PROSPECTING FOR ANTIBACTERIAL COMPOUNDS FROM A BASIDIOMYCETE JO5191

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A thesis submitted to the Graduate School in partial fulfilment for the requirements of the Master of Science Degree in Chemistry of Egerton University

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DECLARATION

This thesis is my original work and has not been presented for the award of any other degree elsewhere.

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DEDICATION

To my parents Mr. and Mrs. Munialo, brothers and sisters for their love and moral support they have provided towards my education.

God Bless!

ABSTRACT

The need for new antibacterial compounds with different modes of action to manage drug-resistant bacterial strains is urgent. The molecular basis of most antimicrobial agents is now fairly well known and thus it is now possible to develop new compounds with improved antimicrobial properties. Though there are several drug targets that have been identified and upon which most antibacterial agents act, there is still great need and potential to prospect from novel sources for new drug candidates. Basidiomycetes have proved to be excellent producers of secondary metabolites with vast activities. Preliminary screening of crude extracts from a basidiomycete JO5191, revealed strong antibacterial activity against two Gram-negative bacteria: *Escherichia coli* and *Salmonella typhimurium*. This research project involved the purification of the compounds using column chromatography and semi preparative HPLC followed by structure elucidation of the compounds responsible for the observed antibacterial activity. The fungus was cultivated in 25 replicates of 1 litre-scale in nutrient liquid media for 22 days and crude extracts prepared from both the mycelium and the culture filtrate immediately after stopping growth. The crude extracts were screened for anti-bacterial activity and the compounds purified by bioassay-guided fractionation on silica gel and reverse phase high-pressure liquid chromatography. The chemical structures of the purified compound(s) were determined using spectroscopic techniques: infrared (IR), nuclear magnetic resonance (NMR) in conjunction with MS and CD and found to be flavanones. The anti-bacterial activities were determined by the calculation of minimum inhibitory concentrations (MIC), which were found to be in the range 3 μg/ml - 12 μg/ml, agreeing considerably well with those from the literature.

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ABBREVIATIONS and ACRONYMS

^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
23S	Twenty-three sequences
30S	Thirty sequences
50S	Fifty sequences
70S	Seventy sequences
80S	Eighty sequences
AICAD	African Institute for Capacity Development
ARSA	Association of Rhodes Scholars in Australia
ATP	Adenine triphosphate
CD	Circular Dichroism
COLOC	Correlation via Long-Range Couplings
COSY	Correlation Spectroscopy
DEPT	Distortionless Enhancement by Polarisation Transfer
DIAION HP21	Manufacturers name for resin
EDTA	Ethylenediaminetetraacetic acid
Fr	Fraction
GPR	General-purpose reagent
HMBC	Heteronuclear Multiple-Bond Correlation
HSQC	Heteronuclear Single Quantum Correlation
HMQC	Heteronuclear Multiple-Quantum Correlation
IBRL	Integrated Biotechnology Research Laboratory
INEPT	Insensitive Nuclei Enhanced by Polarization Transfer
IR	Infra red
Kex	Culture filtrate extract
Mex	Mycelium filtrate extract
MIC	Minimum inhibitory concentrations
MRSA	Methicillin-resistant *Staphylococcus aureus*
Mur NAC	Murein-N-acetyl glucosamine
NA	Nutrient Agar
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect

PBPs	Penicillin binding proteins
PDA	Potato dextrose agar
TLC	Thin Layer Chromatography
tRNA	Transfer ribonucleic acid
UV	Ultraviolet

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Antibiotics are chemical compounds, which are capable of inhibiting the growth of or killing other micro-organisms. Antibacterial compounds are a class of antibiotics that inhibit the growth or proliferation of bacteria or kill them directly. They are secondary metabolites or their derivatives, which are obtained from established biochemical pathways in various organisms from different phyla in the universal tree of life (Rosa *et al.*, 2003). The significance of antibiotics to health and medicine is immeasurable considering their effectiveness against harmful Gram-positive and Gram-negative bacteria.

There are various sources of antibacterial compounds, which include: micro-organisms, fermentation and synthesis. Many antibiotics in clinical use have been developed from secondary metabolites produced by bacterial and fungi and actinomycetes. During the last five decades several pathogenic micro-organisms have developed resistance to the available antibiotics (Evans *et al.*, 2006; Singh *et al.*, 2006). Infections by multi-drug resistant isolates of *Candida* sp., *Staphylococcus epidermidis*, *S. aureus*, *Streptococcus* sp., *Enterococcus* sp. and *Escherichia coli*, among others, have become more frequent thus stimulating the search for new antibiotics with novel mechanisms of action (Kotra & Mobashery, 1998; Morschhaeuser *et al.*, 2000; Sandven, 2000; Thomson & Moland, 2000; Singh *et al.*, 2006; Srivastava *et al.*, 2007).

There is an ever-increasing need for new antimicrobial agents because of emerging as well as re-emerging diseases and their resistance to known antibiotics (Levy; 1998; Burtler, 2004; Zhang *et al.*, 2007). Some bacteria resist not only single but also multiple antibiotics and therefore are extremely difficult to control. In part because of the rise in resistance to antibiotics, resurgence in death rates from some communicable diseases such as tuberculosis have started to increase again after having declined in the industrialized nations (Talaro & Talaro, 2002).

Since human and fungi share common microbial antagonists, humans can benefit from the natural defensive strategies of fungi that produce antibiotics to fight infections from micro-organisms such as *Staphyloccocus aureus, E. coli* and *Pseudomonas aeruginosa.* Hence, it is not surprising that most significant antibacterial drugs have been derived from fungi (Hardman *et al.*, 2001; Kralj *et al.*, 2006).

Basidiomycetes are important in nature, and due to their chemical components especially the biologically active polysaccharides. They produce and accumulate a variety of secondary metabolites, including phenolics, polyketides, terpenes and steroids (Turkoglu *et al.*, 2007), which have demonstrated some interesting biological properties such as antibiotic, immunomodulatory, anti-tumour, insecticidal, antioxidant, anticarcinogenic and antiviral activity (Longvah & Deosthale, 1998; Teissedre & Landrault, 2000; Guterrez *et al.*, 2005; Peng *et al.*, 2005; Zheng *et al.*, 2005; Smiderle *et al.*, 2006).

The phenolics including flavonoids, have been found to exhibit strong antibiotic and antioxidant activities. The chloroform and ethyl acetate extracts of the basidiomycetes have been shown to have antibacterial activity against *Streptococcus mutans* and *Prevotella intermedia* (Hirasawa, *et al.*, 1999). Both fruiting body and the mycelium contain compounds with wide-ranging antimicrobial activity (Teissedre & Landrault, 2000; Martini *et al.*, 2004). *Laetiporus species* (Polyporales, Fungi), which are basidiomycetes have been shown to contain N-methylated tyramine derivatives (List, 1958; Lee *et al.*, 1975; Rapior *et al.*, 2000), polysaccharides, a number of lanostane triterpenoids, laetiporic acids, flavonoids and other metabolites (Davoli *et al.*, 2005; Weber *et al.*, 2004; Turkoglu *et al.*, 2007).

The anti-bacterial activities of isoflavonoids, flavonoids and glycosides of luteolin and apigenin have been reported. In particular, flavonoids, including phenolic acids, which are the main group of phenols from fungi and many more compounds, are still being detected (Basilea *et al.*, 1999).

1.2 Bacteria as disease causative agents

Bacteria colonize a host system by breaking through the mucus barrier that lines most of the alimentary canal (mouth to anus), or entering through damaged tissue like wounds and bites. Once a bacterium has entered the system it grows and spreads to other parts of the body. Most infectious diseases start out as small-localized infections and only spread through the system if the bacteria gain access to the blood stream (Black, 2002).

Some of the common diseases caused by bacteria include: pneumonia, toxaemia, wound infections and urinary tract infections, which are caused by different genera of bacteria like *Staphylococcus, Streptococcus, Acinetobacter* sp. *Enterococcuss, Escherichia* and *Pseudomonas Salmonella, Helicobacter* among others (Talaro & Talaro, 2002; Rolston, 2005; Rolston *et al.*, 2005; Chamilos *et al.*, 2007).

Bacteria are classified according to characteristic features as Gram-negative and Gram-positive. The two groups differ mainly in cell wall morphology. The Gram-negative

cell wall is more complex because it contains an outer membrane, has a thinner shell of peptidoglycans and has extensive space surrounding the peptidoglycan shell. The outer membrane contributes an extra barrier in Gram-negative bacteria making them more impervious to some antimicrobial chemicals such as dyes and disinfectants. Consequently they are generally more difficult to inhibit or kill than are Gram-positive bacteria. Treating infections caused by Gram-negative bacteria requires different drugs, especially those that can cross the outer membrane (Taiaro & Talaro, 2002).

Gram-negative bacteria develop resistance to drugs faster than do Gram-positive bacteria. A salient example being the Gram-positive methicillin-resistant *S. aureus* (MRSA), which is very rampant, while *S. aureus* took 40 years to develop resistance to methicillin, which had been in use since the 1950s (Srivastava *et al.*, 2007). While, alternative antibiotics are available to treat infections caused by Gram-positive bacteria, and others are being developed. This is not true for Gram-negative bacteria, and currently several resistance mechanisms are emerging in such microbes, enabling them to become virtually untreatable (Poole, 2003; Bootsma, *et al.*, 2006; Josephson, 2006). Furthermore, there are currently no new drugs being developed for Gram-negative bacteria (Josephson, 2006).

Examples of Gram-negative bacteria that have rapidly developed resistance to β-lactam antibiotics are *Acinetobacter baumannii* and some species of *Enterobacter*. *A.cinobacter baumannii* (*Enterobacteraceae*), are opportunitic hospital pathogens that causes skin and soft-tissue infections and blood poisoning in immunocompromised patients (Siroy *et al.*, 2006; Chamilos *et al.*, 2007).

1.3 Secondary metabolism in fungi

Fungi constitute a group of micro-organisms united by their mode of nutrition: absorption, have the ability to exploit an almost infinite diversity of nutritional micro-niches. In their natural habitat, fungi are faced with competition and hence the need for survival. Competitors comprise of all the organisms that are fighting for space, nutrients, water, oxygen and the ability to express their development possibilities (Richardson, 1990). Fungi like any other organisms have found ways to exploit their substrate in the face of competition and have to design adaptive processes to increase the probability of survival. Once the life style and the developmental pattern have become established as a result of adaptation, the organism will retain the necessary characteristics, perhaps becoming even more specialized as a result of continuing competition (Kettering *et al.*, 2004a).

Like plants, fungi have evolved a rich and extensive source of secondary metabolites (Lewer *et al.*, 2006). The reason for which organisms produce these compounds remains a controversially interpreted and intensely debated topic in natural sciences (Adrio &Demain, 2003). However, before it was possible to speculate freely about the role of secondary metabolism, the boundaries of credible ideas were circumscribed more by the experience and the creativity of the people proposing them than by conformity with a knowledge base (Zähner *et al.*, 1983). Progress in establishing the biosynthetic pathways of secondary metabolites and more particularly the insights provided through molecular genetics into the organization and structure of genes directing the process have shed some light on the mechanisms by which metabolic products are formed (Boros *et al.*, 2006; Bartlett & Entzeroth, 2007). With this knowledge it is now possible to list some features of secondary metabolism that are no longer matters of speculation:

(i) Secondary metabolites are not essential for growth and tend to be strain specific;

(ii) They have a wide range of chemical structures and biological activities;

(iii) Unique biosynthetic pathways for primary intermediates and metabolites control secondary metabolite formation. These pathways are long and have complex multiple enzymes with substrate specificity different to those of primary metabolism;

(iv) The formation of secondary metabolites is directed by organised sets of genes associated with special regulatory mechanisms that seem to control both the timing and the level of gene expression;

(v) The control mechanisms are well integrated with the physiology of the producing organisms;

(vi) The production of secondary metabolites is of great interest, both ecologically and industrially (Hornbogen & Zocher, 2005; Weber *et al.*, 2006).

1.3.1 Biosynthesis of secondary metabolites by fungi

Secondary metabolites (idiolites) are special metabolites usually possessing unique chemical structures and although not essential for the producing organism's growth in pure culture, they have survival functions in nature. They are produced only by some species of a genus and possess unusual chemical functionalities, such as β-lactam rings, cyclic peptides made of natural and modified amino acids, unsaturated bonds of polyacetylenes and polyenes, and large macrolide rings (Jiao, R. *et al.*, 2006; Jiao, P. *et al.*, 2006). Idiolites are produced typically as members of a particular chemical family because of the low specificity

of some enzymes involved in secondary metabolism (Demain, 1986; Jiao, P. *et al.*, 2006). Specific mechanisms regulating the onset of idiolite synthesis include repression by sources of carbon, nitrogen and phosphate and enzyme induction. However, in batch cultures containing nutritionally rich media, high levels of idiolites are usually produced only after most of the cellular growth has already occurred. Timing depends on the nutritional environment presented to the culture (Demain, 1986).

All classes of known fungal secondary metabolites are formed from glucose. The interrelationship between primary and secondary metabolism has been established. The principal pathway of secondary metabolism starts from glucose as well as the broken down units via acetate-malonate, mevalonic acid, shikimic acid and amino acid pathways. The secondary metabolites are often produced during a well-defined time span at a certain stage of the producer's life cycle (Bu'lock *et al.*, 1975).

The main building blocks of secondary products are: acetate units from acetyl coenzyme A, leading to polyketides, polyenes, terpenoids, steroids or caretonoids; shikimate, from which aromatic compounds can be derived; amino acids, which serve as precursors for peptides; and alkaloids and glucose, for the biosynthesis of glycosides and aminoglycosides (Herbert, 1989). In spite of the limited number of pathways leading to the formation of secondary metabolites, nature continues to produce a rich array of natural products that display diverse chemical structures and biological activities (Schilmigann *et al.*, 1995; Zhao *et al.*, 2003). These products are an integral source of pharmaceutical drug leads, as proven by the fact that a great majority of therapeutic agents, especially many antibiotics developed thus far, are products of micro-organisms and/or their derivatives (Zhao *et al.*, 2003).

1.4 Nutrient composition and growth regulation for secondary metabolism in fungal cultures

About half of all basidiomycetes produce antibiotically active compounds when grown in rich media under laboratory conditions. Since secondary metabolism is less strictly regulated than primary metabolism, it is strongly dependent on the available nutrients (Kettering *et al.*, 2004b).

Culture media can be defined in several ways, as for example with the key nutrient present in a form that cannot be rapidly assimilated (Ratledge & Kristiansen, 2002). Laboratory defined media are artificial media that are constituted with defined ingredients to have a controlled growth of a micro-organism. In artificial cultures, the production of these "special" metabolites is sensitive to culture conditions (Weinberg, 1974). While in batch

cultures, processes leading to the production of idiolites are often sequential; the cultures exhibit a distinct growth phase (trophophase) followed by a production phase (idiophase). In some cases, trophophase and idiophase may overlap. In a defined medium supporting only slow-growth, some nutritional factor may be growth-limiting from the very start of cultivation, thus favoring production while slow growth is occurring (Demain, 1986).

There are essential compounds and elements required for expression of growth and the associated metabolism in fungal cultures. Carbon, nitrogen, sulphur, phosphorus, magnesium and potassium are macro-elements required for fungal growth. Calcium, iron, copper, manganese, zinc and molybdenum are micro-elements required in small quantities for growth. Carbon is mostly supplied in the media as organic substrates such as glucose; while in the wild fungi are able to degrade structural material such as cellulose, lignin, chitin, and keratin successfully using their oxidative enzymes like peroxidases (Vining, 1992).

Fungi grow rapidly using glucose (tropophase) and then enter a slower-growing phase (idiophase) favouring secondary metabolism (Bu'lock *et al.*, 1975). In complex and nutritionally rich media, production of secondary metabolites usually, but not always starts after the cellular growth rate has slowed down. It is in this phase that metabolic pathways that are not concerned with producing the compounds necessary for growth become operative and the production of secondary metabolites begins (Adrio & Demain, 2003).

The increase in mycelia dry weight which occurs during the idiophase does not mean an equivalent increase in protoplasm, it is almost certain that this increase is due to carbon incorporated into storage material. In this case, the reduction in growth rate is associated with nutrient exhaustion. Furthermore, the characteristics of the external medium do not necessarily give a proper picture of the internal milieu of the fungus that is responsible for the change to secondary metabolism. Secondary metabolism is enhanced when particular nutritional controls are lifted, for example repression of glucose, nitrogen and phosphate. When growth is limited in an appropriate manner, idiophase activity ensues. The shift-down can usually be programmed in liquid cultures by formulating media in which a suitable key nutrient will be depleted early (Adrio & Demain, 2003).

1.5 Screening for novel biologically active compounds from fungal cultures

Many biological species such as fungi, bacteria, algae, sponges, and plants, produce secondary metabolites with diverse biological activities, thus representing a rich source of potentially valuable pharmacological agents (Boldi, 2004; Koehn & Carter, 2005). Currently,

the majority of drugs derived from secondary metabolites were discovered by random screening of biological samples and subsequent rigorous testing (Zotchev *et al.*, 2006).

Under laboratory conditions, fungi are brought into axenic culture by isolating them from their natural substrates. However not all fungi are easy to isolate and maintain in culture. For example most species belonging to the genera *Inocybe*, *Rhodophyllus* or *Russula* neither display spore germination nor growth from tissue plugs (Lorenzen & Anke, 1998). Conditions under which fungi are isolated and grown in culture are extremely variable, with sample pre-treatments, isolation techniques, types of artificial media and growth conditions such as temperature, light/dark cycles, humidity, and aeration playing crucial roles (Zabel & Terracina, 1990).

The screening for biologically active fungal secondary metabolites involves testing crude extracts obtained from fermentation broths, in established biological test systems (Corley & Durley, 1994). Although secondary metabolism is an intracellular process, a few products may accumulate within the cytoplasm (mycelium). Compounds produced in fermentation processes are part of a complex crude mixture and more often than not, desired compounds with significant activities are often produced only in minute quantities. It is even more challenging, when a biological active compound in the crude extract is unstable due to heat, light, pH, and time in such cases isolation is rarely achieved. An appropriate method for the isolation of a particular secondary metabolite is usually influenced by several factors which include; facilities, the quantity of compound, the degree of purity desired, stability, complexity physicochemical properties and expertise involved (Zhang & Greasham, 1999).

Since most secondary metabolites are complex molecules, they usually do not diffuse readily through membranes, especially in the case of polar substances. Therefore standard techniques exist which help to extract these compounds after the growth processes are complete. For the intracellular compounds, this involves the soaking of the cytoplasm (biomass) in a polar solvent like acetone and agitating for a considerable period of time then carrying out the normal solvent-solvent extraction methods.

Extracellular compounds found in the culture filtrate can be recovered by use of several techniques, which include solvent-solvent extraction and liquid-solid extraction. These techniques enable the researcher to get crude extracts that are further processed to give pure compounds allowing their structure to be determined. Consequently, most water-soluble secondary metabolites produced in low yield in the filtrate or supernatant solutions are recovered after separating the cell material from the culture. To increase the yield, or

facilitate production on a large scale, the produced compound is synthesised using chemical techniques (Setti & Micetich, 1998).

1.6 Natural products as templates for organic synthesis

The avalanche of new natural products appearing on the scene as a consequence of the advent and development of new analytical techniques demand new and more systematic approaches to strategy design. Organic synthesis is considered to a large extent to be responsible for some of the most exciting and important discoveries of the 20th century in chemistry, biology and medicine, and continues to fuel the drug discovery and development with myriad processes and compounds for new biomedical breakthroughs and applications (Jehnings & Lysek, 1999; Walsh & Wright, 2005).

New methods are often incorporated into the synthetic schemes towards the target molecule and total synthesis is an opportunity for the invention and discovery of new chemistry (Kim *et al.*, 2004). The gradual sharpening of this tool is demonstrated by considering its history through the pre-World War II, the Woodward and Corey eras, and into the 1990s, and by accounting major accomplishment along the way. Today, total synthesis of natural products is associated with prudent and tasteful selection of challenging and preferably biologically important target molecules. The discovery and invention of new synthetic strategies and technologies, and explorations in chemical biology through design and mechanistic studies have improved and enhanced the process (Nicolaou *et al.*, 2000a). In modern natural products chemistry, bioactivity-guided purification, followed by structure elucidation is wrapped up with partial or total synthesis of the target compounds.

1.7 Natural products as templates for drug discovery

Natural products have proven to be the best source of therapeutic compounds and lead structures for medicinal chemistry (Njardarson *et al.*, 2004; Sorensen, *et al.*, 2007). Many current drugs are either natural products or their derivatives. Additionally, natural products have been extensively used to elucidate complex cellular mechanisms, including signal transduction and cell cycle regulation leading to the identification of important targets for therapeutic intervention (Nicolaou *et al.*, 2000b; Sorensen *et al.*, 2007). By combining the drug quality of natural products with the efficiency of high-throughput chemistry, the synthesis and screening of small molecule libraries based on natural products as templates has attracted growing attention in the past few years, despite being increasingly neglected in the lead-finding process by the pharmaceutical industry (Song *et al.*, 2004).

However, the modern drug discovery process and the trend toward high-throughput screening (HTS) and structural scaffolds amenable to the rapid synthesis of analogues have constrained the role of natural products (Sorensen *et al.*, 2007). Novel approaches are clearly needed for natural products to successfully run the gauntlet of the industrial drug discovery process. While modified structures may have superior properties to the natural products themselves, the modified analogues may not be accessible from inducible biosynthetic pathways (Bianco *et al.*, 2000; Bianchi *et al.*, 2006; Bruce *et al.*, 2007).

Timely dereplication, isolation, and structural elucidation of compounds are initial prerequisites, which can be accomplished by the emerging hyphenated spectroscopic techniques like GC-IR, GCMS, LC-MS and LC-NMR (Bartlet & Entrzeroth, 2007). Fungi being an interesting source of secondary metabolites have historically led to the important discoveries of various natural products like β-lactams, macrocyclic/actonespolyenes, polyethers, ionophores, petides, phenolics, glycopeptides, quinolones and tetracyclines. Existing scientific knowledge and novel technology present the opportunity to conduct chemotaxonomy-driven studies of these biological small-molecule libraries (Mang *et al.*, 2006). Thus, the search for novel scaffolds for high-throughput synthesis of natural products and libraries is a relevant and timely pursuit.

1.8 The basidiomycete, JO5191, to be studied in this proposed research project

From a previous screening programme for antibacterial activity from crude extracts prepared from about 200 strains of fungi collected from Mt. Kenya Forest, a basidiomycete coded JO5191, showed significant and reproducible activity. JO5191 was collected on the slopes of Mt. Kenya in a natural forest at the foot of a bamboo shrub. The fresh morphological features as well as microscopic data confirmed that the fungus is a basidiomycete. Microscopic examination of the hyphae showed clear clamp connections. The basidiomycete was brought into culture at the site of collection by trapping the basidiospores onto a potato dextrose nutrient agar and the hyphal growth allowed to develop into visible mycelium under sterile conditions. Once the mycelia developed on the plate, corresponding agar slants were prepared and kept in a culture collection in the Integrated Biotechnology Research Laboratory (IBRL) Egerton University. The herbarium material of the JO5191 is shown in Figure 1.

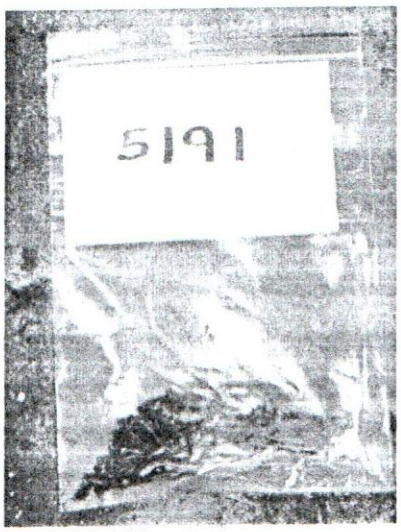


Figure 1: Herbarium material of JO5191

In this research project, the basidiomycete JO5191 was cultured, the antibacterial compounds purified and the structures determined using spectroscopic techniques.

1.9 Hypothesis

The basidiomycete JO5191 produces antibacterial compounds in liquid cultures that are stable enough to be purified and chemical structures determined.

1.10 Statement of the problem

There has been a rapid increase in cases of resistance to antibiotics currently in use. Therefore, there is need to search for more new antibiotics and/or antibacterial agents from unexplored natural genetic sources like tropical fungi to assist in fighting bacterial diseases.

1.11 Justification of the study

There is increasing evidence of pathogenic bacteria, which are resistant to conventional antibiotics. This is stimulating the development of strategies targeting new or unexplored sources of antibacterial compounds with unique mechanisms of action. Accordingly, there should be an increasing effort to try and identify potential targets for the development of new antibacterial agents. Therefore, the need for new and novel antibacterial compounds to manage various diseases justifies this search for compound(s) with antibacterial activity from the basidiomycete JO5191.

1.12 Objectives

(i) To culture and optimize the production of antibacterial compounds by the basidiomycete JO5191 in liquid submerged culture.

(ii) To fractionate crude extracts from the basidiomycete JO5191 using solvent-solvent extraction and solid-liquid adsorption techniques.

(iii)To purify the antibacterial compound(s) from the basidiomycete JO5191 using chromatographic techniques.

(iv)To elucidate the structure(s) of the purified antibacterial from the basidiomycete JO5191 compound(s) using modern spectroscopic and physico-chemical techniques.

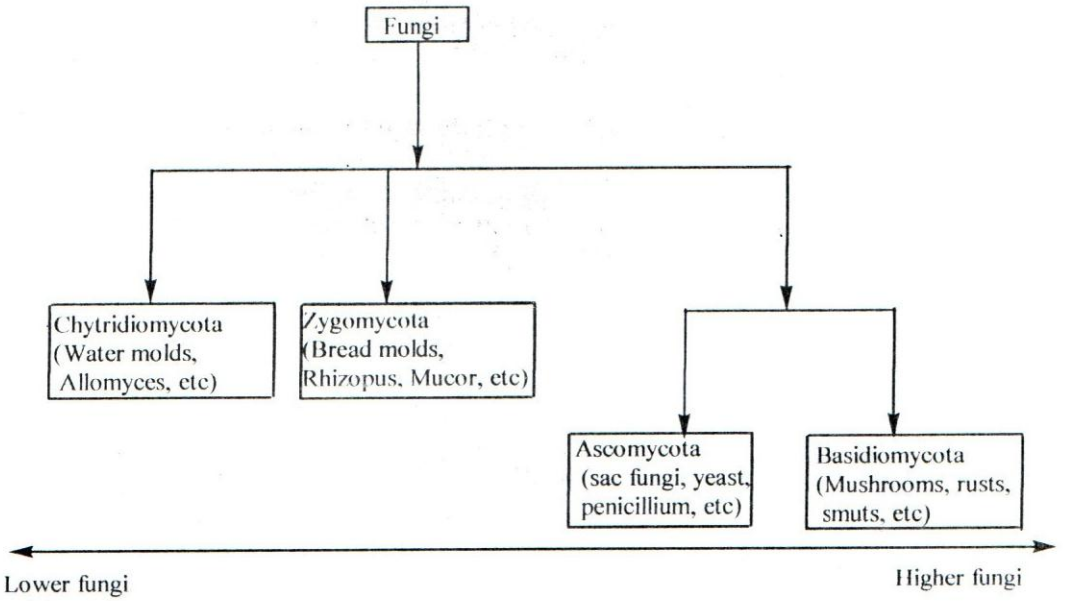
(v) To undertake structure activity studies of simple derivatives and analogues of the isolated antibacterial compounds.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Fungi classification and habitat

About 80,000 species of fungi have been described (Isaka *et al.*, 2005). However, this represents about 5% of those estimated to exist (Hawksworth, 1991; Hawksworth *et al.*, 1995; Hawksworth & Rossman, 1997; Isaka *et al.*, 2005). Of these, only a fraction are utilised as food or medicine, both modern and traditional or as biological control agents (McCoy, 1990; Steinkraus & Whitfield, 1994). The organisms of the fungal lineage include mushrooms, rusts, smuts, puffballs, truffles, morels, moulds and yeast as well as many lesser known organisms (Alexopoulus *et al.*, 1996). Fungi are classified into major divisions (Bruns *et al.*, 1993) as illustrated in Scheme 1.



Scheme 1: Classification of fungi into major divisions

Filamentous fungi grow on rich nutrient agar plates as dense colonies, but in nature the filaments can be much longer and the colonies less dense. The entire colony mobilises and reallocates resources to exploit new food sources when one of the filaments contacts a food supply. However, upon depletion of food, sporulation is triggered. Although the fungal filaments and spores are microscopic, the colony can be very large with individuals of some species rivalling the mass of the largest animals or plants (Meletiadis *et al.*, 2001)

Fungal spores may be actively or passively released for dispersal by several effective methods. An example is the air which is filled with spores of species that are air dispersed. There are usually species that produce a large number of spores. Examples include many species pathogenic on agricultural crops and trees. Other species are adapted to dispersal within or on the surface of animals (particularly arthropods). Some fungi are rain splash or flowing water dispersed. The function of some spores is not primarily for dispersal, but to allow the organisms to survive as resistant cells during periods when the conditions of the environment are not conducive for growth (Carrol & Wicklow, 1992).

As a result of their uniqueness, fungi have proved to be the diverse source of most antibiotics and antibacterial compounds in particular. The antibiotics have been classified by their different modes of action on different targets.

