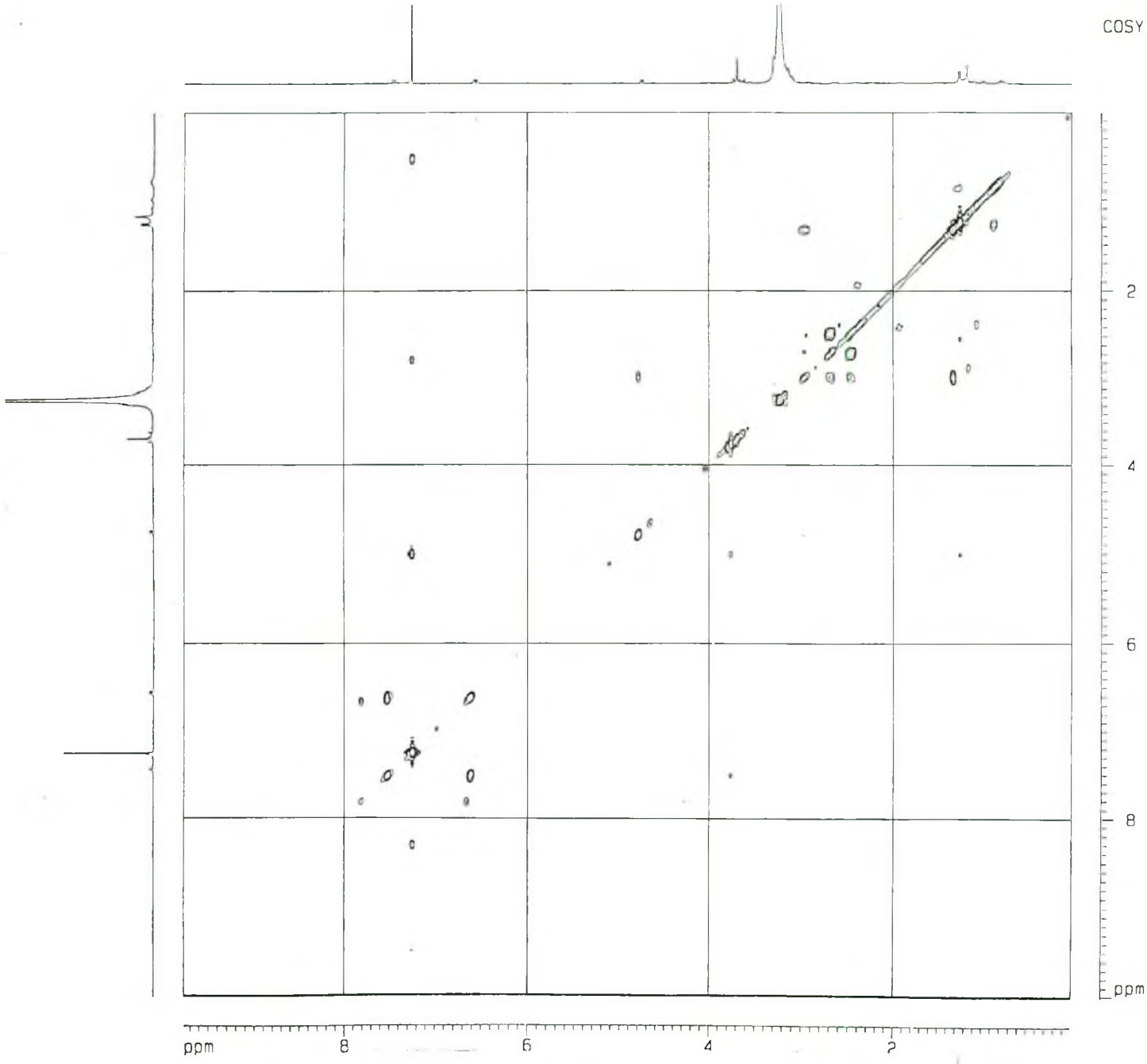
F2 - Processing parameters

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

1D NMR plot parameters

CX	20.00 cm
F1P	12.152 ppm
F1	4862.64 Hz
F2P	0.218 ppm
F2	87.31 Hz
PPMCM	0.59671 ppm/cm
HZCM	238.76674 Hz/cm

Figure-24: ^{1}H NMR spectrum of compound S-16C



COSY 45, S-16C in CDCl3, Shahanara, DU

Current Data Parameters

NAME	A4227
EXPNO	4
PROCNO	1

F2 - Acquisition Parameters

Date_	20000610
Time	16.35
INSTRUM	cpx400
PROBHD	5 mm Multinuc
PULPROG	cosy45
TD	1024
SOLVENT	Aceton
NS	12
DS	16
SWH	4005.410 Hz
FIDRES	3.912510 Hz
AQ	0.1278452 sec
RG	256
DW	124.800 usec
DE	6.00 usec
TE	310.0 K
d0	0.00000300 sec
D1	3.00000000 sec
IN0	0.00024960 sec