2.2 Scope of fungal antibacterial compounds

The increasing evidence of pathogenic bacteria, which are resistant to conventional antibiotics is stimulating the development of strategies that target new or unexploited mechanisms of action. Consequently, there are increasing efforts to identify potential novel targets for the development of new anti-microbial agents. Compounds, which are highly selective towards a specific group of bacteria or against organisms/cells other than bacteria such as yeast, fungi, protozoa, algae, viruses, tumours, nematodes, insects, mites and weeds have been detected (Demain, 1992; Vandamme, 1994). Implementation of synthetic strategies to find analogues of anti-bacterial compounds with increased affinity to the modified target is a common approach in overcoming many types of resistance (Josephson, 2006).

2.2.1 Clinically useful antibiotics

Several thousands of compounds with antibiotic activity have been isolated from microorganisms over the years, but only a few of them are clinically useful (Patzer & Dzierżanowska, 2007). Some clinically important antibiotics and their mode of action are summarised in table 1. Most of the anti-bacterial agents act on bacterial cell-wall synthesis or protein synthesis. Peptidoglycan is one of the major cell-wall targets because it is found only in bacteria. Other compounds target bacterial protein synthesis, because bacterial ribosomes (70S) are different from the ribosomes (80S) of humans and other eukaryotic organisms. Similarly, the one antifungal agent shown in the table 1 (griseofulvin) binds specifically to

the tubulin proteins that make up the microtubules of fungal cells; these tubulins are somewhat different from the tubulins of humans (Mohan *et al.*, 2006).

Table 1: Some clinically important antibiotics and their mode of action

Antibiotic	Producer organism	Activity	Site or mode of action
Penicillin	*Penicillium chrysogenum*	Gram-positive bacteria	Wall synthesis
Cephalosporin	*Cephalosporium acremonium*	Broad spectrum	Wall synthesis
Griseofulvin	*Penicillium griseofulvum*	Dermatophytic fungi	Microtubules
Bacitracin	*Bacillus subtilis*	Gram-positive bacteria	Cell wall synthesis
Polymyxin B	*Bacillus polymyxa*	Gram-negative bacteria	Cell membrane
Amphotericin B	*Streptomyces nodosus*	Fungi	Cell membrane
Erythromycin	*Streptomyces erythreus*	Gram-positive bacteria	Protein synthesis
Neomycin	*Streptomyces fradiae*	Broad spectrum	Protein synthesis
Streptomycin	*Streptomyces griseus*	Gram-negative bacteria	Protein synthesis
Tetracycline	*Streptomyces rimosus*	Broad spectrum	Protein synthesis
Vancomycin	*Streptomyces orientalis*	Gram-positive bacteria	Protein synthesis
Gentamicin	*Micromonospora purpurea*	Broad spectrum	Protein synthesis
Rifamycin	*Streptomyces mediterranei*	Tuberculosis	Protein synthesis

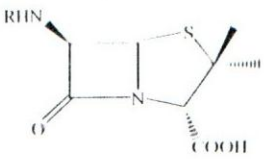
2.2.2 Classes of antibiotics

There are different chemical classes of antibiotics, originating from various microorganisms (Monaghan & Tkack, 1990). Some of these classes with their characteristic mode of action are reviewed below.

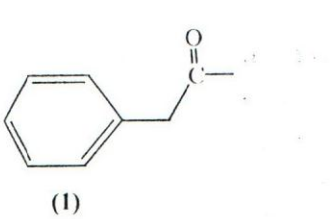
β–Lactam antibiotics: - were first isolated from the fungus *Penicillium notatum* (penicillin G) with the characteristic β-lactam functional group, which is responsible for the anti-bacterial activity. The β-lactam antibiotics were the most important therapeutic agents in the 20th century (Mishra *et al.*, 2007). Their mode of action involves inhibition of the cell-wall synthesis thus killing the bacteria. They inactivate penicillin-binding proteins (PBPs) that are membrane-bound proteins involved in the catalytic activity of the final cross-linking reactions of the peptidoglycan synthesis. Binding of β–lactam antibiotics to essential PBPs results in cell–wall lysis, disturbance of cell shape and inhibition of cell division, respectively (Neu, 1985; Monaghan & Tkack, 1990; Healy *et al.*, 2007; Hung *et al.*, 2007).

The largest group of β-lactam antibiotics is the penicillins [penicillin G (**1**), methicillin (**2**), ampicillin (**3**), floxacillin (**4**), cloxacillin (**5**), amoxicillin (**6**), azlocilin (**7**), penicillin V (**8**) and ticarcillin (**9**)].

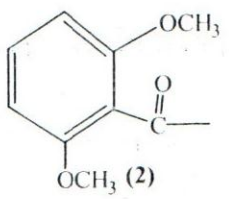
The general structure of penicillin's is given as:



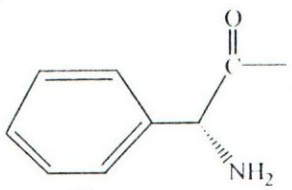
Penicillin G (**1**) and penicillin V (**8**) are the two naturally occurring compound while the others (**2-9**) are synthetic derivatives and analogues.



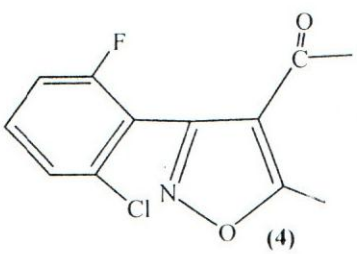
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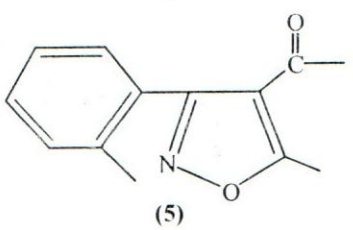
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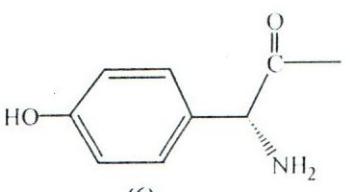
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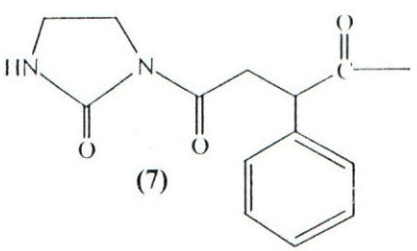
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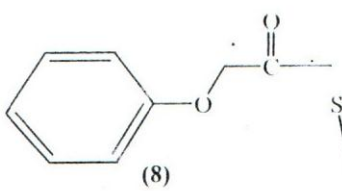
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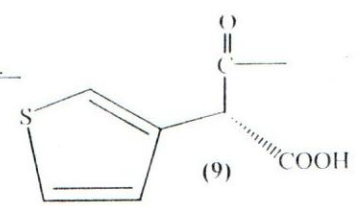
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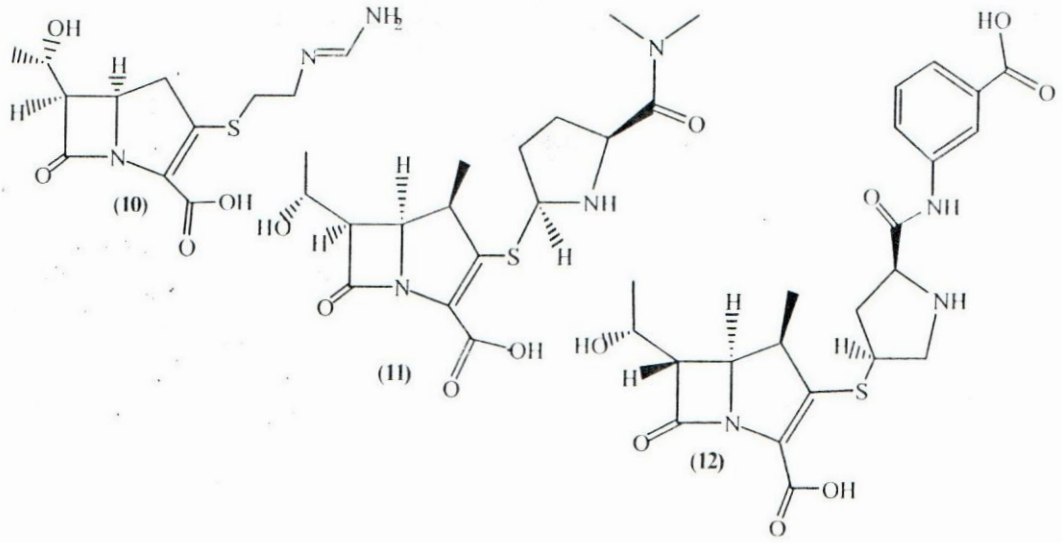


(9)

The basis of penicillin resistance in *S. aureus* was shown by Kirby (1944) to be production of β-lactamase, referred to at that time as penicillinase. This led to the development of the synthetic derivatives and analogues. The first semi-synthetic penicillin to be derived that was stable to staphylococcal penicillinase and clinically effective against penicillin resistant *S. aureus* was methicillin (**2**). This was followed by the development of the other analogues, cloxacillin (**5**), flucloxacillin (**4**), ampicillin (**3**), amoxicillin (**6**), ticarcillin (**9**) and azlocillin (**7**) respectively (Rolinson & Geddes, 2007). An alternative

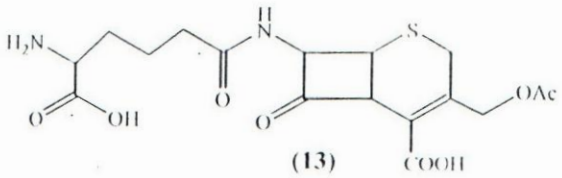
approach to the problem of a β-lactamase mediated resistance to penicillin was the use of *β*-lactamase inhibitor, which led to the development of the other classes of *β*-lactam antibiotics, carbaphenems and cephalosporins.

Carbapenems are hydrophilic antibiotics that are very effective against Gram-negative bacteria. Resistance against carbapenems has been known to be caused by alterations in membrane permeability due to decreased influx and/or increased efflux. (Nordmann & Poirel, 2002; Siroy *et al.*, 2006). Common examples of carbapenems are imipenem (**10**), meropenem (**11**) and ertapenem (**12**) (Rosen *et al.*, 1999; Makoto *et al.*, 2006). Carbapenem resistance is a significant problem. Imipenem and meropenem are potent agents for the treatment of infections due to multi-drug resistant *P. aeruginosa*. However, the prevalence of carbapenem-resistant *P. aeruginosa* has recently been on the increase (Nordmann & Poirel, 2002; Patzer & Dzierżanowska, 2007). Moreover, the emergence of carbapenem-resistant *Acinetobacter* meningitis is a major problem which complicates therapy. New antimicrobials that are effective against these organisms and penetrate into the cerebrospinal fluid with high concentrations are urgently required (Metan *et al.*, 2007).

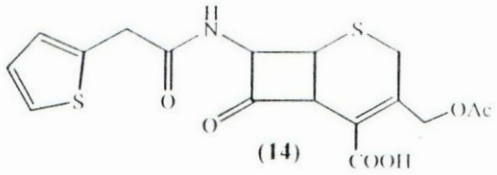


Cephalosporins were first isolated from cultures of *Cephalosporium acremonium*. Cephalosporin C (**13**) was the first in this class to be isolated. It is stabile to β-lactamases but is not sufficiently potent for clinical use (Vialta *et al.*, 1997; Fenoll *et al.*, 2007). The cephalosporin nucleus, 7-aminocephalosporanic acid (7-ACA), is in from cephalosporin C is analogous to the penicillin nucleus, 6-aminopenicillanic acid. Modification of the 7-ACA

side-chains has resulted in the development of useful antibiotic agents like cephalothin (cefalotin) (**14**).



(13)

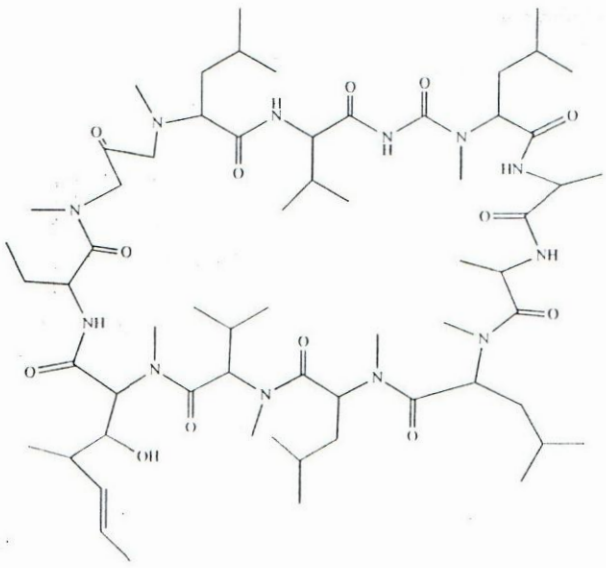


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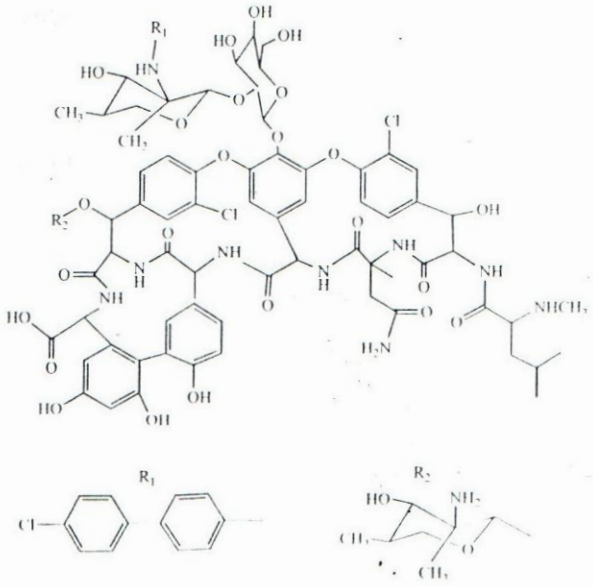
The key feature of penicillin and its well-known relatives, the cephalosporins, penems, carbapenems, clavulanic acids, and monobactams, is the *β*-lactam nucleus. Gram-negative bacteria have at their disposal a plethora of resistance mechanisms that they can sequester and/or evince, eluding the actions of penicillin, carbapenems, cephalosporins and other β-lactam antibiotics (Mishra *et al.*, 2007). The common form of resistance is through lack of drug penetration (outer membrane protein mutations and efflux pumps), hyper production of a β-lactamase and/or carbaphenem-hydrolyzing β-lactamases (extracellular enzymes), which hydrolyze the *β*-lactam moiety to inactive ring-opened amino acids (Walsh *et al.*, 2005; Hung *et al.*, 2007). β-Lactamases are the most important mechanism of β-lactam drug resistance in Gram-negative bacteria (Ishii *et al.*, 2007). This resistance has spawned a renewed interest in identifying new families of antibiotics (Mishra *et al.*, 2007).

Glycopeptide antibiotics: These constitute macrocyclic peptides some of which are linked to at least one sugar ring. Two basic examples are cyclosporin A (**15**) and vancomycin (**16**). The glycopeptide group of antibiotics act by preventing the transglycosylation and cross linking of the peptidoglycan layer in Gram-positive pathogens by complexing the peptidyl D-ala D ala termini (Grdadolnik *et al.*, 1998). However, certain species in the genus *Enterococcus* have been shown to withstand the killing action of the gylcopeptide antibiotics through the biosynthesis and use of a uridine diphospho- MurNAC–pentadepsipeptide containing a D-lactic acid instead of D-alanine at the terminal position. This substitution reduces drastically the binding affinity of this group of antibiotics to the depsipetidyl terminus making the biosynthesis of the peptidoglycan possible even in the presence of these drugs. There is therefore need for new effective antibiotics with unique modes of action especially since the appearance of resistance strains like the vancomycin resistant strains (VRSA) (Borel *et al.*, 1991; http://www.news.cornell.edu/releases/Sept96/cyclosporine.hrs.html; Stähelin, 1996; CDC, 2003; Chang *et al.*, 2003; Mongodin *et al.*, 2003; Evans *et al.*, 2006). Ever-increasing

resistance of MRSA to vancomycin however, underscores the need to urgently expand the existing arsenal of anti-MRSA agents.

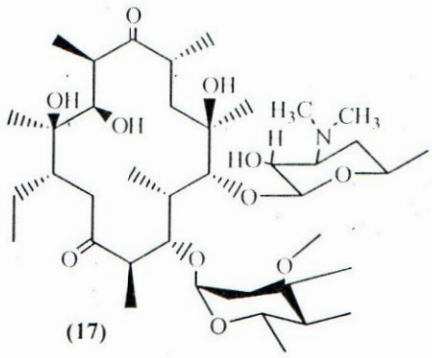


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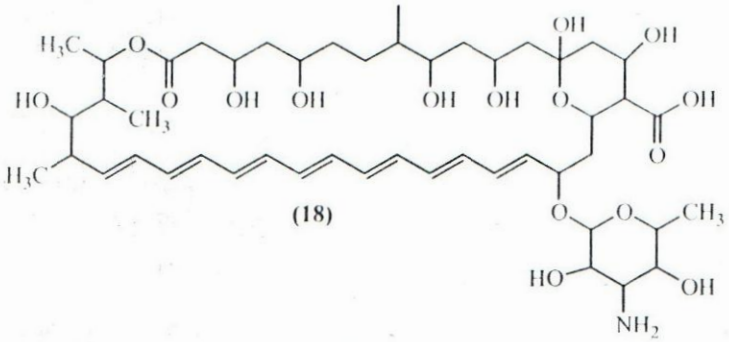


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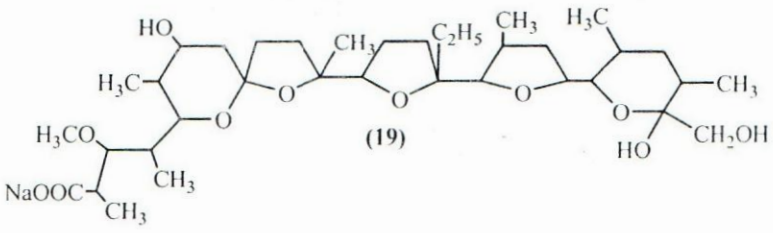
Macrolides: - They inhibit RNA–dependent bacterial protein synthesis by reversibly binding to the 50S sub-unit of the ribosome and consequently blocking protein synthesis (Katz & Ashley, 2005). Resistance to macrolides is due to the presence of the enzyme methylase, which is responsible for the dimethylation of the adenine 2058 in the 23S ribosomal RNA sequence (Lucier *et al* 1995; Pereyre *et al.*, 2002; Pereyre *et al.*, 2004; Pereyre *et al.*, 2006; Pereyre *et al.*, 2007). This enzymatic modification results in 50S ribosomal sub-units, which do not bind the antibiotics (Dinos & Kalpaxis, 2000). They are classified as polyketide antibiotics. The main subclasses include: macrocyclic lactones, polyethers, polyenes and ionophores. Examples include erythromycin A (**17**), (Tennakoon *et al.*, 2006), amphoterecin B (**18**), and monensin sodium A (**19**), (Bryskier & Bergogne-Berezin, 2005).



(17)

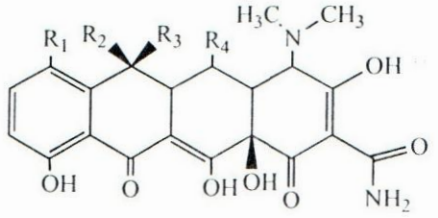


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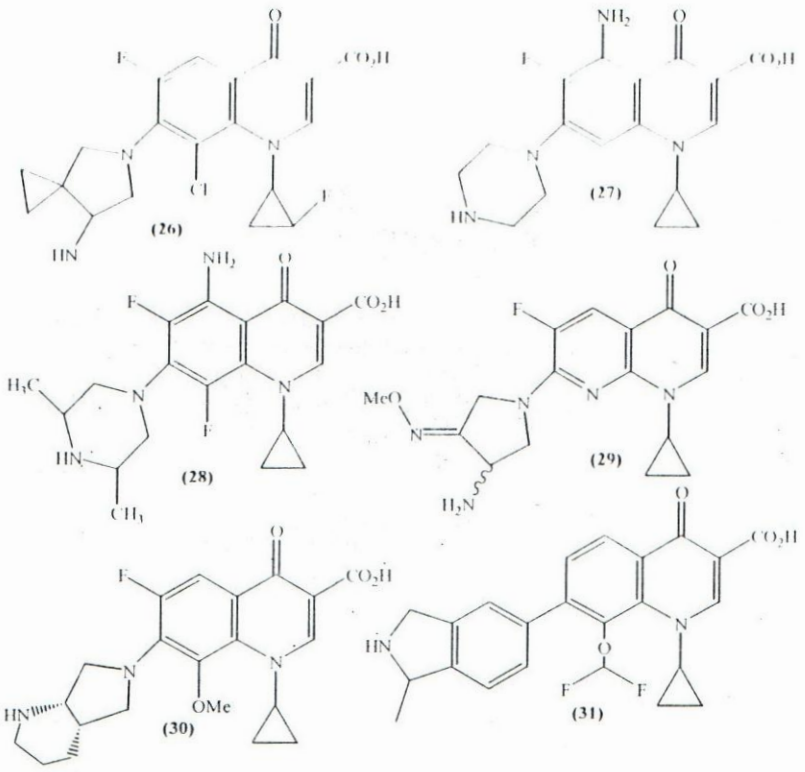
(19)

Tetracyclines: - Belong to polyketide class of antibiotics. They prevent bacterial growth by inhibiting protein synthesis by binding to a high-affinity site on bacterial 30S ribosomal unit, therefore preventing the aminoacyl-tRNA attachment to the ribosome A-site. Two bacterial resistance mechanisms to tetracyclines have been identified: (a) the production of cytoplasmic proteins that reduce the sensitivity of the ribosome to tetracyclines; and (b) the reduction of the intracellular concentration of the antibiotics due to the intracellular concentration of the membrane-associated protein pumps that efflux the compounds in an energy dependent manner (Smith *et al.*, 2007). The first tetracycline to be discovered, chlortetracycline (CTc) (**20**), was isolated from *Streptomyces aurefaciens* (Koza, 2000). Subsequently, numerous tetracycline analogues have been introduced including oxytetracyline (OTc) (**21**), tetracycline (Tc) (**22**), demeclocycline (DEM) (**23**), minocycline (MIN) (**24**) and doxycycline (DOX) (**25**), (Olack & Morisson, 1991; Tymiak *et al.*, 1993). In the past, tetracyclines have been the antibiotics of choice against a wide variety of microorganisms. The overuse of this class of antibiotics has led to widespread bacterial resistance and eventual replacement with more effective antibiotics such as fluoroquinolones and penicillins (Hamscher *et al.*, 2002). However, tetracyclines have many distinct advantages despite current bacterial resistance. Clinically, tetracyclines are much safer than other antibiotics and chemical modification has lead to compounds with enhanced antibiotic properties, low toxicity, and clinical effectiveness against a broad spectrum of microorganisms (Othersen *et al.*, 2006). Thus, synthetic modification of tetracyclines continues to hold promise in the development of antibiotics effective against resistant microorganisms (Tymiak *et al.*, 1993).



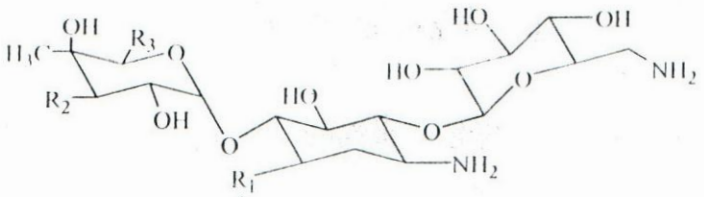
(20)	$R_1 = Cl$	$R_2 = OH$	$R_3 = H$	$R_4 = CH_3$
(21)	$R_1 = H$	$R_2 = OH$	$R_3 = CH_3$	$R_4 = OH$
(22)	$R_1 = H$	$R_2 = OH$	$R_3 = CH_3$	$R_4 = H$
(23)	$R_1 = Cl$	$R_2 = OH$	$R_3 = H$	$R_4 = H$
(24)	$R_1 = NMe_2$	$R_2 = H$	$R_3 = H$	$R_4 = H$
(25)	$R_1 = H$	$R_2 = H$	$R_3 = CH_3$	$R_4 = OH$

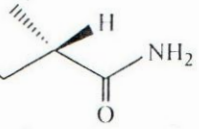
Quinolones: - These are mainly synthetic compounds that are in wide use for the treatment of infections in humans and display the greatest activity and range of any oral antibacterials currently in clinical use (Anderson *et al.*, 2000). They act by inhibiting bacterial DNA gyrase and topoisomerase IV. These enzymes are responsible for bacterial growth by mediating the ATP–dependent crossing of one DNA duplex through a transient enzyme–bridge double strand break in another DNA segment (Pan & Fischer, 1997). Some quinolones also act by increasing the levels of covalent enzyme-cleaved DNA complexes that are normal intermediates in catalytic cycles of both type II enzymes. The action of quinolones on bacteria brings about a decreased introduction of negative super twists into DNA, decreased decatenation of interlocked DNA circles, damage to DNA and inhibition of DNA synthesis. They have been established to help in prevention of diseases caused by several bacterial agents both in human and animals because they are highly active against a wide spectrum of Gram-negative bacteria but also moderately active against Gram-positive bacteria (Lara *et al.*, 2006; Rodrı´guez-Dı´az *et al.*, 2006). Synthesis of quinolones with increased activity against resistant clinical isolates has been accomplished. Examples of quinolones include flouroquinolone **(26)**, sparfloxacin **(27),** ciproflaxin **(28),** gemifloxacin **(29),** moxifloxacin **(30)** and garenoxacin **(31)** (Volmer et al. 1997; Ellsworth *et al.*, 2006; Wang *et al.*, 2007).

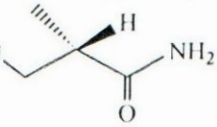


Aminoglycosides: - Belong to an important group of broad-spectrum natural antibiotics that act by inhibiting the bacterial protein synthesis (Miller *et al.*, 1995; Kudo *et al.*, 2005; Magnet & Blanchard, 2005; Hirayama *et al.*, 2006). They bind specifically in the bacterial ribosome at the A-site of the decoding region of 16S rRNA leading to bacterial cell death. Recently growing resistance to aminoglycoside antibiotics has been observed among bacterial species such as methicillin-resistant *S. aureus* (MRSA) (Minowa *et al.*, 2006). The primary mechanism of aminoglycosides resistance is due to aminoglycoside-modifying enzymes (AMEs), which modify the antibiotic compounds by acylation, nucleotidylation, adenylation and phosphorylation. These enzymes include; aminoglycoside acetyltransferases (AATs), aminoglycoside nucleotidyltransferases (ANTs), aminoglycoside adenylyltransferase (AADs) and aminoglycoside phosphotransferase (APHs). Isepamicin **(32)** is a relatively new broad-spectrum aminoglycoside that is active against endemic or epidemic strains of Gram-negative *Bacilli* and *Staphylococci*. Its superior spectrum of action compared to amikacin **(33)** and other aminoglycosides is attributed to it's stability to acylation (Magnet & Blanchard, 2005).

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(32) R_1 =  R_2 = —NHCH$_3$ R_3 = H

(33) R_1 =  R_2 = —NH$_2$ R_3 = CH$_2$OH

2.3 Resistance mechanisms

The rapid emergence of bacterial strains resistant to all existing antibiotics has made it imperative that new approaches to the discovery of more effective antibiotics be developed. (Vlahovic-Palcevski *et al.*, 2001; Rice, 2003; Hsieh, 2005; Petrikkos *et al.*, 2007). Moreover, this has lead to the rapid increase of multi-drug resistance, simultaneous resistance of cells to several structurally and functionally different cytotoxic compounds owing to their increased efflux from the cells (Broccolo *et al.*, 2006). This defence mechanism has been conserved in evolution from bacteria to humans and results in decreased efficacy of antimicrobial therapy and tumour treatment (Cernicka *et al.*, 2007; Petrikkos *et al.*, 2007). Consequently, there is a need to enhance the development of novel types of antimicrobial agents and/or classes of antibiotics targeting unique mechanisms and pathways to fight the problem of drug resistance (Gibbons *et al.*, 2005; Mishra *et al.*, 2006; Shiu & Gibbons 2006). The ability of a microorganism to survive in the presence of antibiotics depends mainly on its capability to: (a) modify the original antimicrobial compound in such a way that the drug no longer binds to intended target; (b) reduce the accumulation of the drug at the action site; (c) synthesize enzymes that inactivate the antimicrobial agent; (d) select for genes that ward off destruction by giving rise to enzymes that degrade antibiotics or that chemically modify and so inactivate the drugs; (e) eliminate entry ports for the drugs or manufacture pumps that export antibiotics before they have a chance to find their intra-cellular targets; (f) exporting the antibiotic agent, minimizing its concentration to a level that does not interfere with the functioning of the cells (McManus, 1997; Zhao *et al.*, 2003; Walsh *et al.*, 2005; Siroy *et al.*, 2006; Zhang *et al.*, 2007).

Bacterial resistance at times involves more than one of the mechanisms listed. This is particularly noticeable in the case of Gram-negative microorganisms, which often use their outer membrane as an additional resistance mechanism against drugs whose targets are

located inside the bacterial cell wall (Setti & Micetich, 1998; Zhang *et al.*, 2007). The organisms become resistant by acquiring genes encoding proteins that protect them from the effects of the antibiotic. In some cases the genes arise by mutation; in others, they are acquired from other bacteria that are already resistant to the antibiotic (Hinman *et al.*, 2006). The situation has now become alarming, with the emergence of pathogenic strains that show multiple resistance to a broad range of antibiotics. It is the biggest challenge to drug development and the main cause of mortality (McManus, 1997; McClerren *et al.*, 2005).

The search for new classes of antibacterial agents to overcome growing bacterial resistance focuses largely on two approaches. The first is to identify novel targets where no pre-existing resistance exists (Sanders, 1989; Kahne *et al.*, 2005). This approach, however, has been met with little success in identifying quality lead compounds due to target validation and low hit rates from high throughput screening. The second approach involves exploring existing clinically proven targets for new chemical entities or modifying existing drugs with limited or no cross-resistance to existing agents, eliminating the risk of target validation. This approach has previously been used successfully to identify new series of antibacterial agents (Projan, 2002; Ellsworth *et al.*, 2006). It has been particularly effective toward the generation of newer classes of macrolide, tetracycline, and cephalosporin antibiotics and leading significantly to the discovery of newer generations of vancomycin derivatives (Wise & Andrews, 1994; Chu, 1999; Ellsworth *et al.*, 2006).

2.4 Potential of tropical fungi for discovery of new and novel antibacterial compounds

Traditional targets such as the cell wall, nucleic acid, and protein synthesis remain robust candidates for identifying new antimicrobial compounds and new targets. However, these likely under-represent the potential targets available for antibiotic discovery. As a result, there has been a call for exploration and screening of non-canonical targets in antimicrobial drug discovery (Coates *et al.*, 2002; Nathan, 2004; Brown & Wright, 2005; Ejim *et al.*, 2007).

Tropical habitats house the greatest diversity of organisms. The organisms are abundant and more species-diverse than other geographical regions in the world (DeWalt *et al.*, 2006). Fungi live in intense environments, surrounded by other fungal types, bacteria, and all manner of predatory soil-inhabiting invertebrates. Advances in biotechnology, automation and information technology have changed the direction of screening for novel molecules. Sensitive, target-specific, mode of action screens have been designed to search for small molecules that can be developed as new drugs by intervening at critical points in biochemical

pathways (Butler, 2004). The discovery of penicillin by Alexander Fleming in 1928, its commercialisation as well as the development of synthetic penicillins has revolutionalized drug discovery. Following the success of penicillin, drug companies and research groups assembled large microorganism culture collections in order to discover new drugs (Nicolaou *et al.*, 2000a). Despite the outstanding success of penicillins and cyclosporin A, many microorganisms, particularly the higher fungi, have not been systematically investigated for biologically active molecules (Zell, 1991; Rosa *et al.*, 2003).

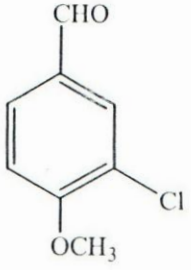
The synthesis of secondary metabolites is often associated with cellular differentiation and the complex life cycles of many fungi indicate a considerable degree of differentiation. It is possible that highly differentiated fungi such as basidiomycetes may produce a rich array of secondary metabolites (Trina, 1992). Fungi are exceedingly diverse and abundant, a fact often overlooked by conservationists interested in global biodiversity (Hawksworth, 2001), a high proportion of which has not yet been discovered and less than 1.0% of which are held in culture collections. The potential for new medicines from natural sources can best be illustrated by the fact that more than 25% of prospective drugs in current use originate from plant sources (Newman *et al.*, 2003). Fungi are ten times more abundant than plants and produce as rich an array of secondary metabolites as plants (Vinning & Chatterfee, 1982).

2.5 Antibacterial compounds from fungal sources

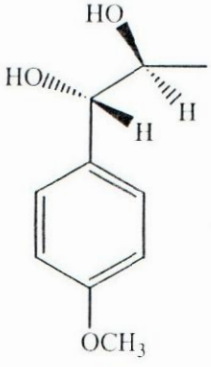
Many compounds that possess antibacterial activity have been isolated by fermentation of fungi. Examples are *p*-anisaldehyde (**34**), 3-chloro-anisaldehyde (**35**) and (4-methoxyphenyl)-1,2-propanediol (**36**), which were isolated from fermentations and from natural substrates of general common-wood and forest-litter degrading fungi like *Pleurotus pulmonarius*, *Bjerkandera adusta*, *Hypholoma fasciculare* and *Pholiota squarrosa*. *p*-Anisaldehyde and (4-methoxyphenyl)-1,2-propanediol have weak anti-fungal activity (Dejong *et al.*, 1994).



(34)

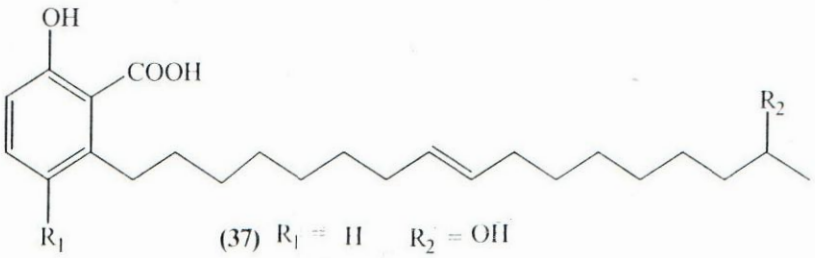


(35)



(36)

Merulinic acid A (**37**), B (**38**), and C (**39**) were isolated from the fruiting bodies of *Merulius tremellosus* and *Phlebia radiata*. They are structurally related to the skin irritant acids isolated from acardiaceae (Giannetti *et al.*, 1978). Merullinic acids showed antibacterial effects (MICs 4-10 μg/ml) while fungi were not affected. Moreover, these metabolites had strong haemolytic effects on erythrocytes.

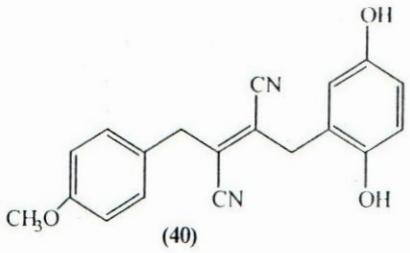


(**37**) R_1 = H R_2 = OH

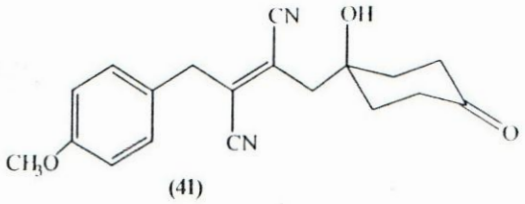
(**38**) R_1 = OH R_2 = H

(**39**) R_1 = H R_2 = H

Darlucin A (**40**) and B (**41**) are xanthocillins, isocyanides, obtained from mycophilic fungus, *Sphaerellopsis filum* (*Darluca filum*) (Zapf *et al.*, 1995b). They are active against yeast and filamentous fungi with low MIC values (2.5-20 μg/ml) for both Gram-positive and Gram-negative bacteria.

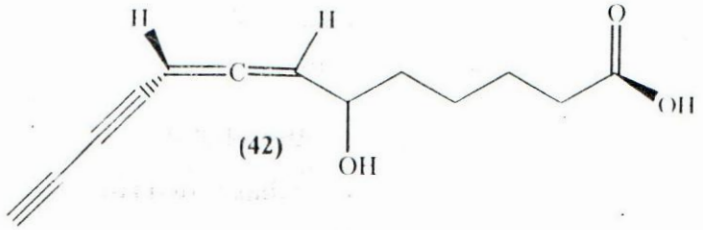


(**40**)



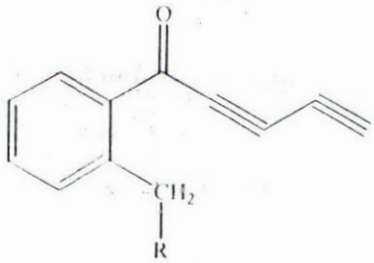
(**41**)

Basidiomycetes produce polyacetylenes for example 3, 4, 5, 6 - tetrahydro-6-hydroxymycomycin (**42**) is an allenic polyacetylene active against *S. aureus* and *B. subtilis* (MIC 2-4 μg/ml) and *Candida albicans* (10 μg/ml) (Schilmigann *et al.*, 1995). However this is very unstable (Zapf, *et al.*, 1995a).



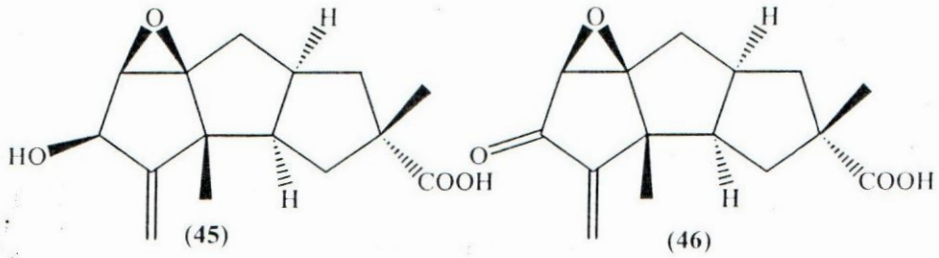
(**42**)

The peniophorines A (**43**) and B (**44**) (MIC 0.5 μg/ml) from *Pheniophora affinis*, have strong antibacterial activity against *Proteus vulgaris* and several *Staphylococcus* species (Mori *et al.*, 1987).



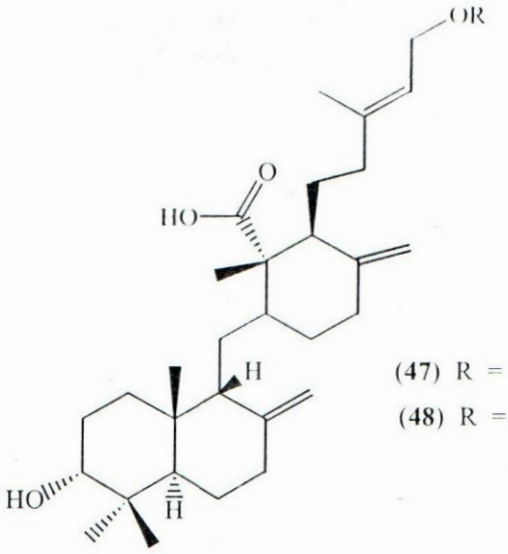
(**43**) R= $COCH(CH_3)CO(CH_2)_2OH$
(**44**) R = CO_2H

Stereum species are known to produce sesquiterpenes with hirsutane, sterepolide, and sterpurene skeletons, benzaldehydes, and benzofurans (Yun, 2001; Liu *et al.*, 2006). Hirsutic acid C, (**45**), and complicatic acid, (**46**), were isolated from the timber decomposing basidiomycete, *Stereum complicatum*. Their biosyntheses were investigated using [2-^{14}C] mevalonate as precursor (Mellows *et al.*, 1973). Complicatic acid exhibits moderate anti-microbial activity against *S. aureus* probably due to theα, β-unsaturated ketone group (Kupka *et al.*, 1983).



(**45**) (**46**)

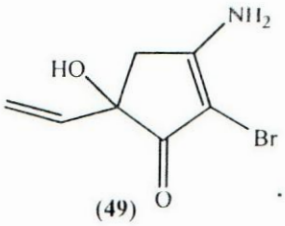
Basidiomycetes of the genus *Tricholoma* occur ubiquitously, with several species highly valued due to their medicinal properties or as culinary delicacies. The genus is known to produce a multitude of different secondary metabolites derived from the polyketide, fatty acid, alkaloid, and terpenoid biosynthetic pathways (Francia *et al.*, 1999). Trichomycins A (**47**) and B (**48**) were isolated from *Tricholoma* species (Tricholomataceae) by activity-guided fractionation following their antibacterial activity. The two compounds were found to have a hitherto unreported triterpenoid skeleton. The structures and relative stereochemistry of the trichomycins were determined through extensive 2D NMR spectroscopy. The two compounds were found to be selective against Gram-positive bacteria, *S. aureus* with activity of IC_{50} =12 μM and *S. pneumoniae*, with activity of IC_{50} = 5 μM for trichomycin A and IC_{50} = 6 μM for trichomycin B (Ovenden *et al.*, 2005).



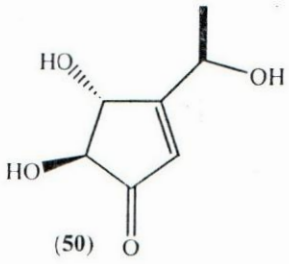
(47) R = CHO

(48) R = H

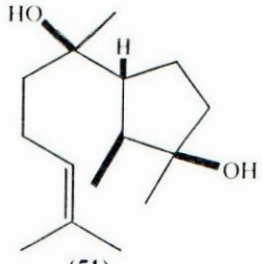
Cyclopentenone derivatives, bromomyrothenone B (**49**), botrytinone (**50**), and cyclonerodiol (**51**) were isolated from the marine derived fungus *Botrytis* sp. These compounds have shown a wide range of biological activities, such as tyrosinase inhibition activities, antibacterial, leishmanicidal, plant growth inhibition, glucose-6-phosphate translocase T1 inhibition and melanogenesis inhibition. The activities have been in the range (IC_{50} and MIC, >100 μg/mL, respectively (Li *et al.*, 2007).



(49)



(50)



(51)

2.6 Flavonoids

Flavonoids are found majorly in the plant kingdom, and are products of secondary metabolism via the shikimate and acetate pathways (Bravo, 1998). Over 5000 flavonoids have been isolated from plants, making it the largest and most widely distributed class of polyphenols (Cook & Samman, 1996). The basic structure of flavonoids is 2-phenylbenzopyran-4-one (Figure 2), which consists of two aromatic rings A and B linked through a heterocyclic pyrone ring (C) (Brown, 1980; Cushnie & Lamb, 2005; Wistuba *et al.*, 2006). The carbons can be substituted from any of the numbered positions. Ring B is the mostly easily oxidised. Flavonoids have common physiochemical properties, and biological

activities amongst them. They have two major UV absorption bands, around 250-285 nm and 320-385 nm, correlating to the rings A and B respectively (Yao *et al,* 2004). They provide pigmentation to the plants, which is important for the organism reproductive purposes. They have also been postulated to participate in energy transfer pollen germination, and resistance against infection (Ross & Kasum, 2002; Witsuba *et al.*, 2006).

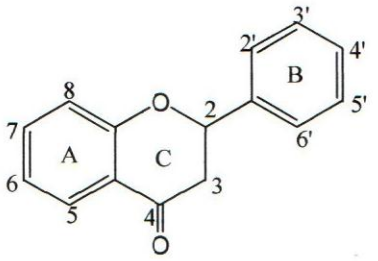
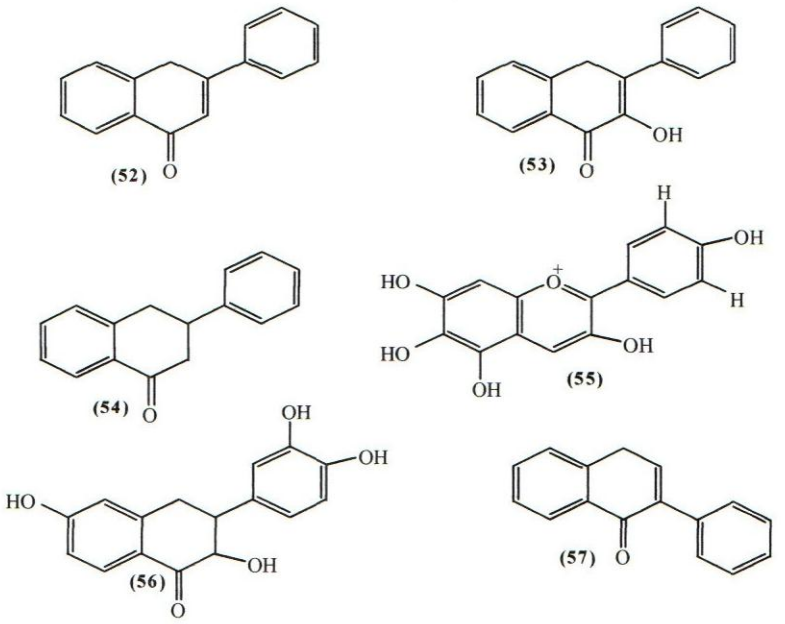


Figure 2: Basic structure of flavonoids

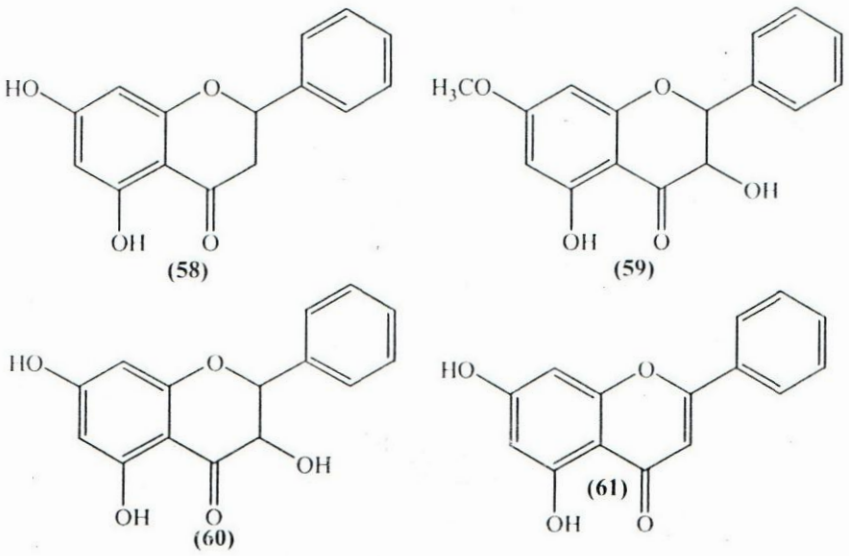
Flavanoids are found as monomers, dimmers or oligomers. They can classified according to biosynthetic origin on the basis of their chemical structure, which include; flavones **(52)**, flavonols **(53)**, flavonones **(54)**, anthocynidines (e. g. aurantinidin) **(55)**, flavanols (catechins) **(56)** and isoflavonoides **(57)**.



Some classes, for example chalcones, flavanones, flavan-3-ols and flavan – 3, 4 - diols, are both intermediates in biosynthesis as well as end products that can accumulate in plant tissues. Other classes are only known as end products

of biosynthesis, for example anthocyanidins, proanthocyanidins, flavones and flavonols. Two additional classes of flavonoid are those in which the 2-phenyl side chain of flavanone isomerises to the 3 position, giving rise to isoflavones and related isoflavonoids (Crouch *et al.*, 1999; Harborne & Baxter, 1999; Silayo *et al.*, 1999).

Flavonones can further be divided into sub groups, which include Pinocembrin **(58)**, pinostrobin **(59)**, pinobanksin **(60)**, and chrysin **(61)** skeletons (Fahey & Stephenson, 2002).



2.6.1 Antimicrobial activity of flavonoids

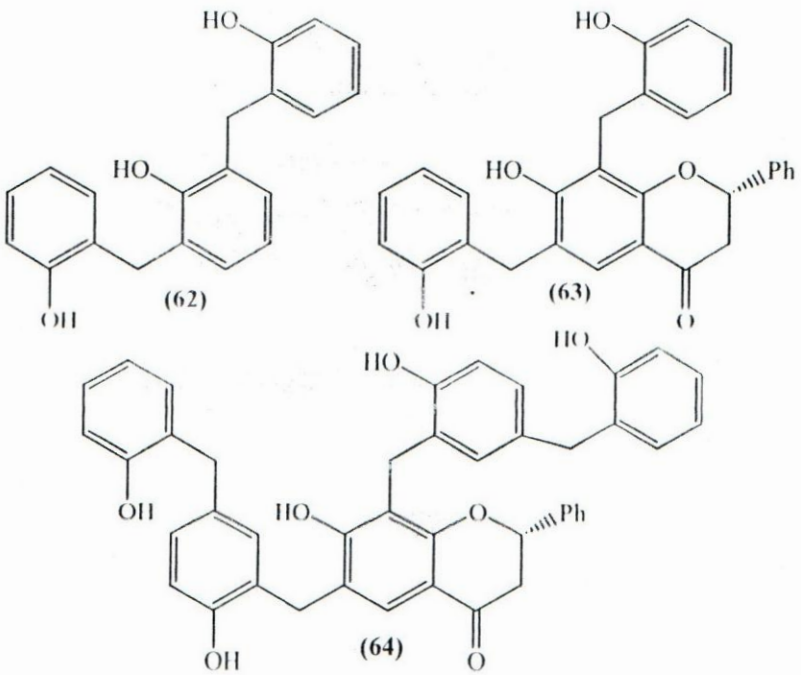
Flavonoids are becoming the subject of medical research. Increasingly, they are thought to be responsible for many biological and pharmacological activities including anticancer (Matsuno, 1995), anti-inflammatory (Wang, *et al.*, 1993), antimicrobial (Havsteen, 1983; Kujumgiev *et al.*, 1999; Koo *et al.*, 2000; Usia *et al.*, 2002; Mohammadzadeh *et al.*, 2007) and antioxidant effects (Basnet *et al.*, 1997; Nieva *et al.*, 2000). The antimicrobial activity has been speculated to be as a result of the flavonoids inhibiting the GTPase activity of FTsZ. This activity is highly related to their polyphenolic structure (Urgaonkar *et al.*, 2005).

Flavanones in particular have shown very interesting activities, which has increased their rate of being investigated. Pinocembrin, pinobanksin, and chrysin, are obtained primarily from propolis or honey, as well as other more omnipresent phytochemicals, including quercetin, kaempferol, myricetin, ellagic acid, and hesperetin (Fahey & Stephenson 2002). Pinocembrin, a flavanone and a potential inducer of phase 2 enzymes, was originally

identified in 1970 by Villanueva and colleagues on the basis of its antibacterial activity (Villanueva *et al.*, 1970). Chrysin is an inhibitor of xanthine oxidase and as such may be effective in combating the symptoms of gout (Nakanishi *et al.*, 1990).

Pinocembrin, pinostrobin, chrysin, pinobanksin and related flavonoids are purported to have antibacterial, ovicidal, and larvicidal activity and local anaesthetic activity in mammals (Del Rayo *et al.*, 1991; Cao *et al.*, 1997; Uda *et al.*, 1997; Bremner & Mayer, 1998; Yannai *et al.*, 1998).

Zantrin, **(62)**, which was discovered in a high-throughput *in vitro* screen for inhibition of FtsZ GTPase activity, possesses a polyphenolic structure reminiscent of several natural products that exhibit potent antimicrobial activity. Dichamanetin **(63)** and 2‴-hydroxy- 5″-benzylisouvarinol-B **(64)**, isolated from *Uvaria chamae* and *Xanthophloea afticana* respectively, have exhibited comparable MIC values to that seen with zantrin when evaluated against a variety of bacterial strains. These compounds show a high level of activity against Gram-positive bacteria (e.g. *S. aureus* (2.5 μM, 1.7 μM, and 10.7 μM respectively) and *B. subtilis* (1.25 μM, 1.7 μM and 2.6 μM respectively), and furthermore, the MIC values are comparable to those of clinically relevant antibiotics (Urgaonkar *et al.*, 2005; Wistuba *et al.*, 2006).



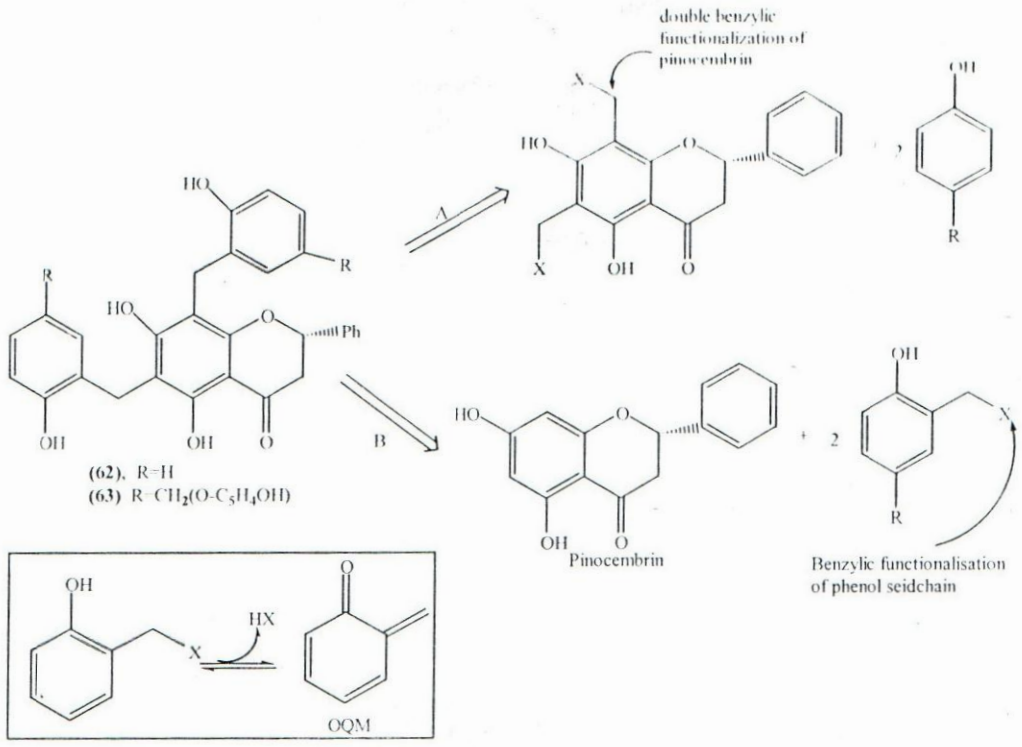
The antibacterial activity of flavonoids is being increasingly documented (Aladesanmi *et al.*, 1986; Sato *et al.*, 2000; Cushine & Lamb, 2005). Many other phytochemical preparations with high flavonoid content have also been reported to exhibit antibacterial

activity (Mahmoud *et al.*, 1989; Torrenegra *et al.*, 1989; Tarle & Dvorzak, 1990; Al-Saleh *et al.*, 1997; Tereschuk *et al.*, 1997; Singh & Nath, 1999; Rauha *et al.*, 2000; Quarenghi *et al.*, 2000). Examples of such flavonoids are apigenin (Palacios *et al.*, 1983), pinocembrin (Palacios *et* al., 1983; Pepeljnjak & Kosalec, 2004), and ponciretin (Basilea *et al.*, 1999). Some cases have reported synergy between naturally occurring flavonoids and other antibacterial agents against resistant strains of bacteria (Hamilton-Miller & Shah, 2000; Cushnie & Lamb 2005).

When reports of the antibacterial activity of flavonoids are compared, the results appear widely conflicting. However, such discrepancies could perhaps be attributed on occasion to different assays being used (Sato *et al.*, 2000). Many different assays are employed in flavonoid research, including the agar dilution technique (Cushnie & Lamb 2005), the paper disk diffusion assay (Osawa *et al.*, 1992), the hole-plate diffusion method (Tereschuk *et al.*, 1997), the cylinder diffusion method, the broth macrodilution technique (Basilea *et al.*, 1999) and the broth microdilution technique (Liu *et al.*, 2000). The antibacterial activity of flavonoids have been associated to the B ring, which may play a role in intercalation or hydrogen bonding with the stacking of nucleic acid bases and that this may explain the inhibitory action on DNA and RNA synthesis (Mori *et al.*, 1987).

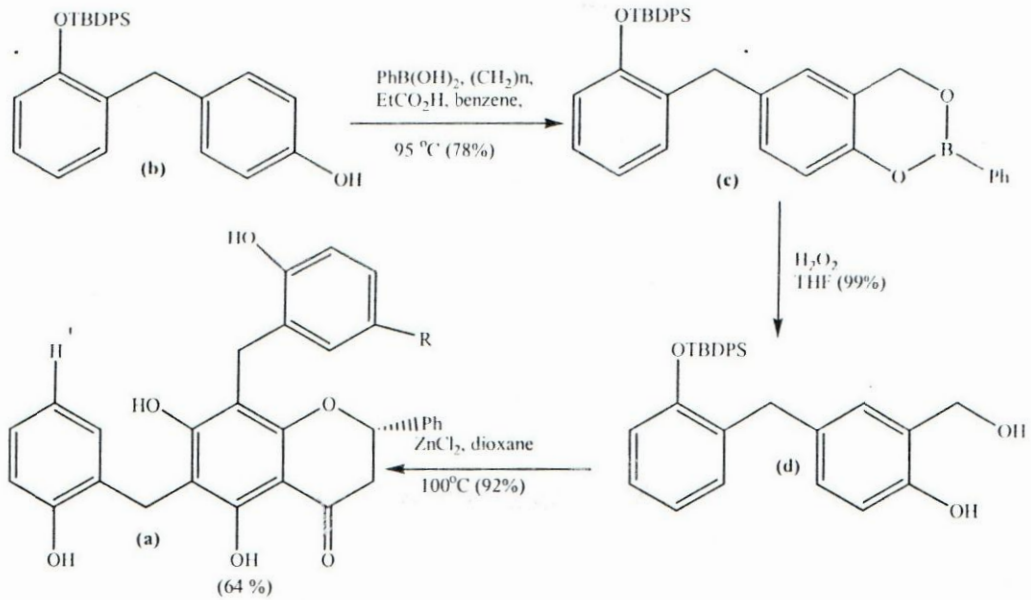
2.7 Total synthesis of 2‴- hydroxy- 5″-benzylisouvarinol-B as an example of natural product compounds as lead templates

While naturally occurring flavanones have attracted the attention of synthetic chemists and biologists alike, benzylated flavanones are quite rare, and as such no efficient syntheses of these flavanones have been reported (Dewick, 1975; Choi *et al.*, 1996; Kim & Lee, 2006). The substituent symmetry of **(62)** and **(63)** suggests that a common core could be elaborated to provide both molecules. This is well illustrated in the retro-synthetic scheme in Scheme 2. The formation of benzylic carbon-carbon bonds with electron rich arenes is often achieved via ortho quinine methide (OQM) intermediates, which can be accessed by a variety of routes.