CHANNEL f1

NUC1	1H
P1	8.30 usec
PL1	-6.00 dB
SFO1	400.1420175 MHz

F1 - Acquisition parameters

ND0	1
TD	235
SFO1	400.142 MHz
FIDRES	17.048553 Hz
SW	10.012 ppm

F2 - Processing parameters

SI	2048
SF	400.1400130 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0
PC	1.40

F1 - Processing parameters

SI	512
MC2	QF
SF	400.1400130 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0

2D NMR plot parameters

CX2	15.00 cm
CX1	15.00 cm
F2PLO	9.771 ppm
F2LO	3909.93 Hz
F2PHI	0.013 ppm
F2HI	5.25 Hz
F1PLO	10.015 ppm
F1LO	4007.75 Hz
F1PHI	0.003 ppm
F1HI	1.34 Hz
F2PPMCM	0.65055 ppm/cm
F2HZCM	260.31235 Hz/cm
F1PPMCM	0.66750 ppm/cm
F1HZCM	267.09402 Hz/cm

Figure-25: ^{1}H-^{1}H COSY spectrum of compound S-16C

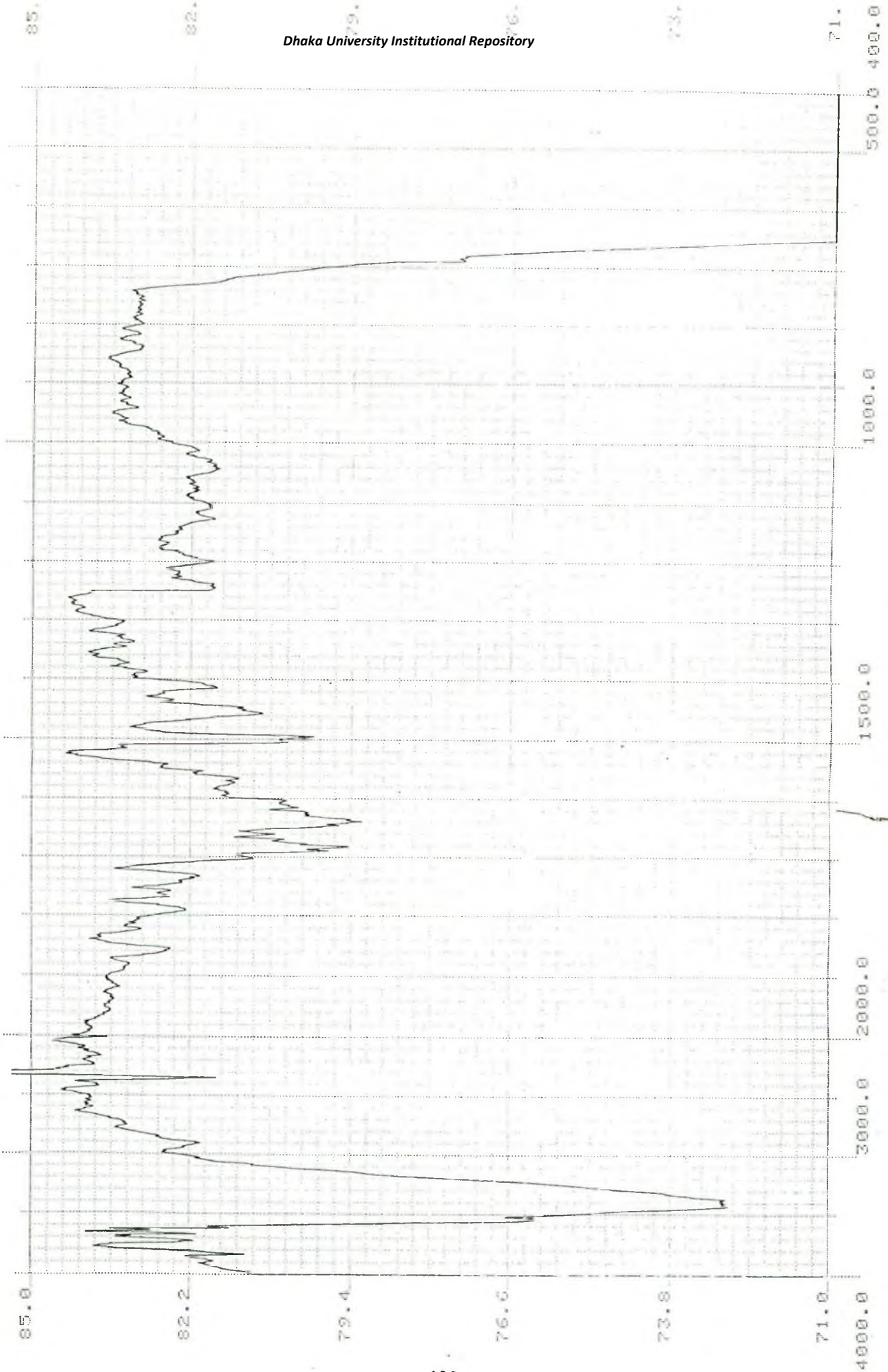


Figure-26: IR spectrum of compound S-16D

Analytical, BCSIR Dhaka, [illegible] Spectrum S-16D in CDCl3, Shahana, DU

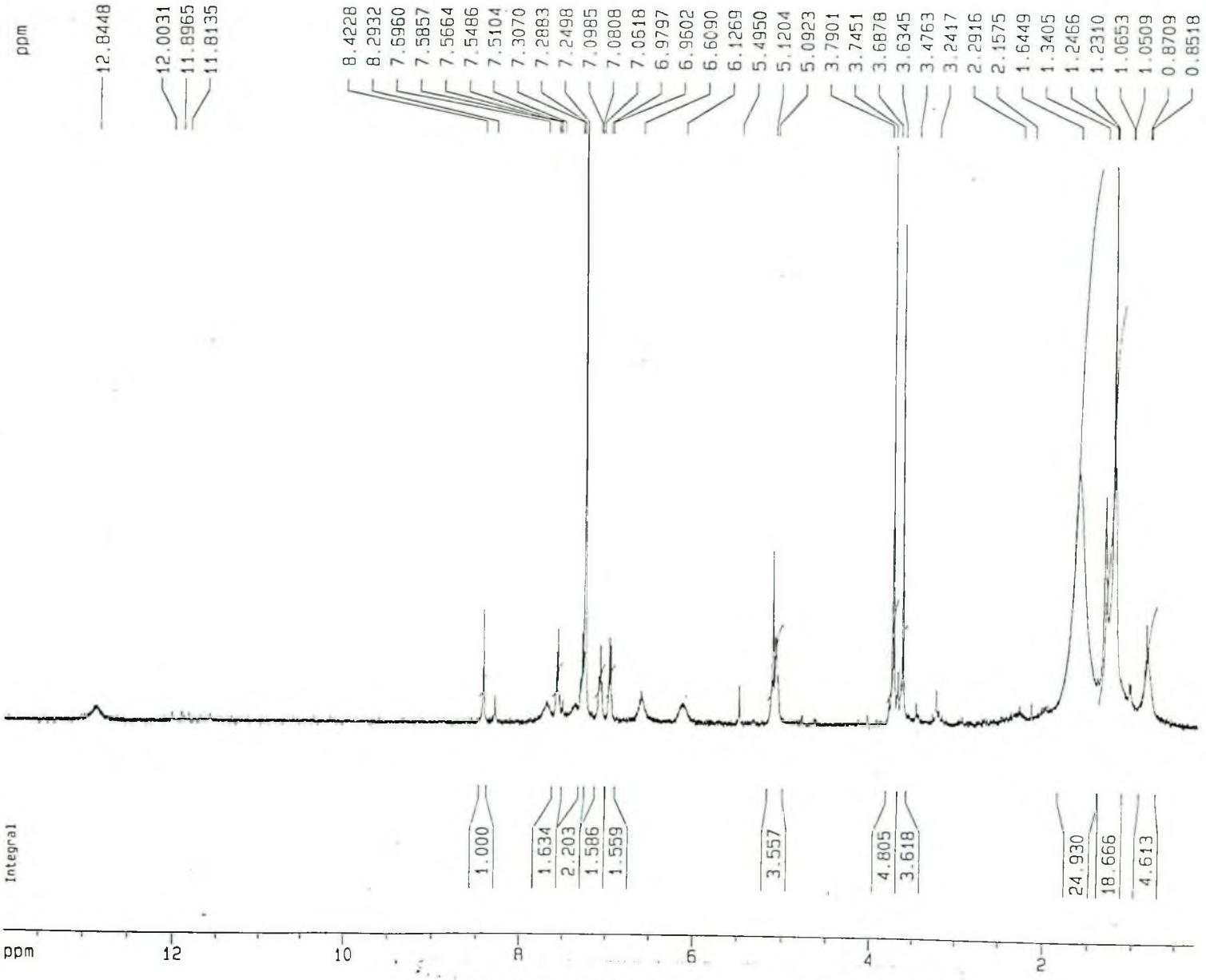


Figure-27: ^{1}H NMR spectrum of compound S-16D

Current Data Parameters

NAME	A4226
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20080417
Time	16.18
INSTRUM	dpx400
PROBHD	5 mm Multinuc
PULPROG	zg30
TD	32768
SOLVENT	CDCl3
NS	128
DS	2
SWH	6410.256 Hz
FIDRES	0.195625 Hz
AQ	2.5559540 sec
RG	812.7
DW	78.000 usec
DE	6.00 usec
TE	310.0 K
D1	1.00000000 sec

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

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

F2 - Processing parameters

SI	32768
SF	400.1400130 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	13.888 ppm
F1	5557.09 Hz
F2P	0.268 ppm
F2	107.35 Hz
PPMCM	0.68098 ppm/cm
HZCM	272.48700 Hz/cm