Scheme 2: Retro-synthetic analysis of (62) and (63)

The synthesis of 2'''-hydroxy-5''-benzylisouvarinol-B **(a),** involves three major steps, (Schem 3) leading to high yields of products. The phenol **(b)** is made by selective monoprotection of commercially available 2,4'-dihydroxydiphenylmethane. Phenol **(b)** is hydroxylated using phenylbromic acid and paraformaldehyde to produce the boronate ester **(c).** Hydrolysis of **(c)** with hydrogen peroxide provide the requisite benyzlic alcohol **(d)**, which is then converted to dichamanetin in high yield after benzylation and deprotection.



Scheme 3: The total synthesis of 2'''-hydroxy- 5''-benzylisouvarinol-B (64% overall yield)

2.8 Application of modern spectroscopic techniques in natural products chemistry

2.8.1 Isotopic labelling

Labelled precursors, enriched in one or more positions with radioisotopes, have been extensively used in biosynthetic studies to detect incorporation, to examine the origin of, and to follow the fate of specific parts of a molecule or atoms in a pathway (Jacobs, 1997). Nuclides that can be incoporatedin biological substrates include carbon, hydrogen, oxygen, nitrogen and sulphur, which are the most abundant (Vederas, 1985; Simpson, 1998). Mass spectrometric and nuclear magnetic resonance (NMR) spectroscopic techniques have been developed to analyse enrichment of heavy atoms in target molecules. Mass spectrometry detects mass differences between natural abundance isotopic distribution and isotopically enriched molecules and it is therefore able to detect any incorporation of a heavy isotope. In contrast, NMR analysis is restricted to nuclei which posses a nuclear magnetic moment (nuclear spin $I \neq 0$). Among the elements in living organisms that can be radio labelled are, ^{1}H, ^{2}H, ^{3}H, ^{13}C, ^{14}N, ^{15}N, ^{17}O, ^{31}P, and ^{33}S. The most significant information gained from the application of selectively ^{13}C-labelled precursors (or multiple labelled precursors in specific positions) in feeding experiments is the location of the label within the target molecule formed during the biosynthetic processes and, in a 'multistep version', being able to follow the flux of the label through biosynthetic sequences (Robins *et al.*, 1994).

A fundamental problem in NMR spectroscopy is its inherent low sensitivity, and even modern high field spectrometers often operate at their detection limits. These limitations are especially true for biosynthetic studies. However, there are several strategies for enhancing sensitivity, that include increasing the magnetic field strength, improving probe head design, miniaturization and cryotechnology. Nevertheless, these methods still need considerable spectrometer time, especially when employed with samples of very low concentrations (Schneider *et al.*, 2003).

2.8.2 Structure elucidation of bioactive secondary metabolites

The advent of new probe technology (Gary *et al.*, 1998; Keifer, 2003) in NMR technology and higher magnetic fields has led to a significant shortening in acquisition time of NMR data, and the structure elucidation of secondary metabolites can be achieved routinely on amounts less than 1 mg (Neri & Tringali, 2000; Reynolds & Enriquez, 2002). Progress has also been made in automated structure solving algorithms (Elyashberg *et al.*,

2004), but presently none can rival the structure elucidation skills of an experienced natural product chemist. However, these programs can be a valuable tool in the search for alternative structures that fit the same NMR data (Butler, 2004; Pauli *et al.*, 2005).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Chemicals and reagents

All chemicals, solvents and reagents/materials used in this work were of pure analytical grade. In cases where general purpose (GPR) reagents/solvents were employed, double distillation was used to remove impurities.

3.2 Preparation of malt liquid media

The growth medium used in this project was malt media. The preparation of the medium was adapted from Anke *et al.*, 1989. The medium constituted 10 g of molasses (Chemilil Sugar Company, molasses C), 4 g of glucose (Kobian Kenya Limited, Nairobi) and 4 g of yeast in 1 litre of tap water. The pH was adjusted to 5.5 using 1.0 M NaOH and 1.0 M HCl and the medium was sterilized immediately by heating in an autoclave twice (to a temperature of 121 °C and pressure of 3 bars) for 15 minutes then left to cool.

3.3 Preliminary preparations

3.3.1 Preparation of test plates for agar diffusion assay

The test plates were prepared by seeding with cells or spores of *Escherichia coli* *Salmonella typhimurium* and *C. albicans*, respectively and kept at 4 °C. Laboratory safety precautions were highly observed during this research work. This included working in a lamina flow hood (under sterile conditions), wearing masks and sterilizing hands every time with 70% alcohol.

3.3.2 Preparation of potato dextrose agar (PDA) media

Potato Dextrose Agar (PDA) plates were used to grow the basidiomycete JO5191 before being cultured in liquid submerged cultures. The plates were prepared by heating 9.75 g potato dextrose agar suspended in 250 ml of distilled water in an autoclave and cooled to 40 °C before dispensing 15 ml per sterile petri dishes under lamina flow hood. The basidiomycete JO5191 was obtained from agar slants in the culture collection at IBRL, Egerton University.

3.4 Cultivation of the basidiomycete JO5191

From the freshly prepared PDA media the basidiomycete JO5191 was sub cultured and incubated at room temperature. From a well-grown plate, four agar plugs having mycelium were aseptically introduced into 250 ml sterile autoclaved liquid malt media. The inoculated liquid culture was left to grow under ambient conditions as still cultures, with regular agitations to equilibrate oxygen demand in the growth culture for seven (7) days. After the growth had picked up, they were transferred in 1 litre scale replicates. The growth of the culture was followed closely by monitoring the glucose levels using glucose-testing stripes while ensuring that there was no bacterial contamination. The cultivation took a period of twenty-two (22) days for glucose levels to be depleted. A total of 25 replicates of 1 litre scale cultures of JO5191 were prepared in this work.

3.5 Preparation of the crude extracts from culture broth and mycelium

Immediately the growth was stopped, culture filtrate was separated from the mycelium by filtration using a Buchner filtration system. The mycelia were soaked in acetone for 12 hours under constant agitation and the acetone removed under reduced pressure. The resulting aqueous solution was extracted with ethyl acetate (four times volume). The combined ethyl acetate extract was dried with anhydrous sodium sulphate, filtered and evaporated to dryness. The residue was transferred into screw-capped glass vials under an inert atmosphere to dry to a constant weight. The weight of the extract was determined and then stored at 4 °C.

The culture broth was extracted by liquid-solid adsorption using Mitsubishi HP21 (DIAION) resin initially pre-treated with 1:1 aqueous methanol. This was then introduced carefully into a vertically mounted glass column, washed with distilled water and drained until all the methanol had been removed. The packed column was always left with 5 cm of distilled water above the resin to prevent drying. The culture filtrate was washed through the resin with 4 column volumes of distilled water, followed by elution with 100% acetone (1 L) and then 100% methanol (1 L). The eluents of the organic solvents were collected separately.

The acetone and methanol eluents were concentrated under reduced pressure at 30 °C. The methanol eluent was dried as above. The aqueous residue from both the methanol and acetone extract was then extracted with of ethyl acetate, dried with anhydrous sodium sulphate and then concentrated under reduced pressure. The concentrates were carefully

transferred into screw-cap glass vials. The concentrated crude extract was immediately tested for antibacterial activity and stored at 4 °C waiting further processing.

3.6 Preparing crude extract for loading onto the chromatographic column

This was done by weighing 2 g of silica gel (Merck KGaA, Germany) in a 50 ml glass beaker and to this the extract added drop wise, then allowing the methanol solvent to evaporate in a running fume hood. This was repeated until all the extract was adsorbed onto the silica gel particles. The evaporation took place in a controlled manner by covering the beaker with multiply perforated aluminium foil. This also helped to avoid spillage of the fine silica gel particles. The silica gel adsorbed extract was dried to constant dry weight.

3.7 Drying of the organic extracts

The actual dry weight of all the crude extract from the 25 replicates of 1 litre scale cultures was determined by transferring the extracts into a pre-weighed container, the extracts were transferred using minimal amount of methanol. The contents were dried completely under an inert atmosphere of nitrogen at 45 °C in a fume hood. Then the net weight of the extract plus cooled container was determined from which the actual weight of the crude extract was determined by difference.

This was the last part of the research work, done at Egerton University, and the remaining work, was done at The University of Sydney.

3.8 Chromatographic analysis and processing of the crude extract

Normal and reverse phase chromatographic techniques were used. The actual antibacterial compounds in the crude extracts and the enriched fractions from column chromatography (CC) were analysed using thin layer chromatography (TLC). Column chromatography was carried out at 1 atmosphere pressure under the influence of gravity while TLC plates were developed in an equilibrated chamber by capillary action. Preparative TLC plates were used for further purifications

3.8.1 Thin Layer Chromatography (TLC)

Analytical TLC was used in this work to determine the best working range of a solvent system to optimise purification of the targeted compound(s) in column chromatography. The technique was also used to detect the purity of the pure compounds. Silica gel pre-coated aluminium plates (Macharey Nagel, 0.25mm, Dueren, Germany, Merck

20 X 20 cm, silica gel 60F KGaA, Germany) and glass pre coated preparative TLC plates (Macharey Nagel, 1.00cm, Dueren, Germany Merck 1 cm, silica gel 60F KGaA, Germany) were used in this work.

Fractions from column chromatography were subjected to thin layer chromatography using aluminium-coated silica gel TLC plates. The TLC plate loaded with samples was developed using a solvent system of hexane and ethyl acetate (70:30), in a saturated TLC development chamber. The developed TLC chromatograms were allowed to dry completely before the spots were visualised by spraying with a freshly prepared anisaldehyde solution and heating with a hot air gun until the spot developed. The spots were also viewed with a UV lamp (λ = 254 nm (short) and 365 nm (long)). On the basis of the R_f values and the colour of the spots, the eluent fractions were pooled to give six and fourteen main fractions for the culture crude extract and the mycelium crude extract, respectively. The six (6) and fourteen (14) fractions were labelled EMK_1- EMK_6 and EMM_1- EMM_{14} respectively. The enriched fractions were tested for antibacterial activity and the active ones further subjected to bioautographic analysis.

Bioautographic analysis involved spotting the fraction on the TLC plates and developing in a solvent system of hexane - ethyl acetate, (80:20). After allowing the developed TLC chromatograms to dry completely, strips were cut longitudinally along the development path of the chromatograms. The strips having the separated spots were placed on agar seeded with the test organisms and incubated at 37 °C for 16-24 hours. An illustration of a developed TLC chromatogram and bioautographic analysis set-up is shown in Figure 3and Figure 4 respectively.

The results were validated by Gram staining. The inoculums were prepared by seeding each of the test organisms (*S. typhimurium, E.coli* and *C. albicans*) into 100ml conical flasks containing sterile nutrient both (10 ml), the flasks were incubated overnight at 37 °C while shaking at 200 rpm. The inoculums were applied to the run TLC plates using a sterile cotton swab. The seeded chromatograms were subsequently incubated overnight at 37 °C in a humidified chamber. The incubated plates were then sprayed with nitro-blue tetrazolium (Sigma Ltd) in order to stain the live test organisms and then re-incubated for 1 hour to develop. The undeveloped areas of the plates indicated the presence of growth-inhibiting compounds.

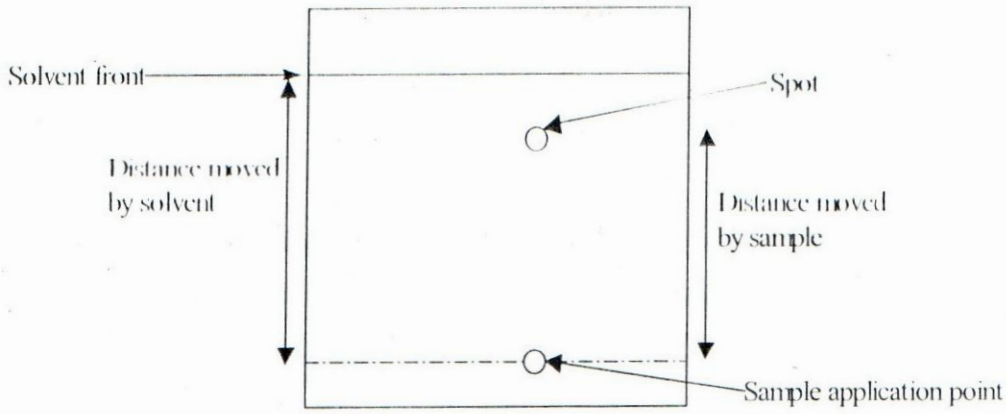


Figure 3: Illustration of a developed TLC chromatogram

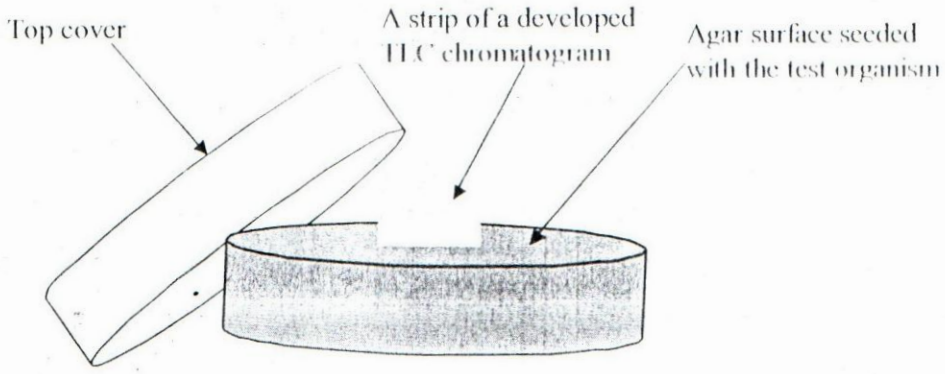


Figure 4: Illustration of bioautographic analysis set-up

The active fractions were further subjected to TLC with different solvent systems in order to determine the best solvent system for elution during further purification with column chromatography. Hexane-ethyl acetate (70: 30) solvent mixture was determined thereafter to achieve good separation of the sample components.

3.8.2 Column chromatography

The technique was used to fractionate the crude extract into enriched fractions and also purification of the compounds. Silica gel adsorbed sample was introduced as uniform disc on a prepared silica gel column. The disc was anchored in place with acid-washed sand, to avoid turbulence while adding the mobile phase. The column was eluted with a mobile phase of a discrete linear gradient with polarity ranging from 100% hexane to 100% ethyl acetate. The gradient ratios that were successively used were: 1:0, 7:3, 1:1, 3:7 and 0:1 of hexane to ethyl acetate, respectively. Each stage was eluted with a total of 200ml of the

mobile phase. The column was finally eluted with 200 ml of 100% methanol to ensure that no sample remained in the column. The eluents were collected as fractions of 20 ml volumes. The total eluent fractions were pooled into main fractions depending on the R_f values of the constituent compounds. The numbering was according to the order in which they were eluted from the column. The collected fractions were concentrated under reduced pressure using a rotary evaporator and immediately transferred into clean screw capped glass vials, before being stored at 4 °C awaiting further processing.

Semi preparative HPLC was used for further purification of the compounds. This was achieved by using isocratic elution on a waters HPLC 501 pump and detector, at λ = 254 nm and at a flow rate of 1ml/min with a mobile phase made of acetonitrile; water; acetic acid (60:40: 0.1) in conjunction with reverse phase Phenomenex jupiter 4μ proteo – 90R (250 x 21.20 mm) and Alltech econosil C_{18} micron (250 x 7.8 mm) columns.

3.8.3 Test for anti-bacterial activity.

The antibacterial activity was used as the main guiding principle in the isolation of the compounds. This was carried out using agar diffusion assay technique for crude extract and enriched fractions. Briefly, by using adjustable (analogue) volume micropipette, 20μl volumes of 1 ml MeOH solution of either the crude extract or the enriched fractions were applied to paper discs (7 mm) and allowed to dry completely through solvent evaporation. The impregnated paper discs were carefully placed onto the agar surface of the test plates with *E. coli,* (ATCC 11775, NTCC 9001), *S. typhimurium* (ATCC074). In order to validate the antibacterial activity of the compounds produced in the cultures, *C. albicans* (yeast, a wild species isolated in Njoro and also a different wild one isolated from Australia) was used to check if there was any antifungal activity. This was followed by incubation at 37 °C for 18 hours. The results were evaluated as inhibition zones around the discs for the active fractions. The results were quantified by measuring the diameter of the inhibition zones.

3.8.4 Determination of miminum inhibitory concentration (MIC)

The MIC for the purified fractions was carried out using agar diffusion assay technique. Different weights of the purified fractions were dissolved in 1 ml MeOH to make solutions of different concentrations (1-20μg/ml). The samples were then applied to paper discs (7 mm) and allowed to dry completely through solvent evaporation. The impregnated

paper discs were carefully placed onto the agar surface of the test plates with *E. coli.* (NTCC 9001), *S. typhimurium* (ATCC074) and *C. albicans* (yeast, a wild one isolated from Australia). This was followed by incubation at 37 °C for 18 hours. The results were evaluated as inhibition zones around the discs for the active fractions. The disc with the least zone of inhibition was used to calculate the MIC with regard to the amount of sample applied.

3.9 Structure elucidation experiments

The compounds obtained were analysed using spectroscopic techniques.

3.9.1 Ultraviolet /Visible spectroscopy

The UV spectra of the purified compounds were determined using a double beam UV/VIS spectrometer (λ_{16}, Perkin Elmer, Langen) in the range 200-600 nm. A quartz sample cell (Perkin Elmer, Langen) with a 3 ml volume and a 1 cm optical path length was used for the reference and the sample beams. Background correction and all spectra were recorded in MeOH. A small amount (microlitre quantity) from a 1 µg/µl solution of a given compound was added to 3 ml MeOH in a sample cell.

3.9.2 Infra Red spectroscopy

The IR spectra of the compounds were recorded using FT-IR Spectrometer (IFS 48, Bruker, Karlsruhe). The samples were prepared as 3% (w/w) KBr pellets, by mixing the dried sample with potassium bromide under anhydrous conditions. The mixture was finely powdered, then pressed under high pressure using a vacuum pump. The pressling was placed in CO_2-free chamber before the spectrum was recorded with a background correction in the range 4000 400 cm^{-1}.

3.9.3 Mass Spectrometry

The molecular weight of the compounds was determined using mass spectrometry. Mass spectrometry was carried out using Liquid Chromatography - Electron Spray Ionisation (LCMS). The samples were run on a Finnigan/MAT TSQ 7000 LCMS/MS (San Jose CA, USA) using an Xcalibur 1.2 Data system, run in Electrospray Ionisation (ESI) mode. Infusion of the solvent, methanol was at 3ul/min, with 2 minute acquisitions and averaging the data over the analysis period. Flow Injection mode method was also used where 1 µl of the samples, were injected into a stream of methanol at 100ul/min and the data processed using the same software.

3.9.4 Nuclear Magnetic Resonance spectroscopy

One-dimensional experiments, ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded at room temperature. ^{1}H NMR spectra were recorded on a Bruker BioSpin GmBh (400 MHz) spectrometer and were reported in ppm from $CDCl_3$ internal standard (7.26 ppm). Data was reported as follows: (s = singlet, d = doublet, t= triplet, q = quartet, m = multiplet, coupling constant(s) in Hz; integration; assignment). Proton decoupled ^{13}C - NMR spectra were recorded on a Bruker BioSpin GmBh (100 MHz) spectrometer and were reported in ppm from $CDCl_3$ internal standard (77.0 ppm).

The Bruker NMR spectrometer had an inverse multinuclear 10 mm probe head equipped with shielded gradient coil. The number of transients acquired (NT) was adjusted according to the sample being run. Composite pulse decoupling was used for ^{13}C NMR measurements.

Two-dimensional experiments: COSY, HSQC, HMBC and NOESY, were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^{1}J_{CH}$ = 400 Hz and $^{n}J_{CH}$ = 10 Hz. The raw data was transformed and the spectra evaluated with standard TopSpin software for acquiring, processing and analysing NMR data.

All spectra were recorded using a flip angle of 40° and a relaxation delay of 1, 2 and 3s alternately. Quadrature detection and phase cycling were applied in all NMR measurements. The resulting free induction decays were processed by zero filling (298K) and multiplication with a Bruker window function lb=-1, gb=0.1, pc= 1 and ssb=0 for the ^{1}H NMR; lb=2, gb=0 ssb=0 and pc = 1, for the one dimensional ^{13}C NMR and lb=0, gb=0 ssb=0 and pc=0 for the two dimensional ^{13}C NMR.

3.9.5 Circular Dichroism spectroscopy

The Circular dichroism spectrum of the fairly enriched pure fraction was determined using a Jasco J-815 Circular Dichroism spectropolarimeter. The data was acquired with windows XP-based data acquisition, and processing computer. The sample was prepared by dissolving a small amount of sample in a buffer solution, (PiBuff: 1-20 mM, EDTA: <1 mM, Salt concentration: <50 mM, Detergent: none). The sample was then run after calibration of the cuvett and the instrument. This was important in determing the absolute stereochemistry of the compounds in corroboration with optical rotation.

3.9.6 Optical rotation

Optical rotation of the compounds was measured with a Perkin-Elmer 141 polarimeter at 22 °C. The specific rotation was determined using D-sodium line (λ= 598 nm) wavelength.

3.10 Melting point

A small amount of the compound was packed into an open capillary melting point tube. The capillary melting point tube was then placed in melting point temperature apparatus chamber (EZ-Melt, Stanford Research Systems) and the melting point determined by observation of the rise in temperature continuously until a constant value was reached.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 The growth of the basidiomycete JO5191

The basidiomycete strain was isolated by trapping basidiospores from a piece of a fruiting of a sprouted mushroom. The fresh morphological features as well as microscopic data confirmed that the fungus was a basidiomycete. Microscopic examination of the hyphae showed clear clamp connections when viewed under a microscope. The mushroom had a cap 4cm wide and had features that fitted quite well to *Collybia* species. However, this needs further confirmation using molecular techniques..The basidiomycete grew on PDA test plates as white cottony mycelium that covered an area of diameter 90 mm in seven days. Cultivation of the basidiomycete in the malt liquid medium, took 22 days for glucose levels to be exhausted and the residual glucose left was evidently not supporting growth.

4.2 Extraction of crude extracts

Preparation of the crude extracts from both the culture filtrate and the mycelium was done using distilled solvents. After extraction, the crude extracts were concentrated, dried and weighed. The actual dry weight of the all the crude extracts from the 25 replicates of 1 litre scale cultures was determined under inert conditions. The net weight of the extracts plus cooled container was determined from which the actual weight of the crude extract was determined by difference. The weight of the culture extract was found to be 9.115g, while that of the mycelium was 1.197g.

The crude extracts were further fractionated into enriched fractions using column chromatography (Section 3.9.2) and their weights determined by difference as given in the Table 2 (culture extract enriched fractions) and Table 3 (mycelium extract enriched fractions). Culture filtrate gave six enriched fractions coded EMK_1- EMK_6 while mycelium crude extract gave 14 enriched fractions coded EMM_1- EMM_{14}.

4.3 Results from column chromatography

Table 2: Weight of the enriched fractions from the culture extract

Fraction No. EMK	Solvent system used (hexane:ethyl acetate)	Weight of sample (mg)
1	100:0	14.7
2	80:20	14.5
3	70:30	14.9
4	50:50	43.8
5	40:60	51.8
6	30:70	1305.9

Most of the fractions were less than a gram except for fraction 6, which was more than 1 gram. Since the polarity of the solvent was increased from fraction 1 to 6, this implied that the crude extract was mostly composed of polar compounds.

Table 3: Weight of the enriched extracts from the mycelium extract

Fraction No. EMM	Solvent system used (hexane:ethyl acetate)	Weight of sample (mg)
1	100:0	8.30
2	100	37.00
3	80:20	0.60
4	80:20	1.00
5	70:30	3.80
6	70:30	2.60
7	50:50	20.10
8	50:50	3.80
9	40:60	35.10
10	40:60	25.60
11	30:70	33.40
12	30:70	1.20
13	30:70	6.40
14	30:70	1215.10

It is also evident from Table 3 that the last fraction (14) had the highest weight and this can be largely attributed to the fact that the crude extract was mostly composed of

polar compounds. The enriched culture fractions were further purified to give one major compound from all the fractions, while fraction EMK_4, consisted of two distinct compounds, which were collected separately. The weight of combined fractions $EMK_{2,3,5,6}$ were very minimal in quantity (**2.6 mg**) and hence they were not processed further. Fraction EMK_4 was further processed, since the amount (**4.8 mg**) could allow for further purification. EMK_1 was not considered for further purification because it was devoid of antibacterial activity.

4.4 Antibacterial activity

The results of the antibacterial activity for the preliminary screening of the crude extracts from the basidiomycete and those for large scale crude extracts during the research work are given in the Table 4 and Table 5, respectively. For every antibacterial test carried out, the inoculums were re-plated on fresh test plates to check the purity of the culture and it was established that all the cultures used in the research work were pure cultures. From the results, it is worth noting that the values of the inhibition zones obtained for the antibacterial activity for crude extracts during the large scale production research work were lower than those obtained for the antibacterial activity of the crude extracts during the preliminary screening. This observation may be attributed to various factors, which include the difference in the processing conditions of the crude extracts and difference in amount of sample applied on the paper disc during the antibacterial activity testing on each occasion.

The preliminary screening of the crude extract was prepared via-solvent-solvent extraction technique while the large scale processes crude extract was prepared by liquid solid desorption. The latter is an efficient method that traps most compounds especially polar secondary metabolites, where antibiotics mostly belong. This has the inherent disadvantage of picking compounds that may not have the desired activity, which may lead to dilution of the active compounds in the crude extracts. The resulting effect leads to low concentrations of the active compounds in the testing aliquots, though the volumes that are being compared are the same.

Table 4: **Anti-bacterial activity from preliminary screening of the crude extracts**

Type of bacterial strain	Diameter of inhibition zone (mm)	
	Kex	**Mex**
S. typhimurium	31	20
E. coli	42	25
C. albicans	34	15

KEY: Kex: crude extracts from culture filtrate (Extracellular secondary metabolites) Mex: crude extracts from mycelium (Intracellular secondary metabolites), (250ml (broth) / mycelium = 2 ml (MeOH) = 20µl per paper disc (Rundfilter, Ø6mm, Schleicher & Schuell, Dasel Germany). For testing from 2ml MEOH solution 20 µl was applied on paper disc.

The values for the inhibition zones from both the extracts were significant, with the culture filtrate extract showing enhanced activity against *E. coli* (42 mm), *S. typhimurium* (31 mm), and *C. albicans* (34mm), while the mycelium extract showed disparate activity against *S. typhimurium* (20 mm), *E. coli* (25 mm) and *C. albicans* (15 mm).

Table 5: Anti-bacterial activity for the crude extracts

Type of bacterial strain	Diameter of inhibition zone (mm)	
	Kex	Mex
S. typhimurium	24	9
E. coli	21	11
C. albicans	+	10

KEY: Kex: crude extracts from culture filtrate (Extra-cellular secondary metabolites) Mex: crude extracts from mycelium (Intra-cellular secondary metabolites), + = 7mm inhibition, (250ml (broth) / Mycelium = 2 ml (MeOH) = 20µl per paper disc (Rundfilter, Ø6mm, Schleicher & Schuell, Dasel Germany). For testing from 2ml MEOH solution 20 µl was applied on paper disc.

From Table 5, it is evident that the culture filtrate extract showed enhanced activity against *S. typhimurium* (24 mm) and *E. coli* (21 mm) while the mycelium extract showed disparate activity against *S. typhimurium* (9 mm), and *E. coli* (11mm). However, the mycelium extract was more active against *C. albicans* (10 mm) compared to the culture filtrate (7mm).

The enriched fractions were also tested for anti-bacterial activity to zero into the actual compounds responsible for the activity. It is worth noting that all the enriched fractions from the mycelium crude extract showed negative antibacterial activity against all the test organisms with no inhibition zones observed on all the test plates, a result that may be attributed to antagonistic effects of other components that were present in the crude extracts. For the culture filtrate enriched fractions activity was observed. Out of the six enriched fractions, five showed strong activity as shown in Table 6.

Table 6: Anti-bacterial activity for the enriched fractions compounds

Fraction number	Inhibition zones of the test organisms (mm)	
	S. typhimurium	*E.coli*
1	-	-
2	21	21
3	16	14
4	19	20
5	24	24
6	21	19

Key: - = no inhibition (250ml (broth) / culture = 2 ml (MeOH) = 20µl per paper disc (Rundfilter, Ø6mm, Schleicher & Schuell, Dasel Germany). For testing from 2ml MEOH solution 20 µl was applied on paper disc.

Fraction 5 showed the highest antibacterial activity, 24 mm, each for the two test organisms while fraction 3 showed the least, 16 mm and 14 mm for *S. Typhimurium* and *E.coli,* respectively. Another important result noted is that all the *C. albicans* test plates were clear after incubation even after changing the type of media, which indicates that probably the fractions had very strong antifungal activity that they inhibited the growth of the micro-organism completely. The inability of *C. albicans* to grow on the test plates during incubation was ruled out re-plating the micro-organism to check the viability several times with the results showing that it was viable on each occasion.

The pure compounds were also tested for antibacterial activity to validate the results obtained from the crude extracts and the enriched fractions. Also the minimum inhibitory concentrations were determined and the results are as shown in Table 7 and Table 8 for the pure compounds and the MIC values respectively.

Table 7: Anti-bacterial activity for the purified compounds

Fraction	Inhibition zones of the test organisms (mm)		
	S. typhimurium	*E.coli*	*C. albicans*
$EMK_{2,3,5\&6}$	15 (c)	19	9
EMK_4	15 (d)	21	23

Key: (c) = clear, (d) = diffuse (Rundfilter, Ø6mm, Schleicher & Schuell, Dasel Germany). For testing from 2ml MEOH solution 20 µl was applied on paper disc.

Table 8: The minimum inhibitory concentrations for the purified compound

Fraction EMK	MIC (µg/ml)		
	S. typhimurium	*E. coli*	*C. albicans*
2, 3, 5 & 6	5	3	2
4	8	12	3

Key: (c) = clear, (d) = diffuse (Rundfilter, Ø6mm, Schleicher & Schuell, Dasel Germany). For testing from 2ml MEOH solution 20 µl was applied on paper disc.

The test compounds exhibited similar activities against individual microorganisms, possibly due to similarities between structures and hence structure activity relationships. There doesn't appear to be a vast difference in inhibitory activity between Gram-positive and fungal organisms. The observed antibacterial activity and the eventual structure proposed for the responsible compounds agree quite well with the literature reports (Martini *et al.*, 2004), and the slight difference may be attributed to the purity of the compound at the time. The minimum inhibitory concentrations for the purified fractions of the proposed structures in the literature was found to agree with the values obtained in the research project, 6-12 µg/ml against *S. typhimurium*, 2-5 µg/ml against *E. coli* and 4 -7 µg/ml against *C. albicans* (Martini *et al.*, 2004). The differences in activities may be attributed to the purity of the fraction at the time of testing or probably due to the different assay techniques being used (Sato *et al.*, 2000). It should also be noted that may be the strong antibacterial exhibited by the fraction may be due to synergistic effect of each of the compounds speculated to be present in the fraction.

4.5 Bio-autographic analysis

This method was used to identify the R_f values of the active compounds. The results for R_f values on the basis of bioautographic analysis are in Table 9.

Table 9: Results for R_f values of the active spots

Fraction number EMK	Number of spots	R_f values	
2	2	0.5098	0.3846
3	2	0.3741	0.2142
4	2	0.5301	0.3155
5	1	-	0.4321
6	1	-	0.2441

The results above corresponded very well to those that were observed under UV light, Table 10, immediately after spotting the TLC plates and before the bio-autographic experiment. This indicated that the compounds responsible for the activity had chromophores that were absorbing the UV-light, hence UV active.

Table 10: Results for R_f values of the UV active spots

Fraction number EMK	Number of spots	R_f values	
2	2	0.5128	0.3846
3	2	0.3784	0.2162
4	2	0.5349	0.3256
5	1	-	0.4412
6	1	-	0.2142

4.6 Gram staining experiment

Gram staining procedure was used to validate the R_f values from the bioautogpahic analysis and also check some of the properties of the active compounds. Gram's method is the most important and fundamental orthodox method for bacterial identification. It classifies bacteria into two groups, Gram-negative and Gram-positive and also reflects the cell wall structure. The mechanism of Gram staining is based on the fundamental structural and chemical attributes of bacterial cell walls. The cell walls of Gram-positive bacteria have a high percentage of peptidoglycan, while those of Gram-negative bacteria have only a thin peptidoglycan layer. Gram-negative bacteria have a periplasmic space between the lipopolysaccharide layer and the plasma membrane. In Gram's method, an insoluble dye-iodine complex is formed inside bacterial cells and is extracted by alcohol from Gram-negative but not Gram-positive bacteria. In this research work, all the plates stained remained blue except for the zones where there was inhibition of the growth of the living organisms, which were clear, and corroborating the results obtained (Table 11) with the ones obtained from the inhibition zones during bio-autographic analysis. It is evident that probably the compounds responsible for the activity are effective against Gram-negative bacteria.

Table 11: Results for R_f values of the active spots on Gram staining

Fraction number EMK	Number of spots	Rf values	
2	2	0.5100	0.3826
3	2	0.3714	0.2152
4	2	0.5331	0.3266
5	1	-	0.4410
6	1	-	0.2132

From the results in Tables 9, 10 and 11, it is also clear that the active compounds were very close since they had related R_f values. Those fractions with two spots showed R_f values which were close to those in the fractions with one spot which indicates that probably the compounds responsible for the activity were likely to be present in all the fractions.

Another observation was that the fractions EMK_2 – EMK_6 that had antibacterial activity were eluted successively from the column. Ideally structures in these compounds have some similarity in their structure and properties. From the similarity of their R_f values, they may differ only due to minor groups in their structures. This difference could be inherent in their natural structural diversity or modifications brought about by processing. These include solvent effects or other physico-chemical effects.

A further look also at the R_f values in Tables 9, 10, and 11 gives some interesting variation in the differences of the active spots in the fractions. The relatively non-polar fractions have two spots while the relatively polar ones have one spot. Such occurrences can be outright structural differences or possibly variations that arise between structural derivatives.

4.7 Purification of crude extracts

The crude extracts from the culture filtrate and the mycelium filtrate were purified by column chromatography, TLC, preparative TLC and HPLC. Silica gel was used as the stationary phase in column chromatography and the compounds were obtained by gradient elution, starting from a less polar solvent, hexane, to a relatively polar solvent, methanol. After TLC analysis, the 45 fractions for the culture filtrate obtained from column chromatography were pooled to six (6) main fractions while the 67 collected fractions for the mycelium extract were pooled to fourteen (14) main fractions. After antibacterial activity testing, all the enriched fractions from the mycelium extract showed no activity and

therefore were not processed further. Five of the six enriched fractions from the culture filtrate extracts, which showed antibacterial activity, were processed by purifying and determining the structures. TLC spotting was used as the main guiding principle in this research work, solvent system hexane: ethyl acetate (70:30). The results of the active fractions based on the R_f values have already been discussed. The spots were characterised according to their R_f values.

Four major fractions were obtained after pooling 24 fractions collected from the enriched fractions. The fractions were pooled on the basis of the mass spectra and spots on the TLC plates, a solvent system of hexane and ethyl acetate (70:30). The weights were determined as in Table 5. Fractions EMK $_2$, EMK $_3$, and EMK $_{5\&6}$ were not processed further since their weights were so small. Fraction EMK_4 was the only one with an amount that could allow for further processing. The fraction was further purified by semi-preparative HPLC, using a C_{18} (micron, 250 x 7.8 mm) column followed by characterisation and the probable structures determined.

4.8 Structure speculation of the fairly enriched pure fraction

The structures of the fairly enriched pure fraction were determined using spectroscopic techniques. One dimensional ^{1}H-NMR, ^{13}C-NMR, were combined with two dimensional COSY, HSQC NOESY, and HMBC spectra were used in conjunction with the MS, IR, CD and UV-VIS spectra to identify the probable structures. The identification was also achieved by comparison of the spectral data, available in the literature.

The fraction EMK_4 was isolated as a brown amorphous powder, mp 226-232 ^{o}C, $[\alpha]_D^{23}$ +30.0 ^{o} (0.25, $CHCl_3$ + MeOH, 1:1). The molecular formulae of four naturally occurring flavanones were assigned based on the protonated molecular ion peak at m/z 342.92 $[M + 1]^+$ in the electrospray ionisation (ESI) mass spectrum. The IR spectrum (Appendix VI) showed characteristic absorption bands for an α,β-unsaturated carbonyl system at 1638.38 cm^{-1}, a bridged ether at 1150.14 cm^{-1} and a broad hydroxyl stretching band at 3274.90–3384.84 cm^{-1}. The UV maximal absorptions at 240 and 301 nm were suggestive of a flavonoid skeleton (Langlois *et al.*, 2005). Bathochromic shifts with NaOMe (+28 nm), NaOAc (+28 nm) and $AlCl_3$ (+12 nm) (Mabry *et al.*, 1970) indicated the presence of an –OH group on either the C-5, C-7 or C-8 and any other position around ring A, but not C-6. The numbering system of a skeleton for a flavanone during interpretation of the spectra was as shown in Figure 5.

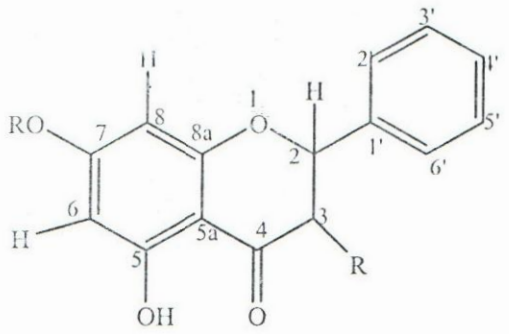


Figure 5: Numbering system of the skeleton structure

The ^{1}H NMR spectrum (Table 12, Appendix III) indicated the presence of two aromatic moieties: a five-proton multiplet between δ_H 7.31–7.48 ppm and a one-proton singlet at δ_H 6.05 ppm The first group of signals indicated the presence of a mono-substituted aromatic ring B at δ_H 7.43-7.48, (2H, *m*, H-3′ and H-5′) and δ_H 7.31-7.41, (3H, *m*, H-2′, H-4′ and H-6′). Also observed was a downfield exchangeable singlets at δ 12.02 (1H, *s*, OH-5), which is a general characteristic for a chelated OH-5 of flavones and flavonones due to strong hydrogen bonding between the OH group at C-5 and the carbonyl oxygen at C-4. The resonance at δ_H 6.05 ppm is typical for an aromatic or olefinic methine proton of a substituted aromatic ring A. Another observable signal at δ_H 6.08 ppm, which is characteristic of a proton at C-8 suggesting that C-8 is unsubstituted. The ^{1}H NMR spectrum also showed resonances for an ABX system at δ_H 1.97 (1H, *dd*, J = 7.4, and 3.3 Hz, H-3), δ_H 3.05 (1H, *dd*, J = 11.3 and 7.4 Hz, H-2), and δ_H 5.32 (1H, *dd*, J = 11.3 and 3.3 Hz, H-3), which is diagnostic for H-2 and H-3 of a flavanone nucleus (Jang *et al.*, 2003). This was corroborated well by the ^{1}H- ^{1}H correlations observed in the COSY spectrum. Differences in the spectra are likely to be due to the overlapping of the peaks from the similarity of the structures. A singlet at δ_H 3.78 indicated the presence of the protons of a methoxyl group (3H, *s*, OMe-7). Based on spectroscopic data, pinocembrin and pinostrobin skeletons with penta-substituted ring A and an unsubstituted ring B were proposed (Trakoontivakorn *et al.*, 2001).

The ^{1}H NMR spectra also contained two aromatic signals at δ_H 6.05 (*s*, H-6) and δ_H 6.08 ppm (*s*, H-8) and two doublet signals at δ_H 5.77 and δ_H 5.80 ppm *J*=7.4 and 3.3 Hz (H-2 and H-3 respectively) suggesting the presence of a pinobanksin moiety (Kumazawa *et al.*, 2002). Furthermore, several aliphatic signals at δ_H 0.88, δ_H 1.23, δ_H 1.61, δ_H 1.77 and δ_H 1.92 ppm in the ^{1}H NMR spectra of the purified fraction were also observed. This speculation was strongly supported by the ^{13}C NMR, which showed 18 carbon atoms even

though some of the peaks were overlapping. Most of the signals in the ^{1}H and ^{13}C NMR spectra were assigned from the $^{1}H - {}^{1}H$ COSY, HSQC and HMBC spectra. In the HMBC spectrum of the purified fraction, the methine signal at δ_H 1.92 ppm and the methyl signal δ_H 1.97 ppm were observed to correlate with a carbonyl carbon at signal at δ_C 169.86 (Figure 6), indicating the possibly that the basic pinobanksin structure has more than one acetyl substituents.

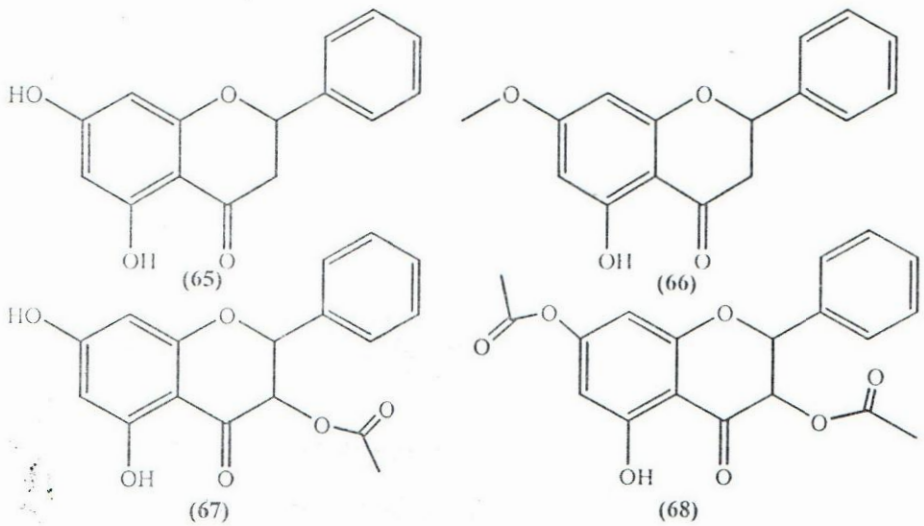


Figure 6: Proposed basic structures for the compounds present in the fraction EMK_4

The ^{1}H (Appendix III) and ^{13}C NMR (Appendix IV), $^{1}H - {}^{1}H$ COSY (Appendix III), and HSQC (Appendix IV) spectra also validated the presence of pinocembrin, pinostrobin and pinobanksin structures. In the HMBC spectrum (Appendix IV), the proton signals at δ_H 3.02 and δ_H 2.78 ppm were seen to correlate to the carbonyl carbon at δ_C 196.1 ppm, indicating the presence of pinocembrin moiety, the proton signals δ_H 4.70 and δ_H 4.31ppm were also seen to correlate to the carbonyl moiety at δ_C 196.1 ppm, which indicated the presence of a pinostrobin moiety, while as it has been discussed above, it was also evident that there was presence of the pinobanksin moiety. The remaining structure was identified on the basis of the MS (Appendix V), HMBC (Appendix IV), HSQC (Appendix IV), and NOESY (Appendix III) spectra.

Table 12: NMR spectroscopic data for the fairly enriched fraction EMK_4

Position	^1H multiplicity (*J* Hz)	^{13}C	$^1H - ^1H$ COSY
1	-	-	
2	5.31, *m* (7.4, 3.3)	43.0, *s*	3α, 3β
3	2.78, *dd* (11.3, 7.4)	76.0, *s*	2, 3β
	3.05, *dd* (11.3, 3.3)		2, 3α
4	-	191.5, *s*	
4a	-	103.2, *s*	
5	-	164.1, *s*	
6	6.05, *s*	95.2, *s*	
7	-	138.4, *s*	
8	5.77	96.1, s	
8a	-	168.2, *s*	
1′	-	135.3, *s*	
2′	7.31-7.41, *m*	128.8, *s*	3′
3′	7.31–7.48, *m*	129.1, *s*	2′
4′	7.31-7.41, *m*	128. 9, *s*	
5′	7.31–7.48, *m*	129.3, *s*	
6′	7.31-7.41, *m*	129.0,*s*	
OH-5	12.09, *s*	-	
OMe	3.78, *s*	56.3, *s*	
OCH_2	3.85, *d*	81.2, *s*	
Me	1.97, s	20.0, s	
$COCH_3$	1.26, *s*	196.1, *s*; 22.6, *s*	

- 400MHz (^1H), 100MHz (^{13}C) in $CDCl_3$; δ values are given in ppm and the *J* values (in parentheses) in Hz. All data were assigned based on the observed 2D NMR spectral correlations.

Consistent with the ^1H NMR spectral analysis, the ^{13}C NMR spectrum of the speculated compounds displayed signals for two oxygenated methines at δ_C 81.3(C-2, *d*), and 72.3 (C-3, *d*), a methoxyl group at δ_C 55.7 (OMe, *q*) the signals of two aromatic rings (with one mono-substituted, δ_C 135.3, C-1′, *s*; δ_C 129.1, C-3′, C-5′ *d*; δ_C 128.8, C-2′, C-4′, C-6′, *d*), and a conjugated ketone at δ_C 196.1, (C-4, *s*). Accordingly, this suggested that the compounds being identified were all flavones with unsubstituted B ring (Kuroyanagi *et al.*, 1982; Ichino *et al.*, 1988; Su *et al.*, 2003).

Since the only methoxyl group was evident in both the ^1H and ^{13}C NMR spectra, had been linked to pinostrobin, all the uncounted for hydroxyl groups in each structure were inferred from the molecular formula. In the HMBC spectrum of the fraction, the correlations gave distinct characteristics of the proposed skeleton structure of flavanones,

Figure 7. The downfield proton singlet of the chelated hydroxyl group at δ_H 12.02 (OH-5) correlated to the aromatic methine at δ_C 95.2 (C-6), and two quaternary carbons at δ_C 164.1 (C-5) and 103.2 (C-5a), indicating that most of compounds were 3, 5, 7, 8-tetrasubstituted flavanones. The location of the methoxyl group at C-7 for pinostrobin was assigned based on the observed NOESY (Appendix III) correlation from δ_H 3.78 (OMe - 7) to δ_H 6.05 (H-6). Also the HMBC and HSQC spectra indicated that δ_H 3.78 (OMe - 7) is directly attached to C-7, δ_C 138.4. The coupling constant between H-2 and H-3 (11.1 and 3.3 Hz) suggested that the B ring and one of the H-3 were equatorially trans-oriented (Gaffield, 1970; Jang *et al.*, 2003). ^{13}C NMR and 2D experiments indicated the presence of 15, 16, 17and 19 carbon atom compounds, which had each six quaternary aromatic carbons and one carbonyl carbon (*s*, 196.1, C-4) involved in an intra-molecular hydrogen bond interaction with the –OH group of C-5 (Agrawal *et al.*, 1989).

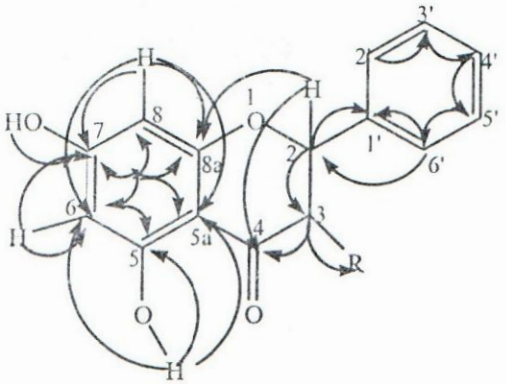


Figure 7: Observed $^1H - ^{13}C$ long range couplings (H-C) for the proposed structures

The positions of ring A substituents were confirmed from the ^1H-^1H COSY and NOESY correlations: –OH groups were assigned to C-5 and C-7 (pinostrobin and pinobanksin), -OH to C-5 and –OMe to C-7 (pinostrobin), and –OH to C-5 and $-OCOCH_3$ for the derivative of pinobanksin. In addition to the correlations of the H-6 singlet (δ 6.05) with C-7 (δ 138.4), C-6 (δ 95.2), C-8a (δ 166.1), C-8 (δ 168.2) C-4 (δ 196.1) and C-5a (δ 103.2). In addition, the long-range correlation observed between the H-2 signal and C-8a was crucial to assign C-2 at δ_C 81.3 and to confirm that the substitution by the phenyl groups is at position C-2 and not C-3. Accordingly the scientific names for the structures were given as 5,7-dihydroxy-2-phenyl-chroman-4-one, 5-hydroxy-7-methoxy-2-phenyl-chromane-4-one, 5,7-dihydroxy-4-oxo-2-phenyl-chroman-3-yl ester and Acetic acid-7-acteoxy-5-hydroxy-4-oxo-2-phenyl-chroman-3-yl ester, Figure 9.

The mass spectra were used to identify the major components of the fragmentation pattern of the fraction, and also the molecular ion peak. The four compounds proposed

from the ^{1}H and ^{13}C NMR data corroborated very well with the peaks in the mass spectra, summarised in Table 13.

Table 13: Summary of the direct probe methane positive ion MS results from purified fraction

MW	$(M+1)^+$	$(M+29)^+$	$(M+41)^+$	Ions	Possible compound identified
256	257	285	297		pinocembrin
270	271	299		255 ($-CH_3$)	pinostrobin
314	315	343	356	273 ($-CH_2CO$), 255, 227,	Pinobanksin-3-O- acetate
356	357			314 (CH_2CO) -273 ($-CH_2CO$), 255, 227,	Pinobanksin - 3,7-diacetate

The result to bring to attention is the presence of a peak at $(M + 1)^+ = 255$ in the MS spectra (Appendix V), which is a characteristic peak for the possibility of chrysin (Figure 8) in the purified compound. However, this was eliminated on the basis of a characteristic peak at δ_H 6.63 ppm, which was absent in the ^{1}HNMR spectra.

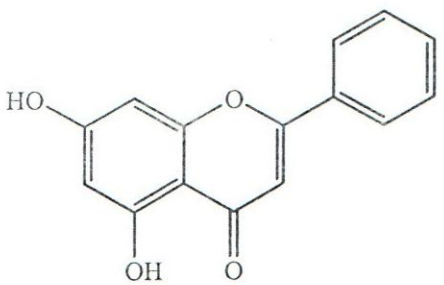
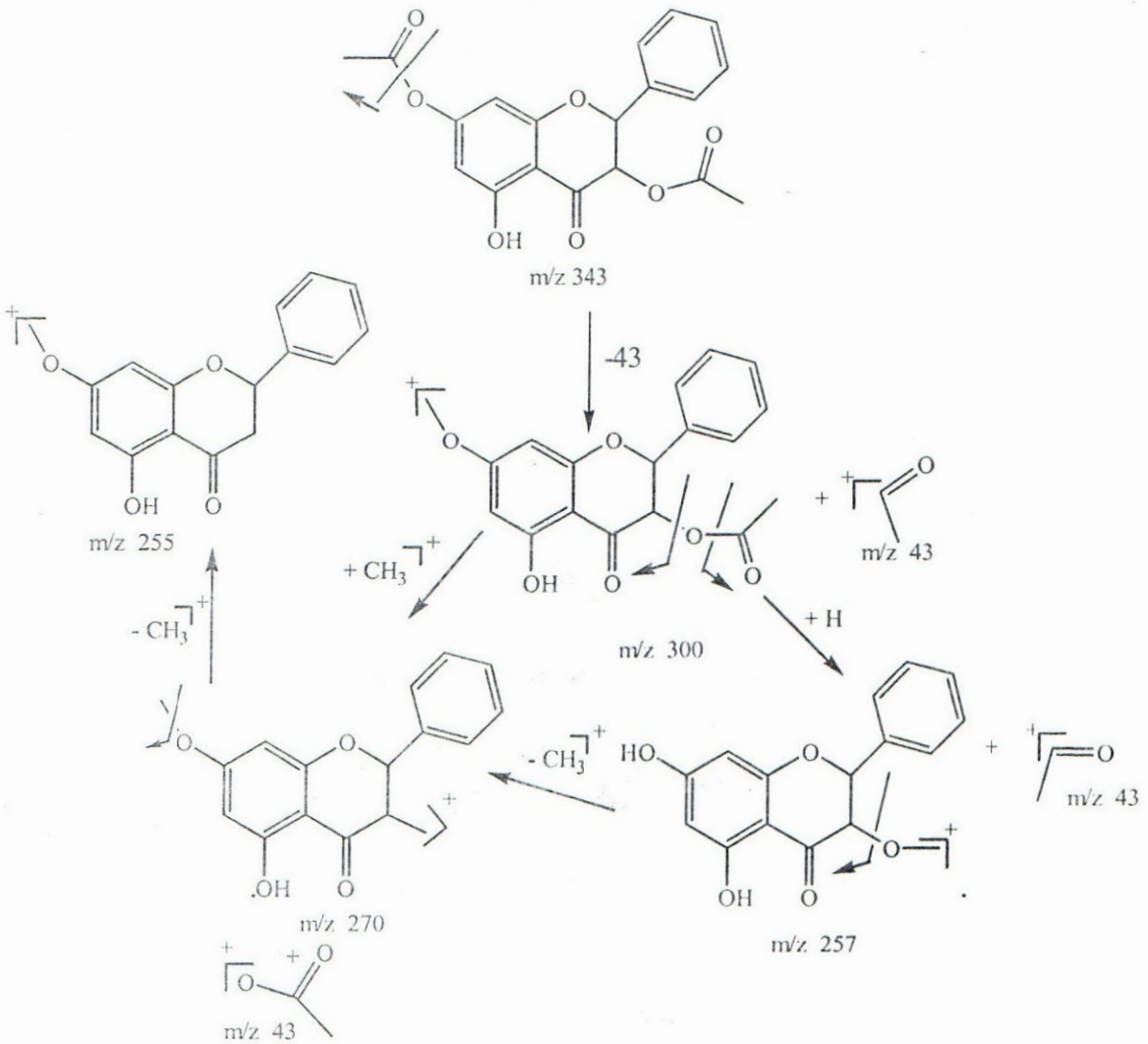


Figure 8: Structure of chrysin

Pinocembrin (5,7-dihydroxy-2-phenyl-chroman-4-one, $C_{15}H_{12}O_4$) forms the basic structure of most of the flavanones and this is also evident from the results. Pinostrobin (5-hydroxy-7methoxy-2-phenyl-chroman-4-one, with a molecular formular of $C_{16}H_{14}O_4$ was a major part of the fraction from the MS (Appendix V) and NMR (Appendix III &IV) data. Pinobanksin-3-*O*-acetate, (acetic acid-5,7-dihydroxy-4-oxo-2-phenyl-chroman-3-yl-ester, $C_{17}H_{14}O_6$) was identified by the characteristic peak at m/z = 314.92. The adducts formed during the ionization and fragmentation of the compound include $(M+1)^+ = 271.08$ and $(M+29)^+ = 343.03$. The fragment ions formed were due to loss of ketene functional group ($-CH_2CO$), and carbon monoxide (CO) respectively from the quasimolecular ion m/z =

314.92. These two fragments indicate that the chemical structure of the substance contains a methoxyl group, and an acetyl group, which enhanced the speculation that the fraction contains more than one compound, basic flavanone structure, pinocembrin, pinostrobin (a methoxy group at C-7), and pinobanksin-3-O-acetate, which is substitute at position 3. More so the fragment m/z =270, which is the base peak, can be found as a fragment from the ionisation and fragmentation of the latter two molecules, Scheme 4.



Scheme 4: Fragmentation pattern of the major peaks in the MS spectrum

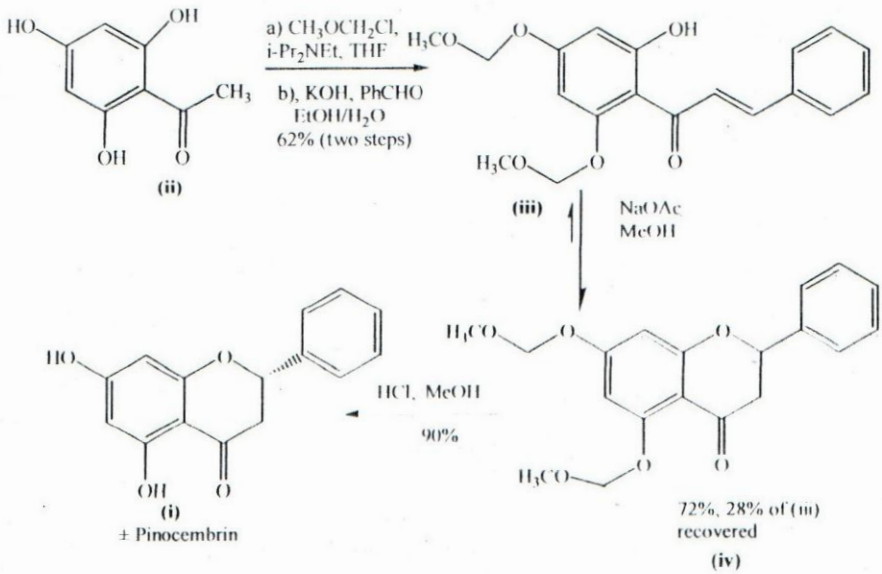
The CD spectrum of compound of the fraction displayed negative and positive effects at 292 and 319 nm respectively. A literature survey revealed that most of the flavanones isolated from natural sources thus far have the 2*S* absolute configuration (Yoshikawa *et al.*, 1998; Kuroyanagi *et al.*, 1982), but 2*R* flavanones have also been reported (Hsieh *et al.*, 1998a, 1998b; Matsuda *et al.*, 2002; Su *et al.*, 2003). However, divergent optical rotation values are occasionally reported for the same flavanones. For example, the specific rotation values of -3.4° (*c* 0.5, MeOH) (Ichino *et al.*, 1988), -47.3 ° (*c*

5.48, MeOH) (Hsieh *et al.*, 1998b) and -52 ° (*c* 0.188, MeOH) (Fukui *et al.*, 1988) were reported for 5,7-dihydroxyflavanone (pinocembrin), and values of -1.6 ° (*c* 0.45, $CHCl_3$) (Ichino *et al.*, 1988), -45 ° (*c* 0.50, $CHCl_3$) (Häberlein and Tschiersch, 1994) and +5 ° (*c* 0.05) (Gonza´lez *et al.*, 1989) were reported for 5-hydroxy-7-methoxyflavanone (Su *et al.*, 2003). Values of -58.5 ° (*c* 0.91, MeOH) and -57.5 ° (*c* 0.80, $CHCl_3$) were obtained for compound pinocemrin and 5-hydroxy-7-methoxyflavanone (Su *et al.*, 2003).

In the CD spectra of the compound, negative and positive Cotton effects, respectively, were observed in the $\pi \longrightarrow \pi^*$ and $n \longrightarrow \pi$ transition regions. These observations indicate 2*S* absolute stereochemistry for flavanones (Gaffield, 1970). The value determined in this research project is in the region of the negative Cotton effect produced by a series of homoisoflavanones (285–297 nm for 3-benzylchroman-4-one skeleton and 333–337 nm for benzocyclobutene- homoisoflavanones) (Adinolfi *et al.*, 1988, 1990; Corsaro *et al.*, 1992; Likhitwitayawuid *et al.*, 2002; Lo´pez *et al.*, 2006). The value of the negative effect coincides very well with the reported values, while the positive does not fully coincide with the reported values. This difference is possibly due to the structural differences between all compounds present in the fraction (an extra chiral carbon in C-9 due to the presence of acetoxy group), prevent making of any definitive conclusion about the absolute configuration of chiral centres.

4.9 Synthetic evaluation of the fairly enriched fraction

Flavanones related to pinocembrin have been prepared in high yield from the reaction of phenols with cinnamoyl chlorides through a Friedel-Crafts or cyclization sequence. This process has been known to be low yielding for pinocembrin, and therefore the development of an aldol condensation or cyclization route that rapidly provides multi-gram quantities of pinocembrin has been necessitated, Scheme 5, 90% yield. Trihydroxyacetophenone **(ii)** was selectively bis-protected as the methoxymethyl ether and then converted to chalcone **(iii)** under standard conditions. The selective bis-protection was achieved by using two moles of the protecting agents. Cyclization with sodium acetate provided an equilibrium mixture of the cyclized product and chalcone starting material. Acidic hydrolysis of the methoxymethoxyl (MOM) groups yielded pinocembrin **(i)** (Urgaonkar *et al.*, 2005).



Scheme 5: Synthetic route for pinocembrin

Synthetic studies were very vital in this research in that they were used to check how high yields for the compounds can be obtained of the proposed compounds, and this can lead to the development of synthetic analogues for this compounds.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Basidomeycetes are known to produce compounds with various activities. The compounds were extracted by using liquid-liquid extraction and solid-liquid adsorption techniques and purified by column chromatography and semi preparative HPLC. Structure determination using a various of spectroscopic techniques led to the identification of the isolated compounds as flavanones: pinocembrin, pinostrobin and pinobanksin derivatives. The observed antibacterial activity and MIC values were in good agreement with the literature data for the isolated compounds. The compounds may be useful as templates for development of novel antibacterial agents. Further biochemical investigations (both *in vitro* and *in vivo*) on the fractions and individual compounds should also be undertaken so as to determine the exact MIC values.

Since flavanoids are widely used as antioxidants, and for other therapeutic functions, their isolation from basidiomycetes opens a new field for future research and represent the compounds in the class very few been identified from the family of fungi.

5.2 Recommendations

The following recommendations were made in relation to this study:

- It was not possible to further purify and process all the pure compounds obtained after the HPLC stage. Careful purification of the fractions should be done considering all the necessary conditions so as to obtain very pure fraction.
- Further taxonomic identification of the basidiomycete should be done so as to determine the exact classification.
- The identified compounds can also be synthesized and processed further to confirm the structures, and their analogues, which can be bioassayed to check their antibacterial activity. Synthesis can also be used to identify the absolute structure and stereochemistry of the compounds.
- Blending of the compounds can be done to investigate which compounds synergize others. Further identification of the possible structure isolated.
- Chiral purification of any possible stereoisomers of the compounds should be undertaken so as to identify the individual stereoisomers in the compounds.
- Production of the compound on a large scale, and de-replication of the results to check the reproducibility and consistency in the results.
- Additional studies including theoretical calculations of the electronic CD spectra for all the four compounds are necessary to compare with the experimental data presented in this research report.
- Use of a wide range of microorganisms so as to asses and validate the antibacterial activity.
- Determination of specific bacterialcidal, and bacterialstatic activities, and the possible mode of action of the compounds.
- Screening of these analogues might lead to the identification of compounds that are sufficiently potent to be useful as antibacterial, antifungal, antiviral or chemotherapeutics.
- In addition to the structural alteration of weak and moderately active antimicrobial flavonoids, investigation into the mechanisms of action of these compounds is likely to be a productive area of research. Such information may assist in the optimisation of a lead compound's activity, provide a focus for toxicological attention and aid in the anticipation of resistance.

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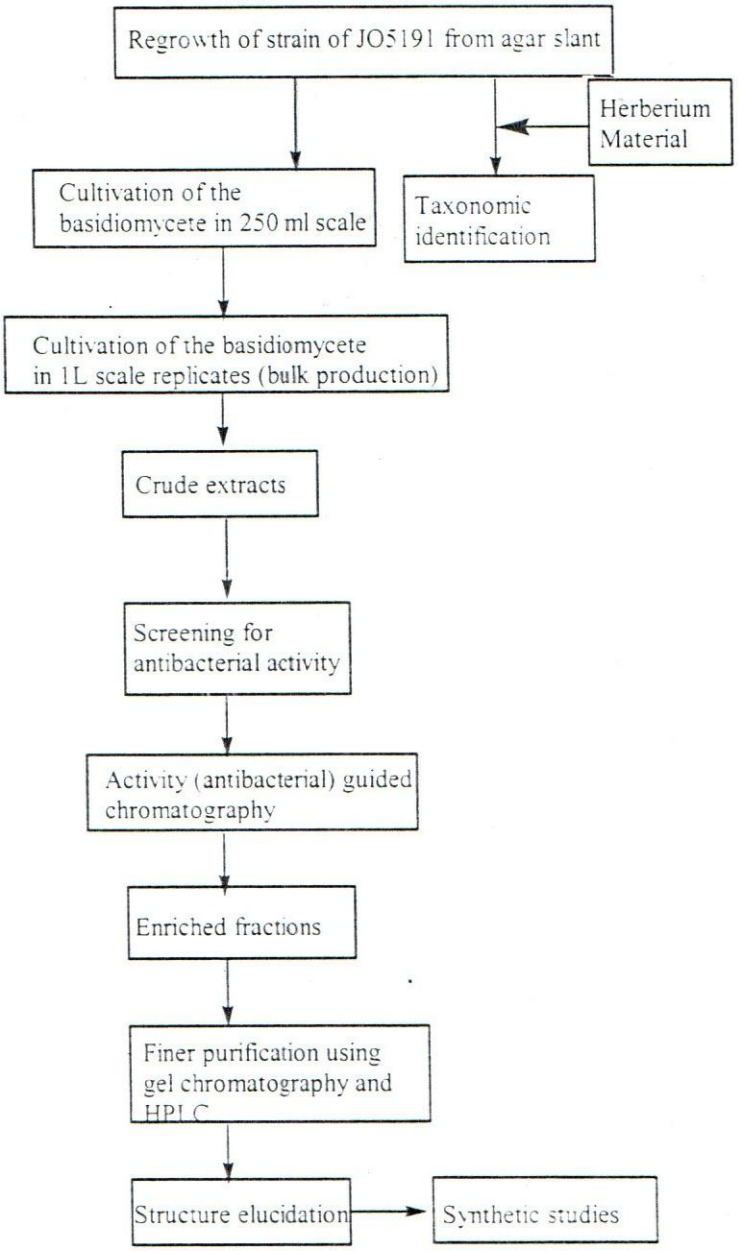
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APPENDIX 1

Scheme of methodology



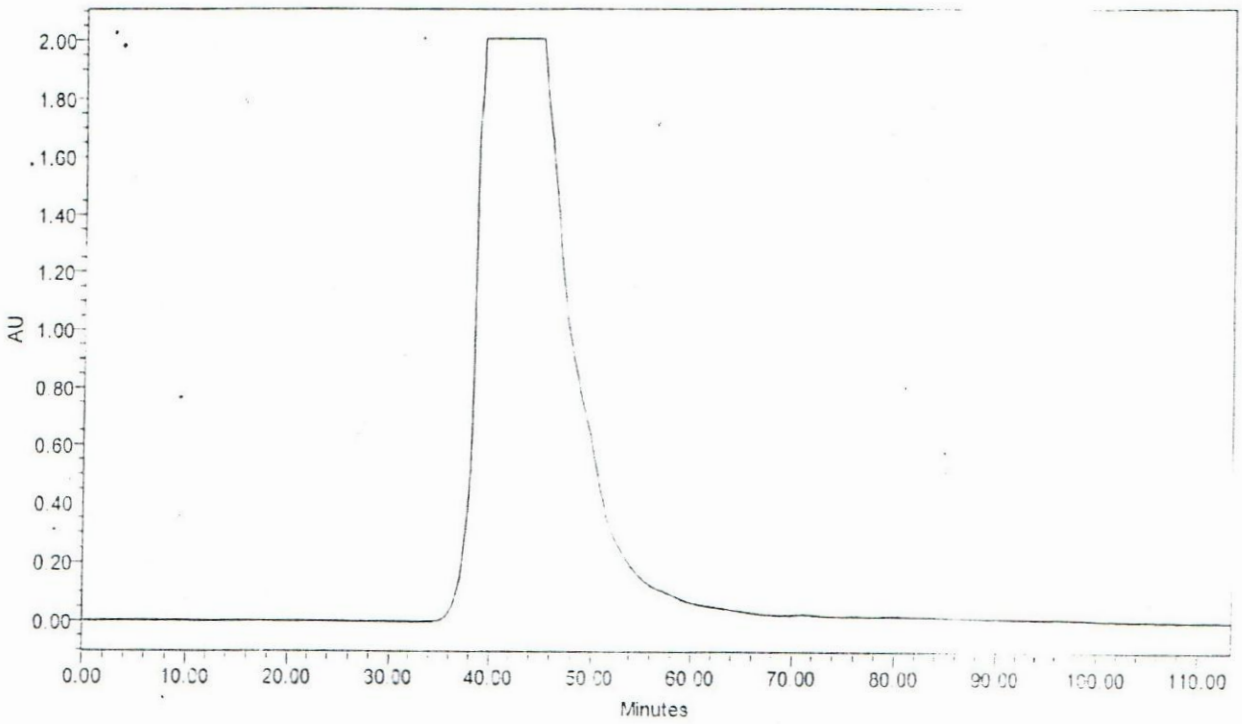
Empower

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Reported by User: System Project Name: Emytrialrun

SAMPLE INFORMATION

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Sample Type:	Unknown	Date Acquired:	2/17/2007 6:48:11 PM
Vial:	1	Acq. Method Set:	test 2
Injection #:	1	Date Processed:	****
Injection Volume:	1000.00 ul	Processing Method:	****
Run Time:	120.0 Minutes	Channel Name:	****
Sample Set Name:		Proc. Chnl. Descr.:	****



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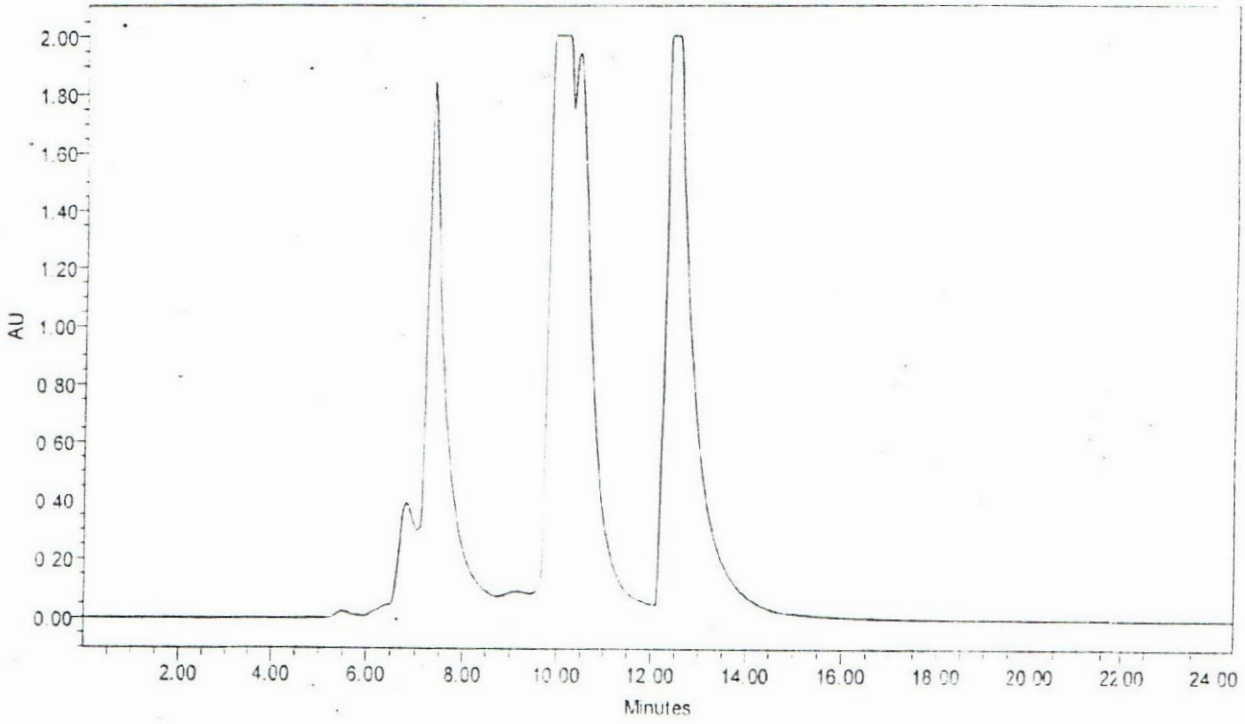
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Reported by User: System

Project Name: Emytrialrun

SAMPLE INFORMATION

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Vial:	1	Acq. Method Set:	test 2
Injection #:	4	Date Processed:	****
Injection Volume:	250.00 ul	Processing Method:	****
Run Time:	140.0 Minutes	Channel Name:	****
Sample Set Name:		Proc. Chnl. Descr.:	****



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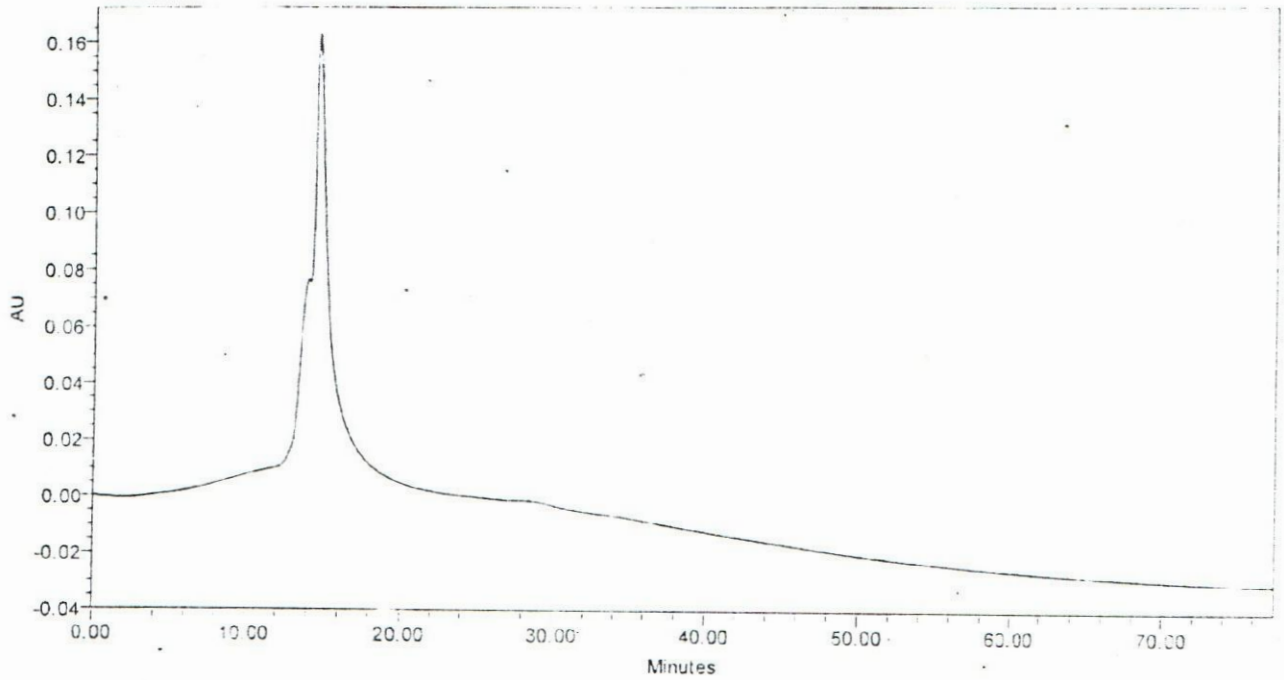
Default Individual Report

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Project Name: Emytrialrun

SAMPLE INFORMATION

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Vial:	1	Acq. Method Set:	test 2
Injection #:	1	Date Processed:	****
Injection Volume:	1000.00 ul	Processing Method:	****
Run Time:	90.0 Minutes	Channel Name:	****
Sample Set Name:		Proc. Chnl. Descr.:	****



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Empower

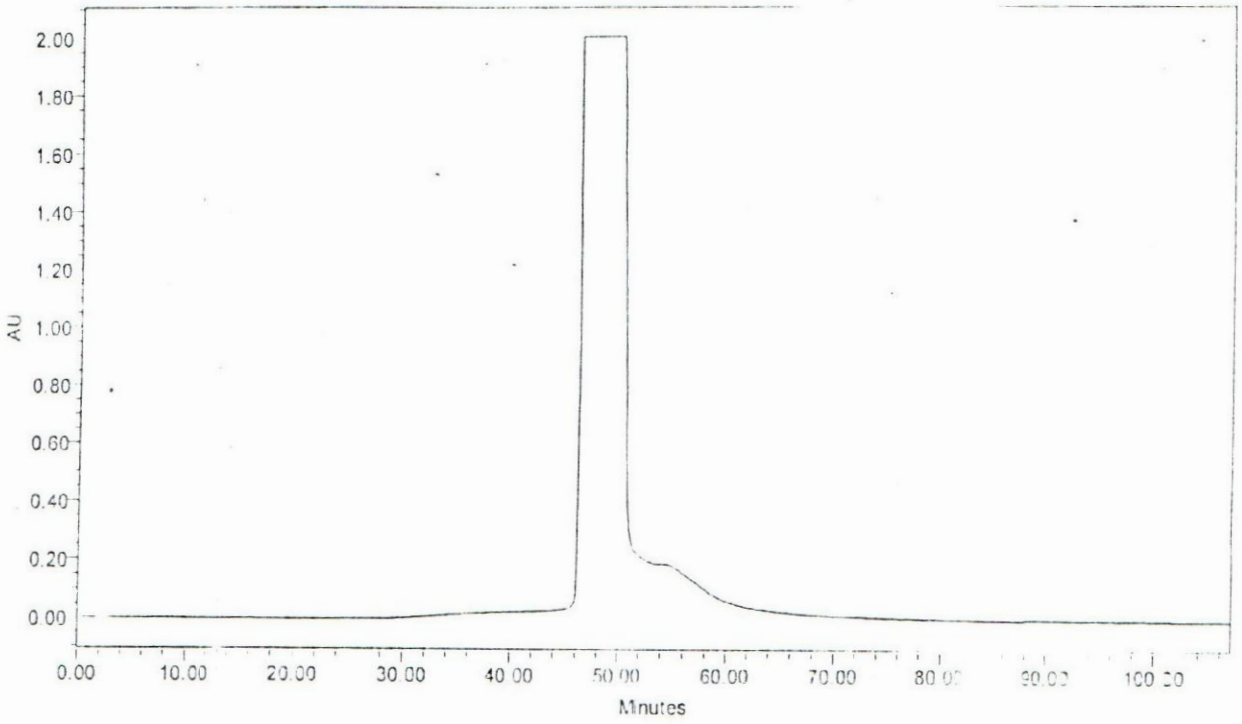
Default Individual Report

Reported by User: System

Project Name: Emytriaton

SAMPLE INFORMATION

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Vial:	1	Acq. Method Set:	test 2
Injection #:	1	Date Processed:	****
Injection Volume:	1000.00 ul	Processing Method:	****
Run Time:	120.0 Minutes	Channel Name:	****
Sample Set Name:		Proc. Chnl. Descr.:	****



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Error Log

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Empower

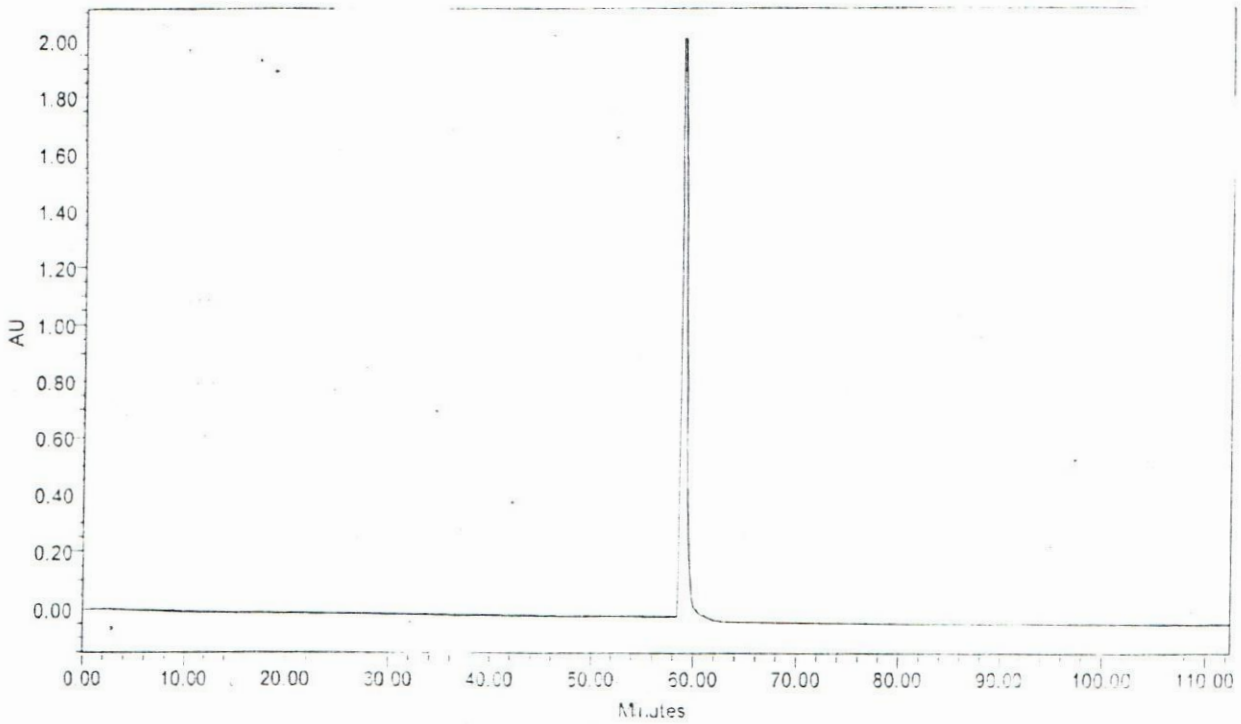
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Project Name: Emytrialrun

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Injection #:	5	Date Processed:	****
Injection Volume:	1000.00 ul	Processing Method:	****
Run Time:	140.0 Minutes	Channel Name:	****
Sample Set Name:		Proc. Chnl. Descr.:	****



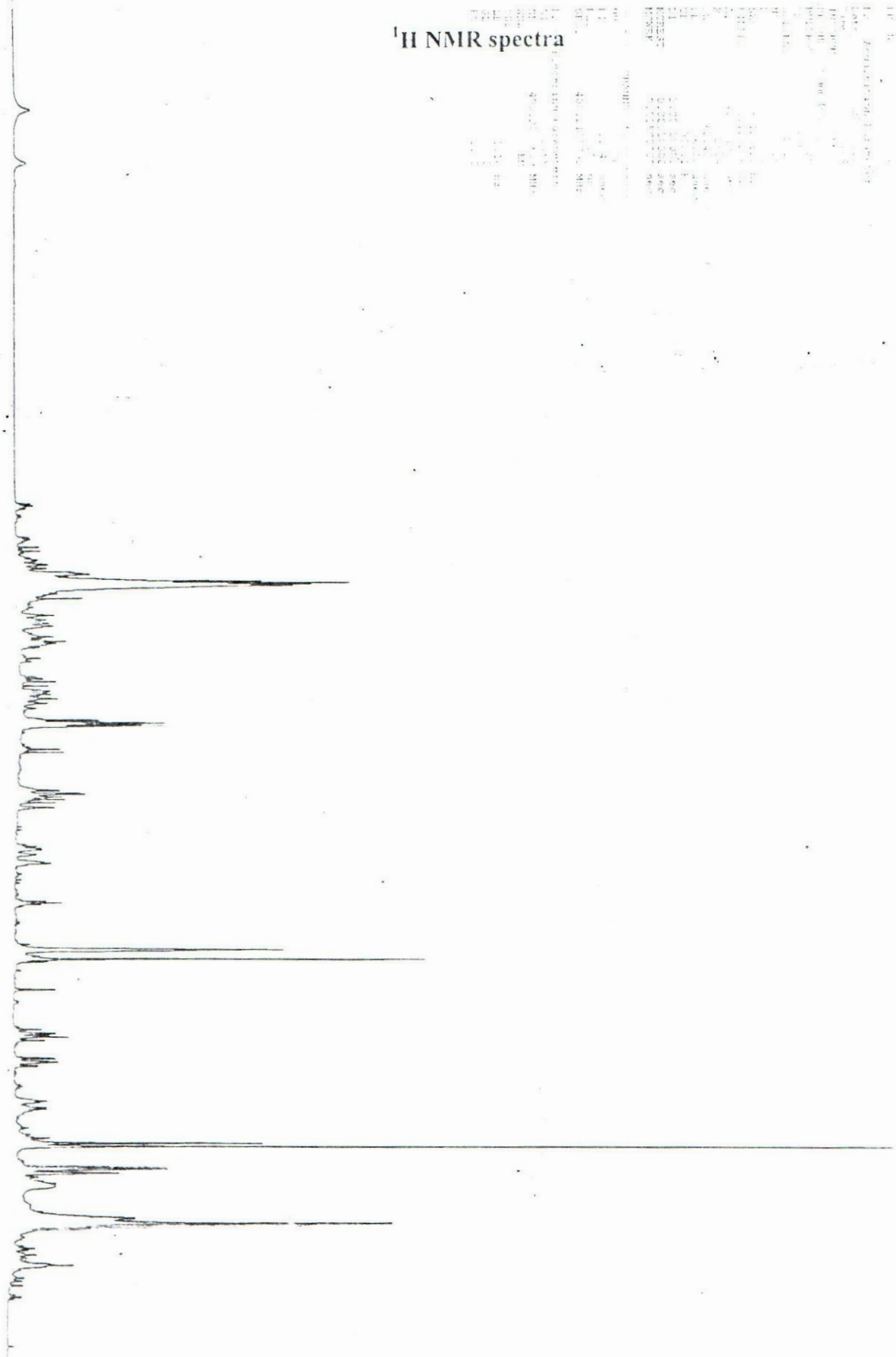
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Error Log

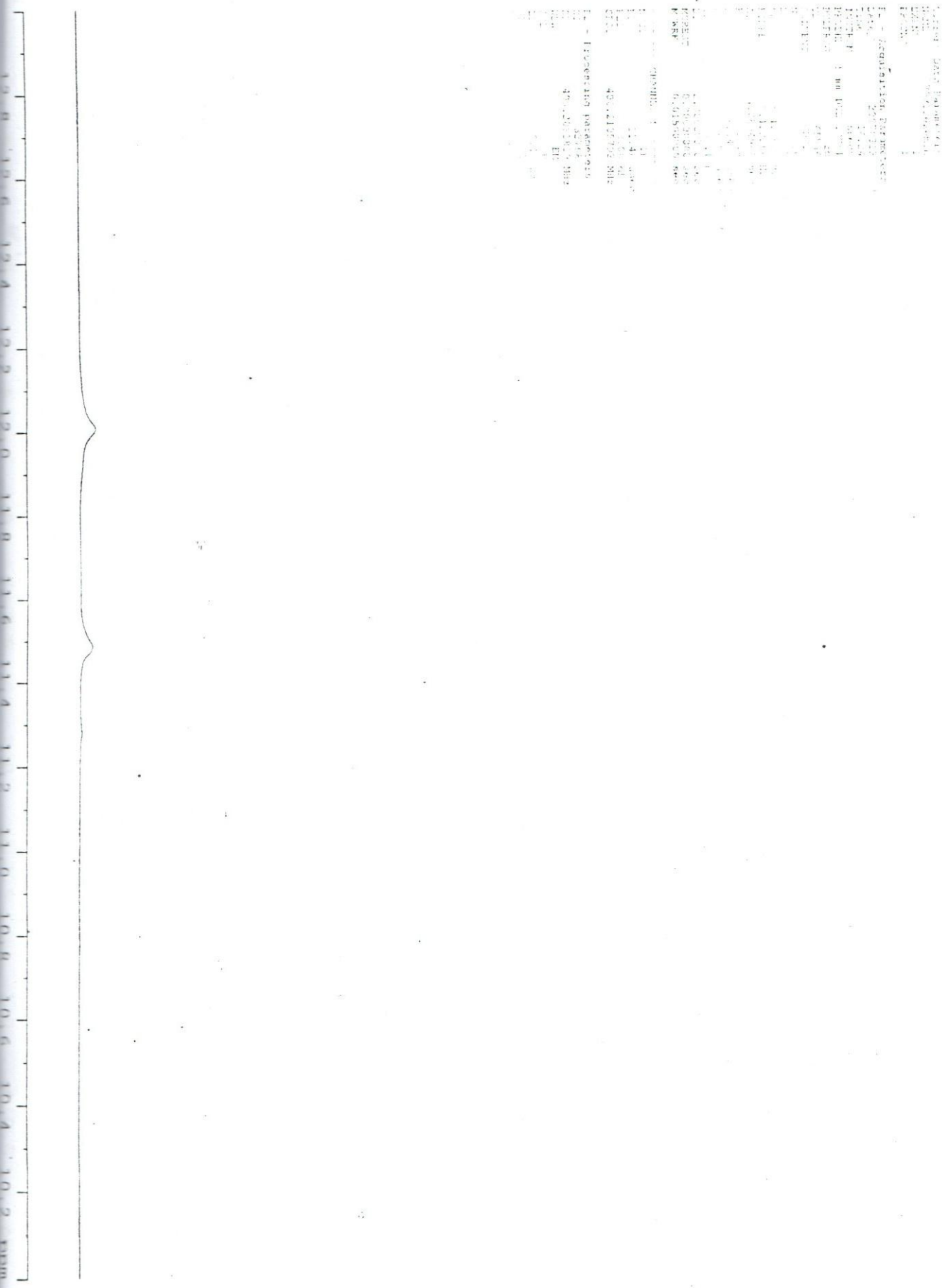
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APPENDIX III

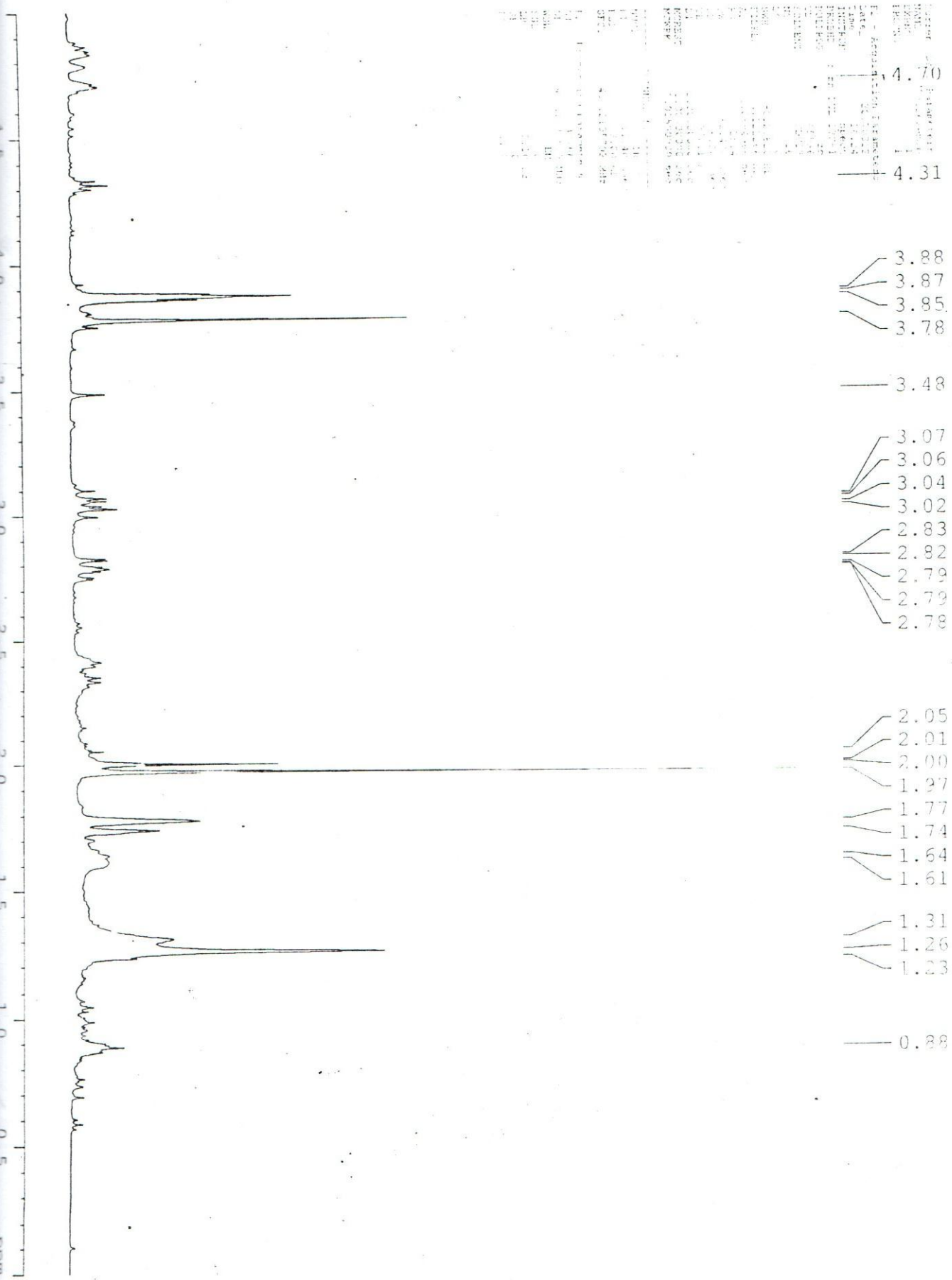
^{1}H NMR spectra



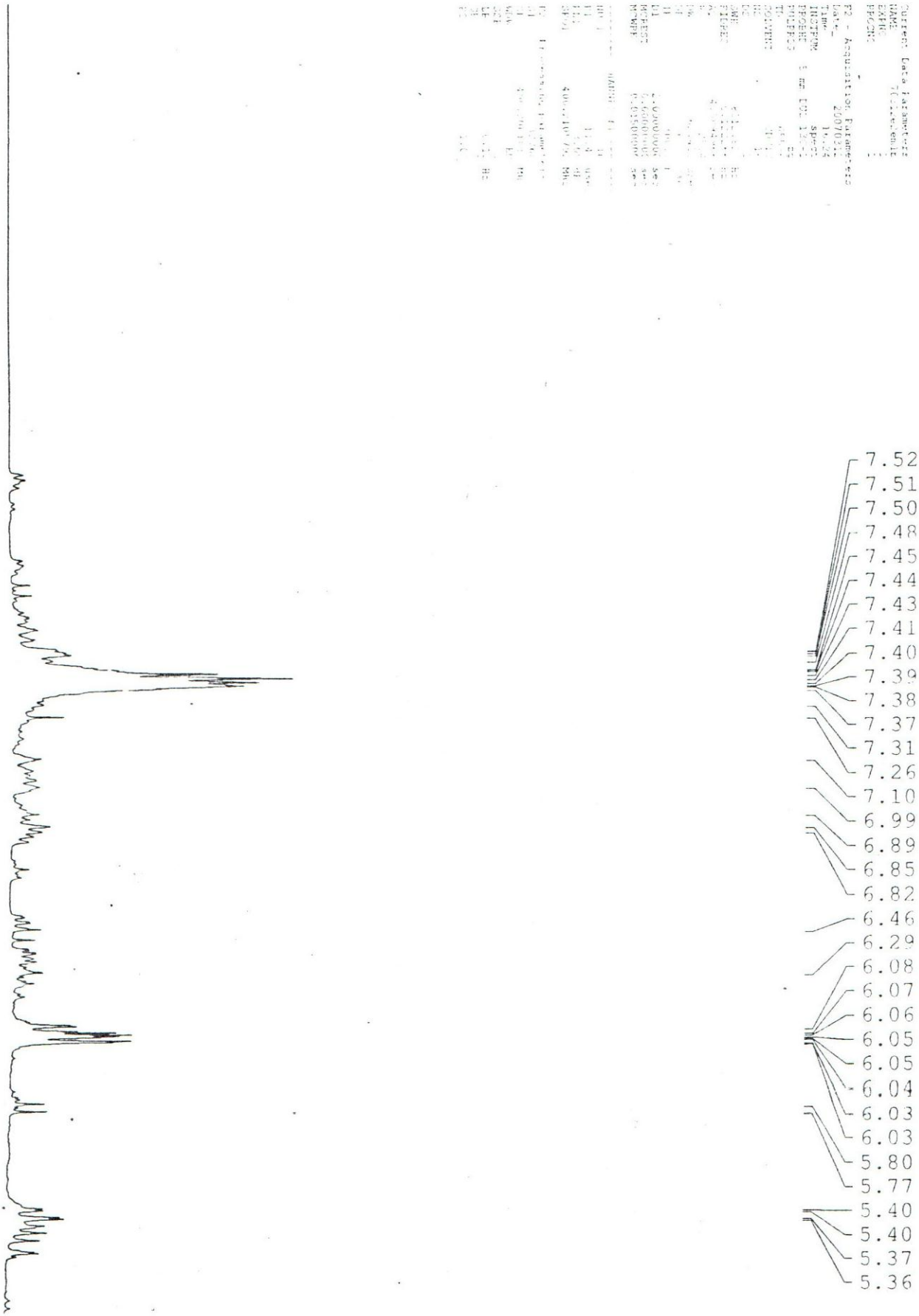
Expanded ^{1}H NMR spectra



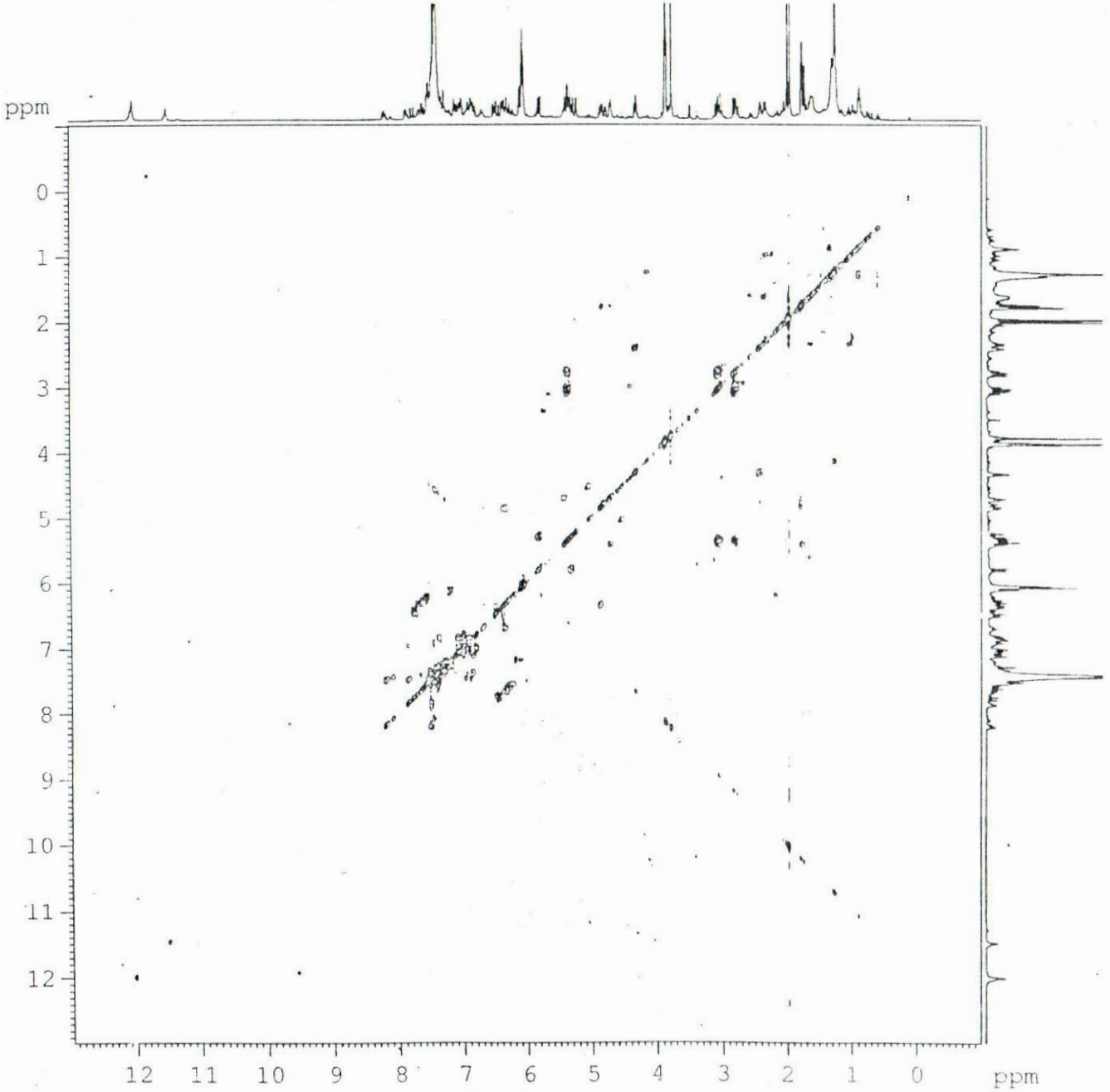
Expanded ^{1}H NMR spectra



Expanded ^{1}H NMR spectra



COSY



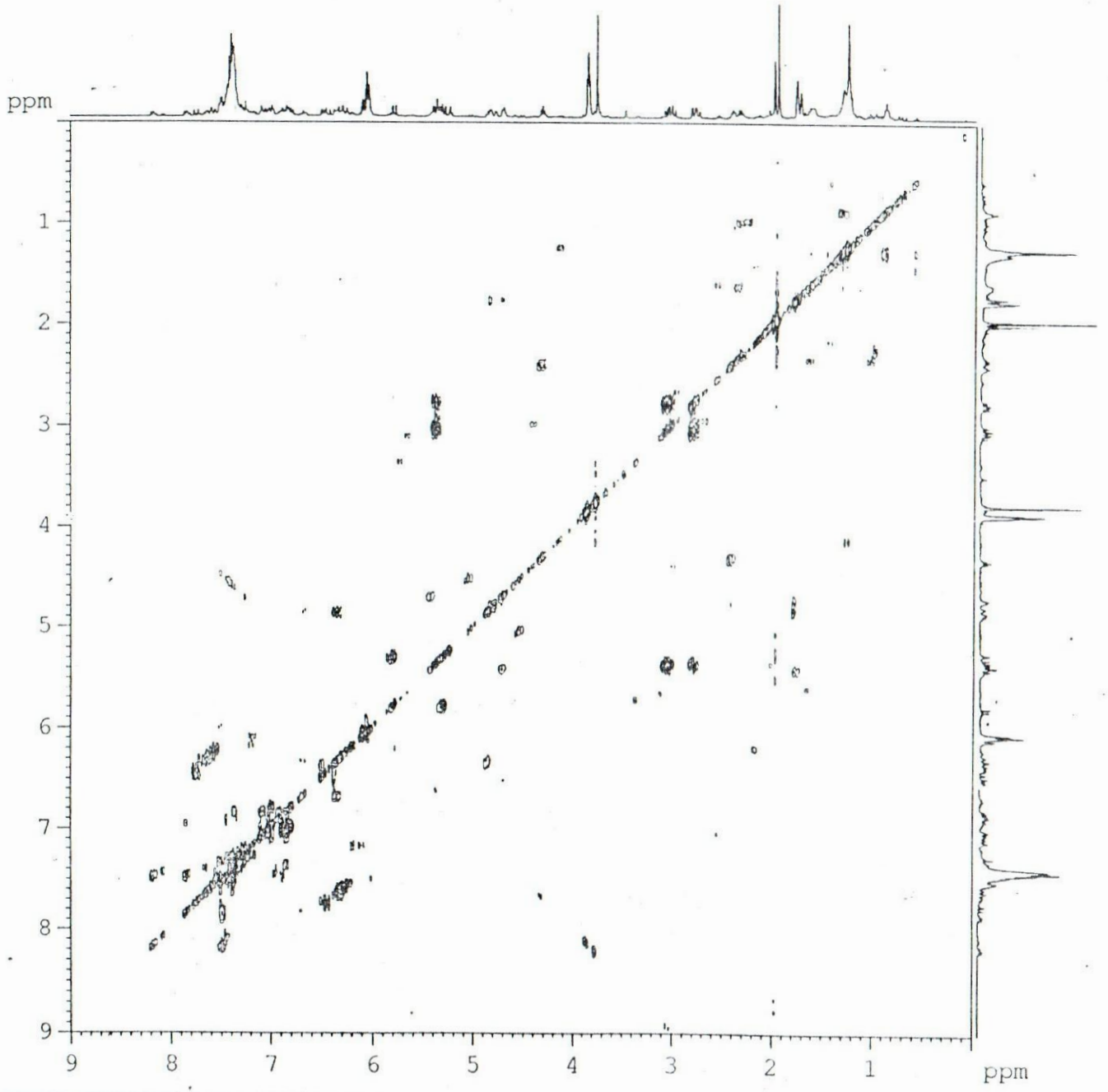

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EXPNO                   1
PROCNO                  1

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INSTRUN              spect
PROBHD    5 mm BBI 1H-BB
PULPROG                 cg
TD                   65536
SOLVENT              CDCl3
NS                      16
DS                       4
SWH              8012.820 Hz
FIDRES           0.122268 Hz
AQ               4.094496 sec
RG                     512
DW                  62.400 usec
DE                   6.00 usec
TE                  300.0 K
D1               2.0000000 sec
MCREST           0.0000000 sec
MCWRK            0.0150000 sec

======== CHANNEL f1 ========
NUC1                   1H
P1                   5.40 usec
PL1                 -3.00 dB
SFO1         400.2105742 MHz

F2 - Processing parameters
SI                   32768
SF         400.2081819 MHz
WDW                    no
SSB                     0
LB                   0.00 Hz
GB                      0
PC                   1.00
```

Expended COSY



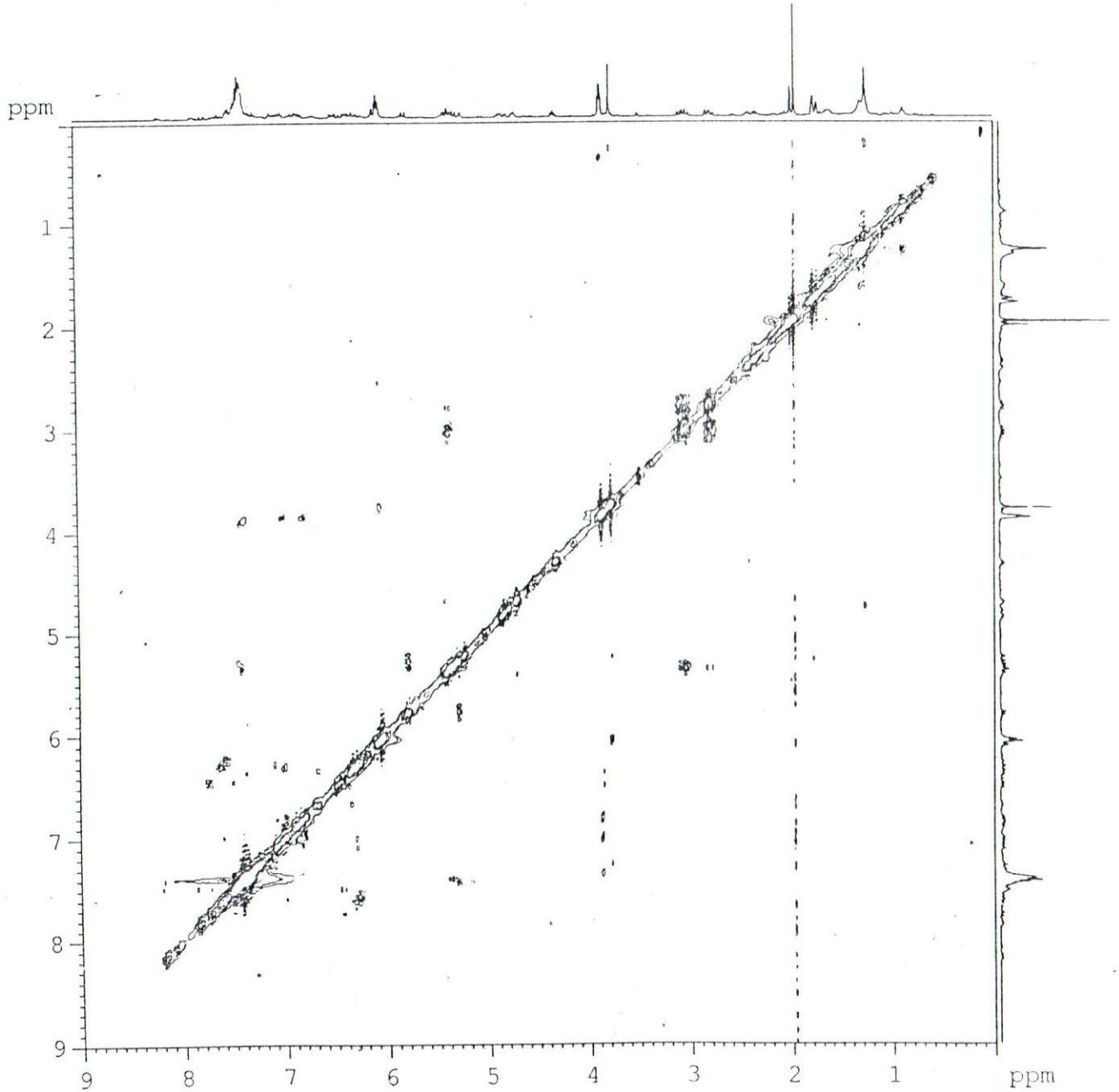

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PROCNO                   1

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PULPROG                zg
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SOLVENT             CDCl3
NS                     16
DS                      0
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FIDRES        0.122246 Hz
AQ            4.0894966 sec
RG                     8
DW              62.400 usec
DE               6.00 usec
TE              301.2 K
D1            2.00000000 sec
MCREST        0.00700000 sec
MCWRK         0.02500000 sec

======== CHANNEL f1 ========
NUC1                   1H
P1                   5.30 usec
PL1                 -3.00 dB
SFO1          400.2105792 MHz

F2 - Processing parameters
SI                 32768
SF          400.2081819 MHz
WDW                   no
SSB                    0
LB                  0.00 Hz
GB                     0
PC                  1.00
```


COSY



EM01
NOESY

```
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PROCNO                     1

F2 - Acquisition Parameters
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PULPROG                zg30...
TD                       ...
SOLVENT                  CDCl3
NS                         ...
DS                         ...
SWH                 5192.441 Hz
FIDRES              1.267440 Hz
AQ                  0.786432 sec
RG                         ...
DW                   96.313 usec
DE                    6.50 usec
TE                    ... K
D1                  1.00000... sec
D11                 0.03000... sec
D16                 0.20000... sec
TD0                         1
P1                   ...  usec
PL1                  ...  dB
SFO1        400.2100... MHz

======== GRADIENT CHANNEL ========
GPNAM1           sine.100
GPNAM2           sine.100
GPZ1                ...  %
GPZ2                ...  %
GPY1                ...  %
GPY2                0.00  %
GPZ1               40.00  %
GPZ2              -40.00  %
P16              1000.00 usec

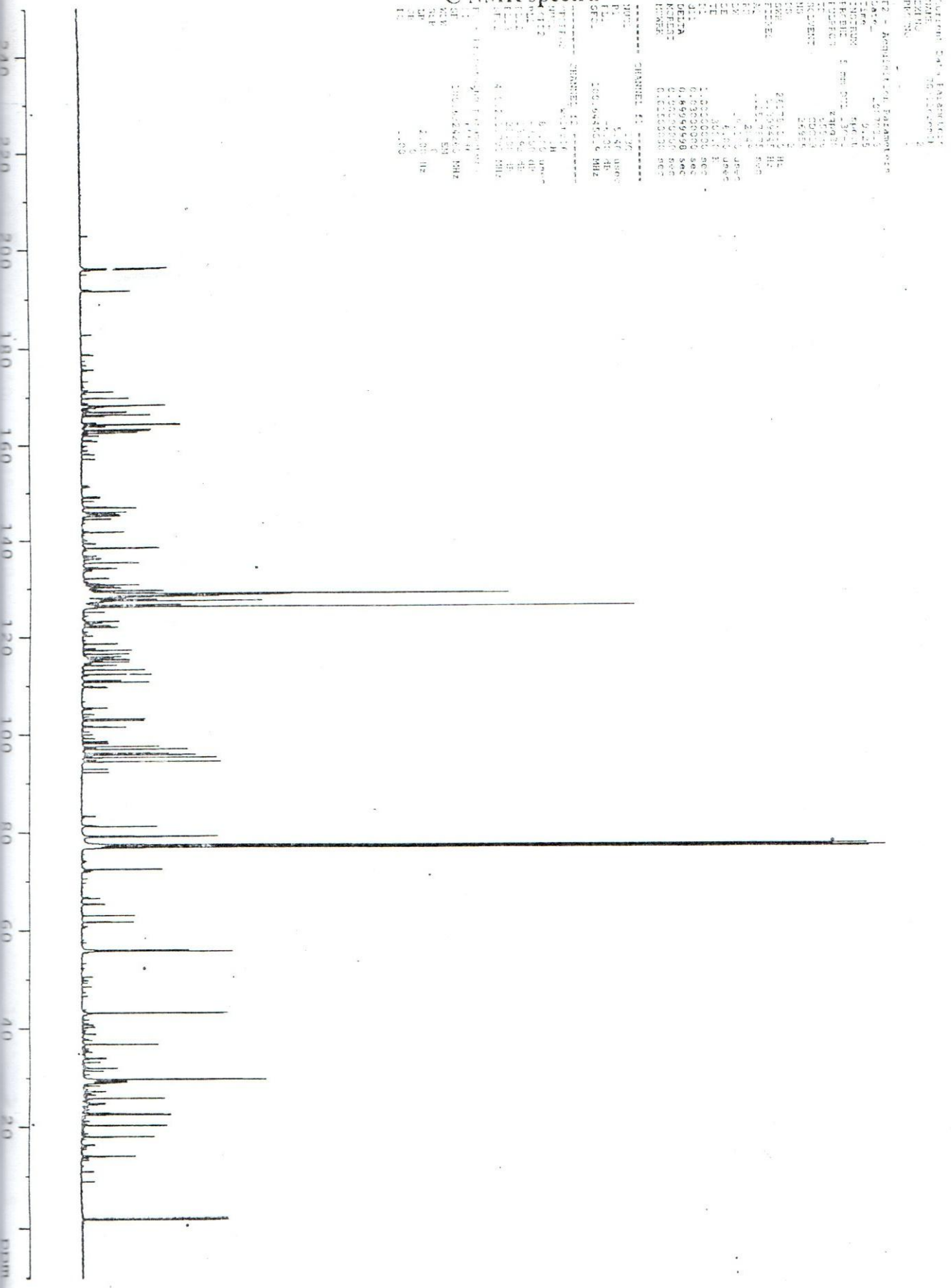
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FnMODE              TPPI

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GB                       0
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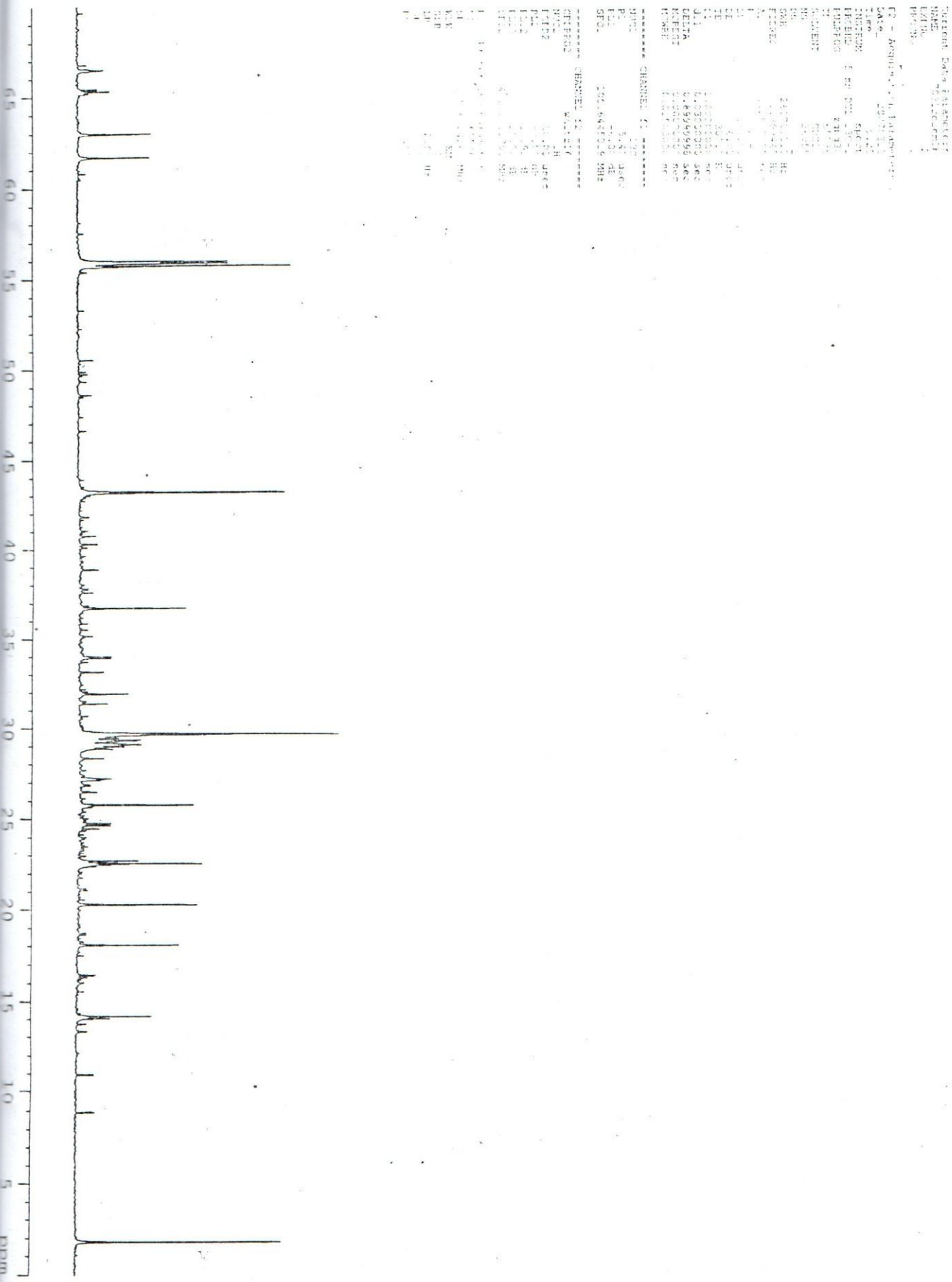
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APPENDIX IV

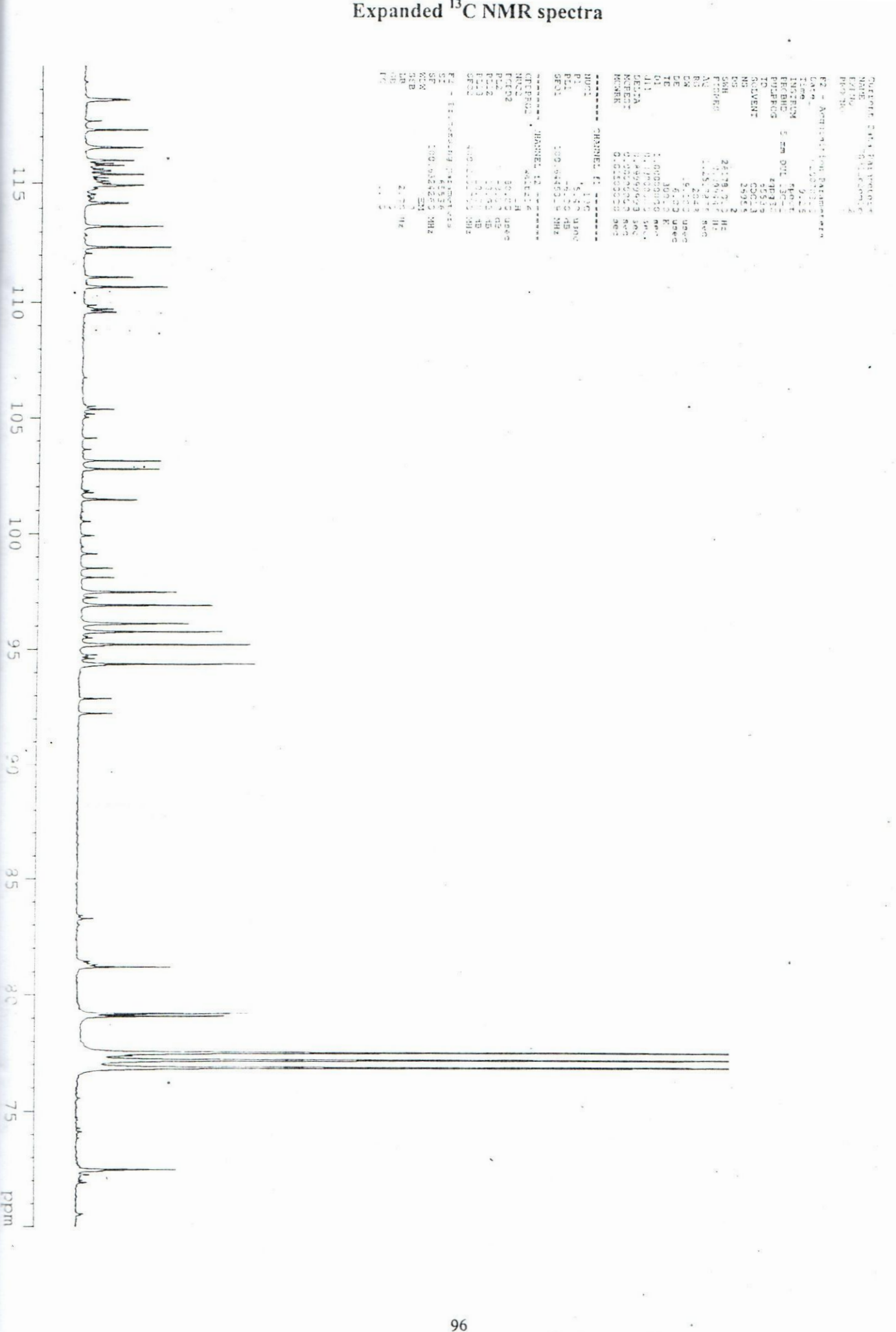
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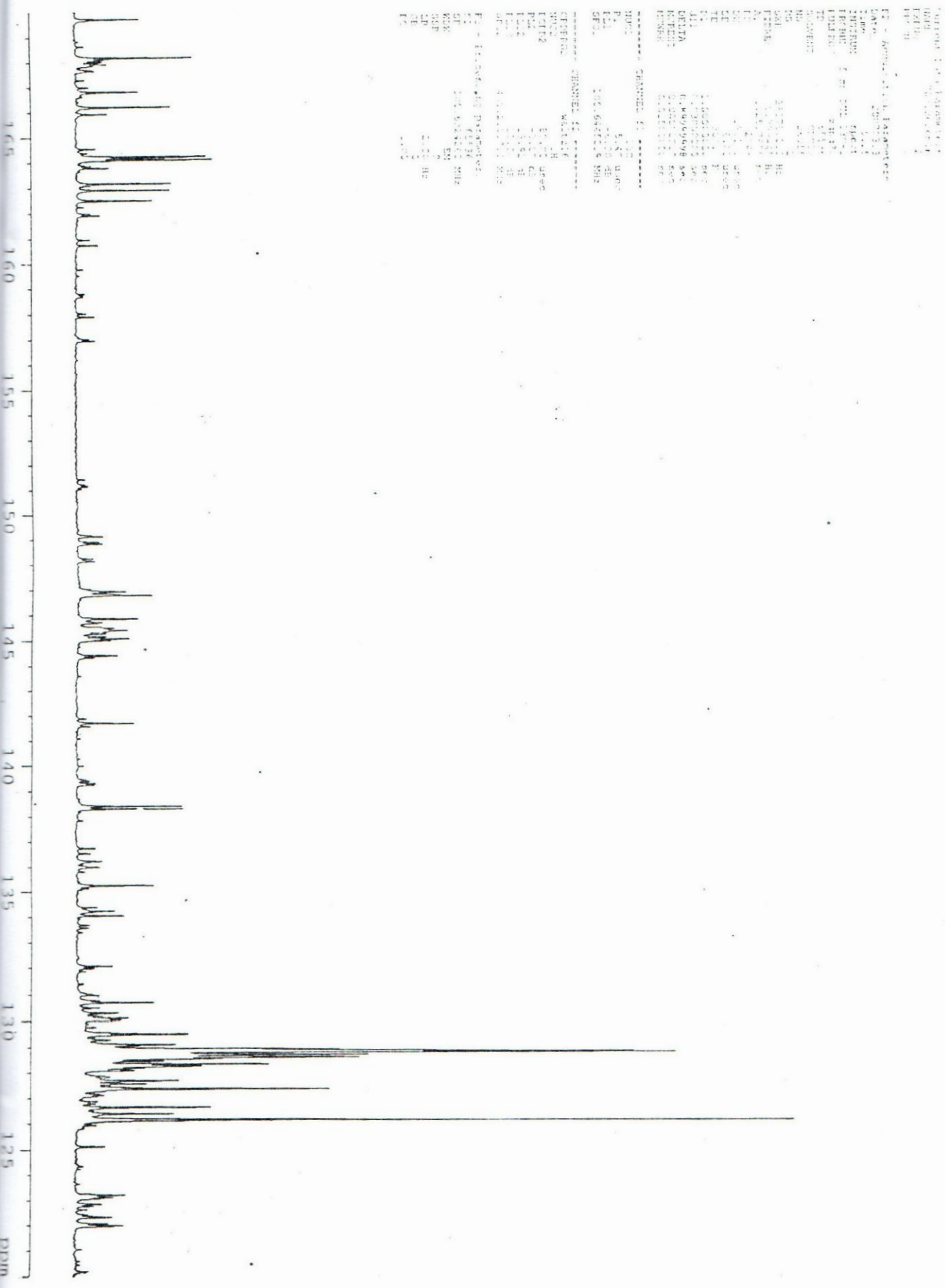
Expanded ^{13}C NMR spectra



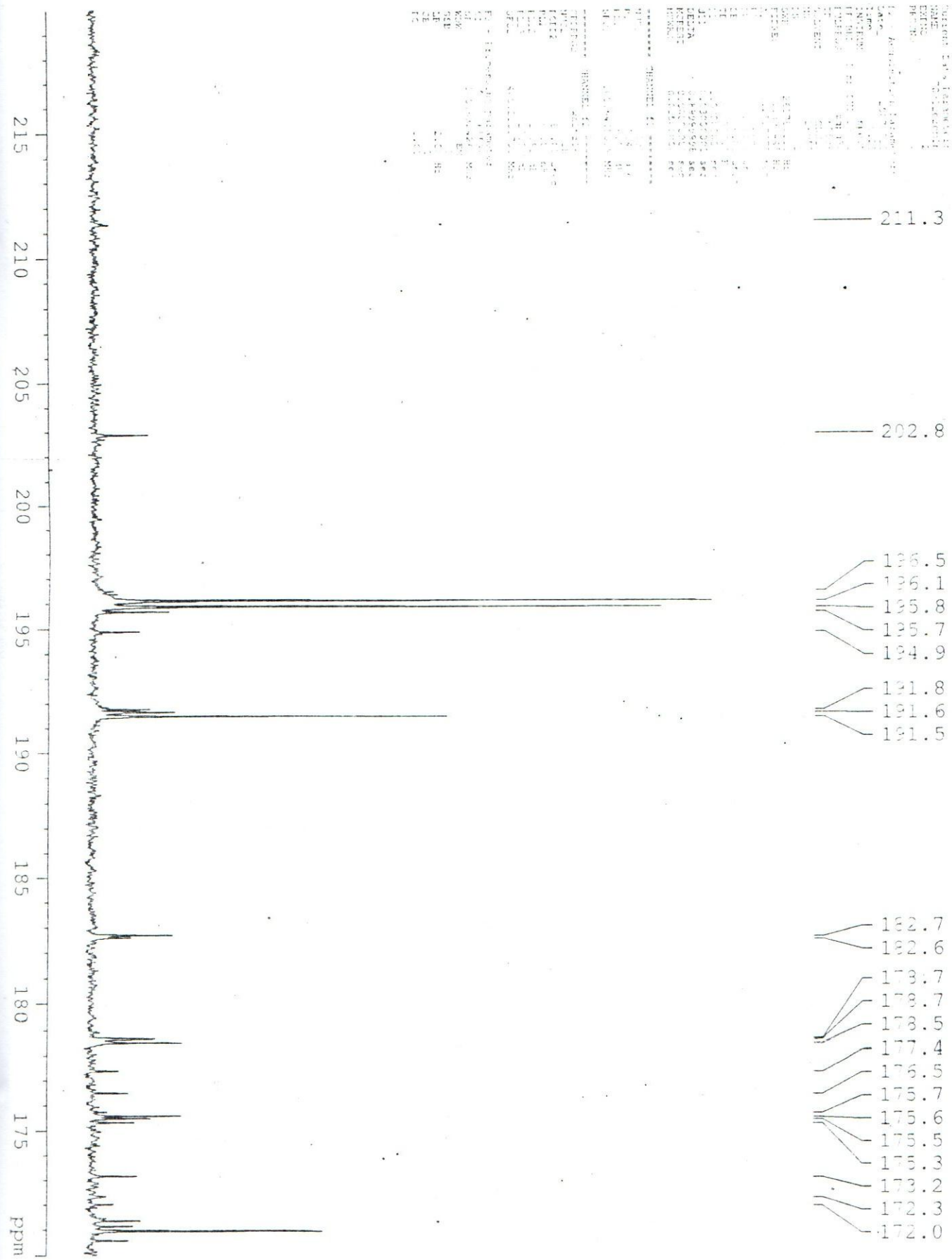
Expanded ^{13}C NMR spectra



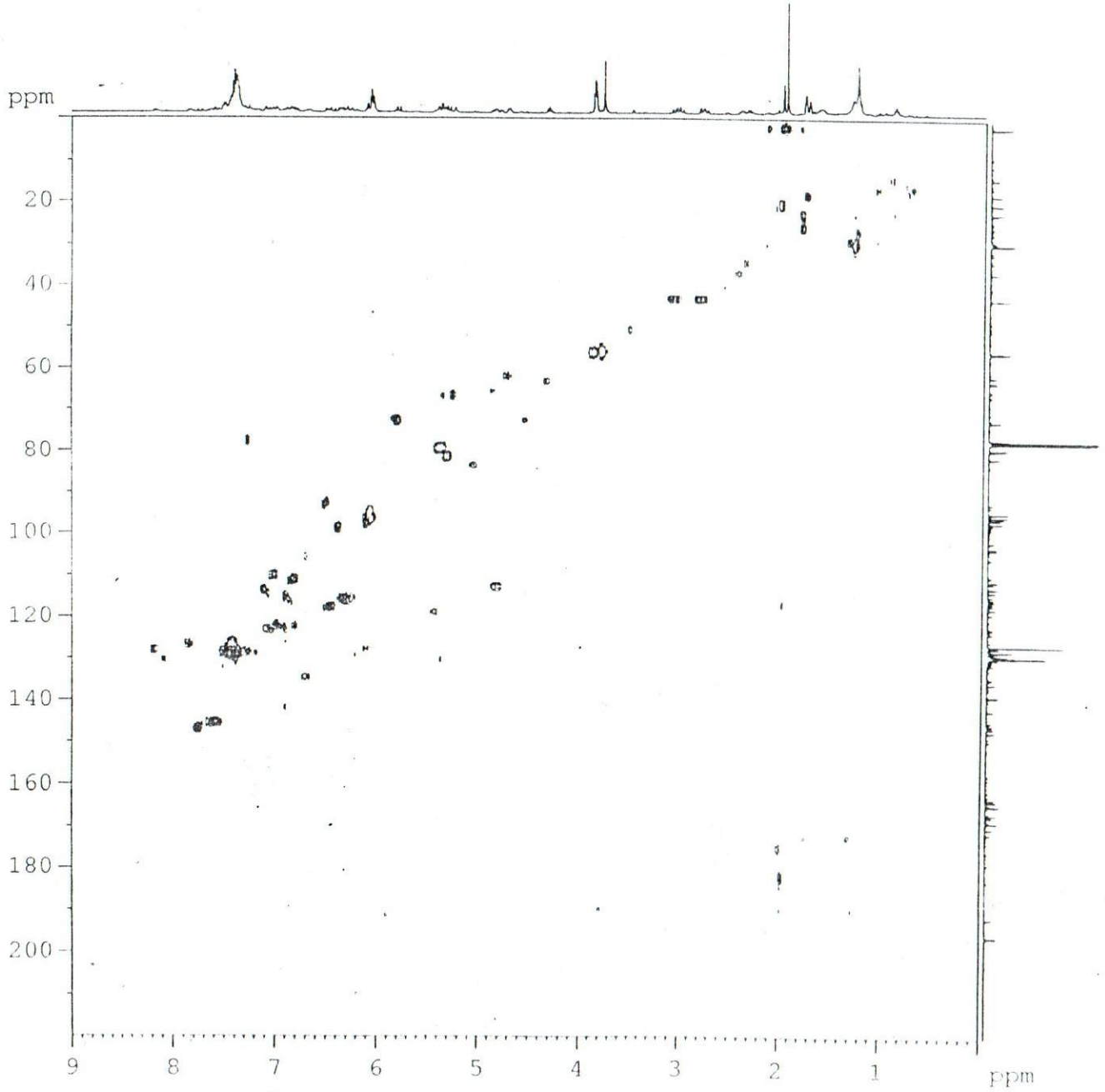
Expanded ^{13}C NMR spectra



Expanded ^{13}C NMR spectra

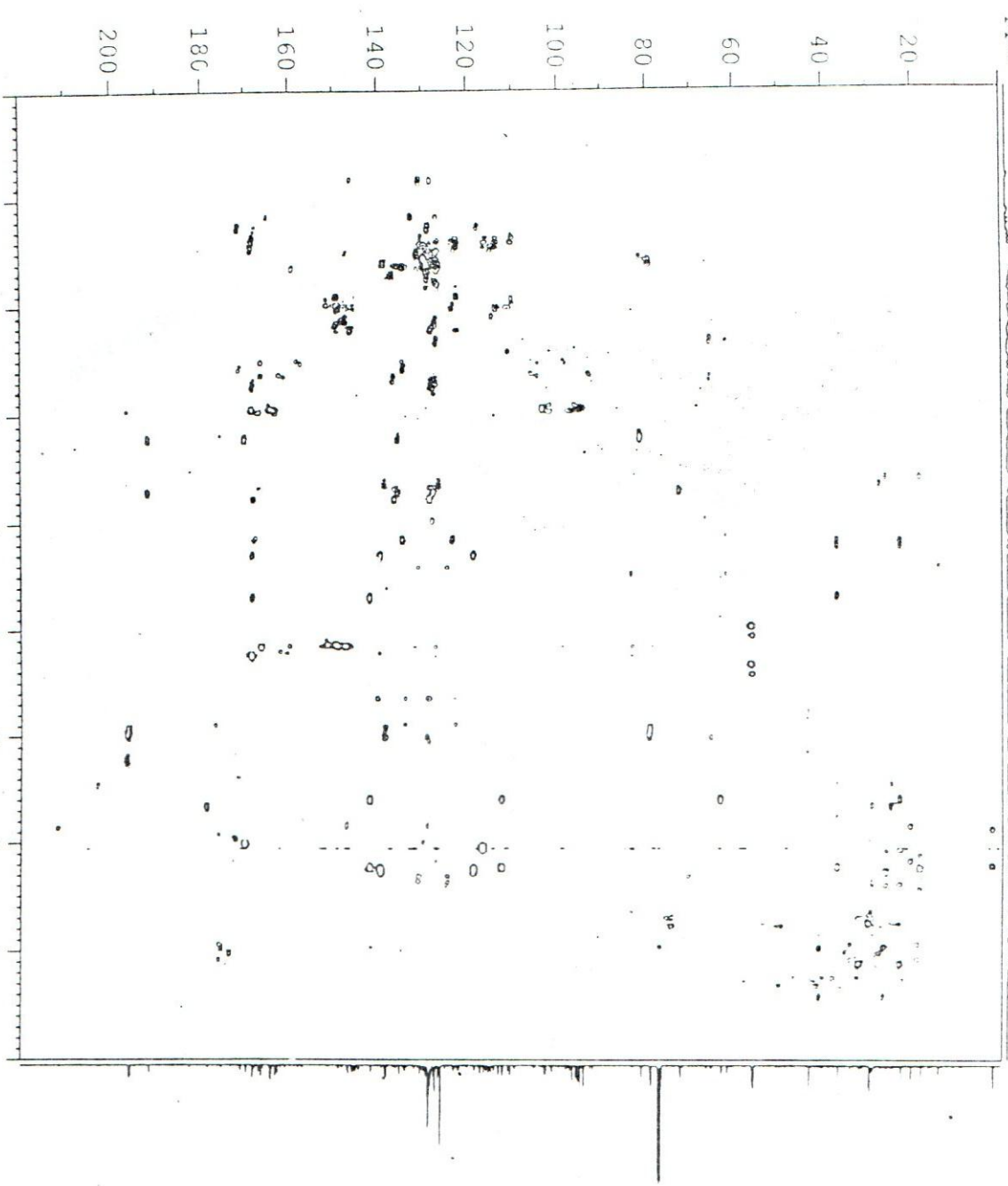


EM01
HSQC



HSQC

HMBC



EM01
HMBC

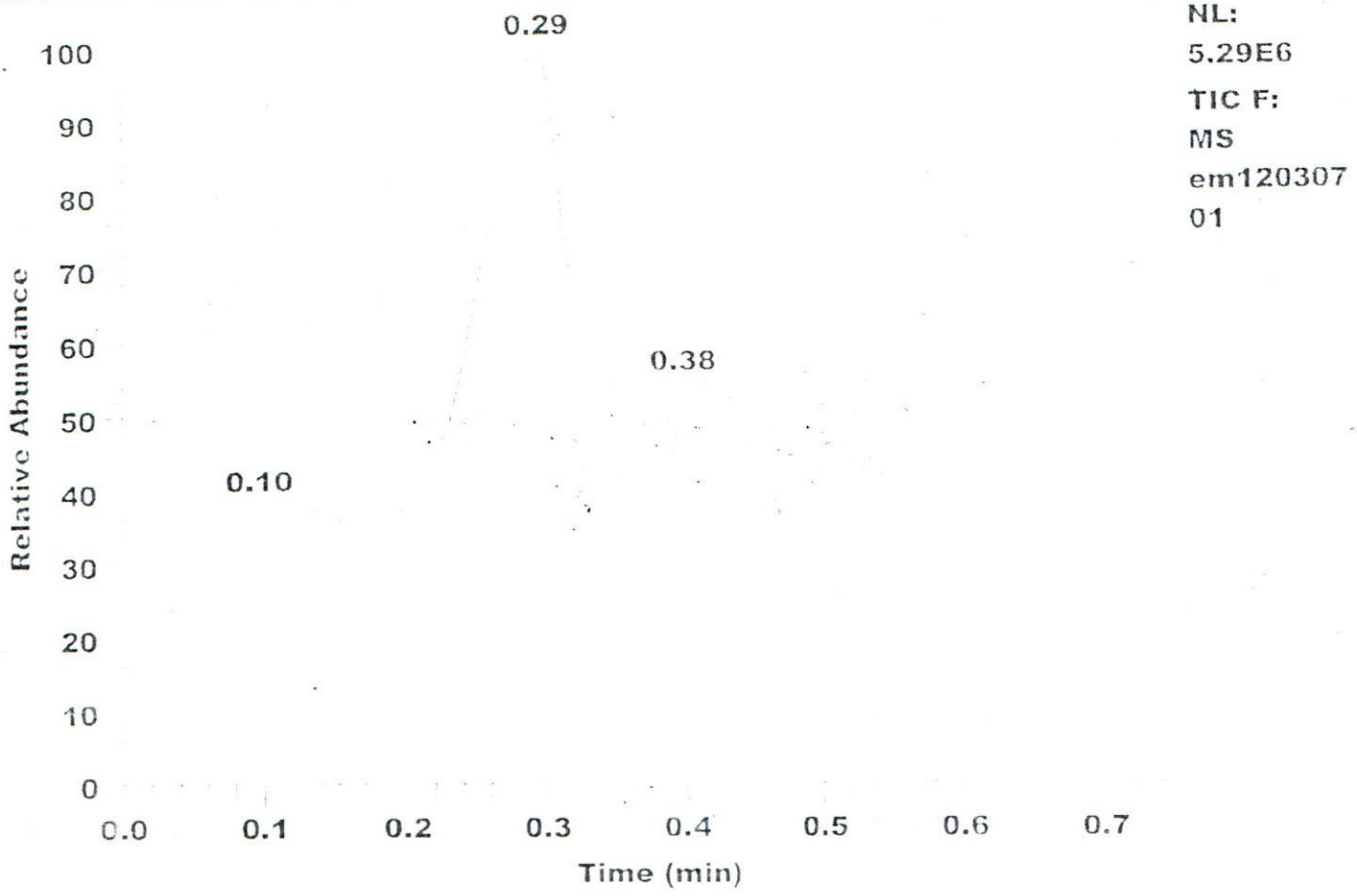
APPENDIX V

MS spectra

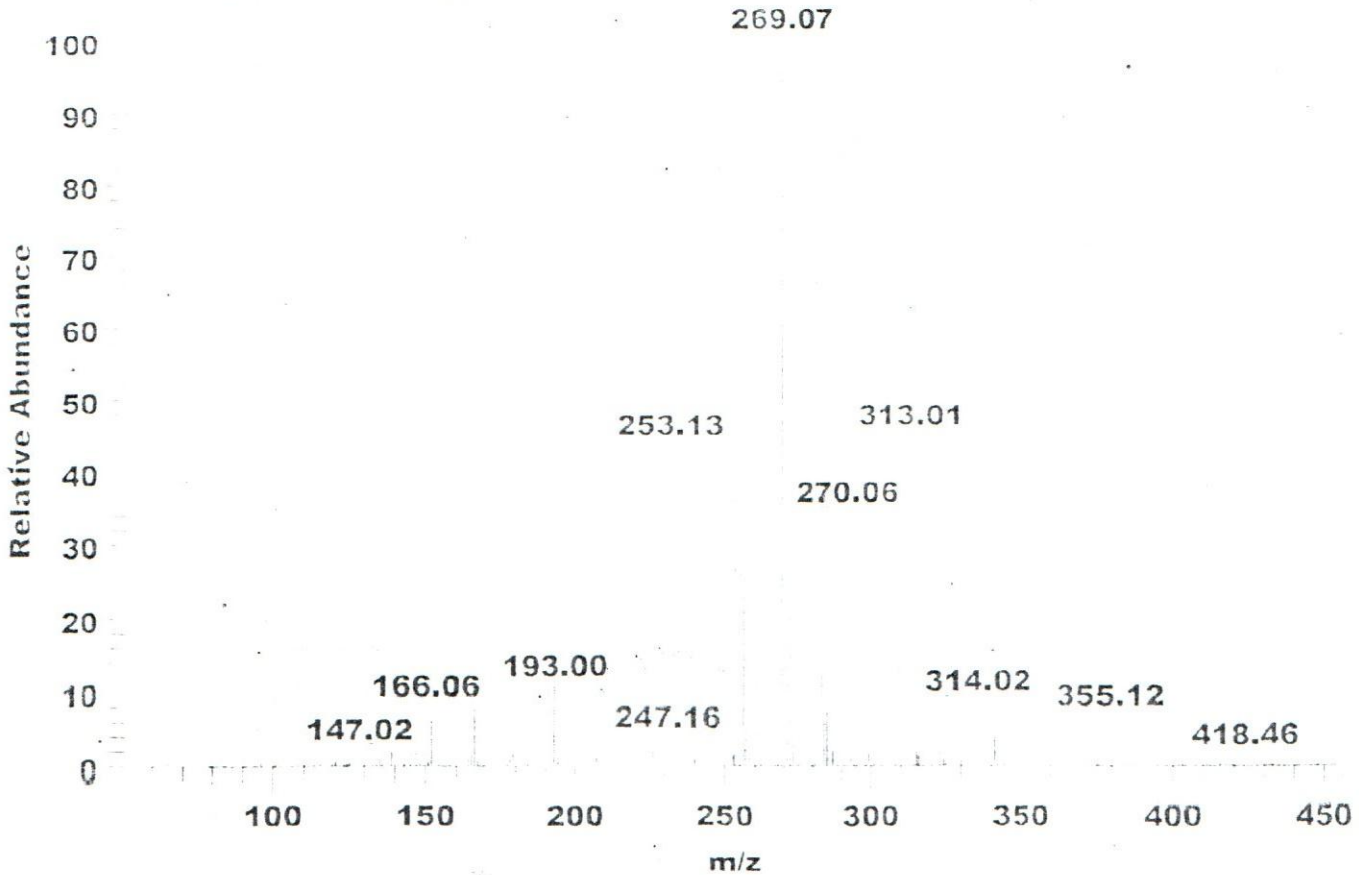
em12030701
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Project:	n/a
Client:	E.Mutanibi
Pol/Mode:	-CI-CH4 Probe
Column:	N/A
Operator:	B.Tattam
Instrument Method:	C:\Xcalibur\methods\Probes\ProbeMeth300to650negion.meth
Processing Method:	
Vial:	1
Injection Volume (ul):	1.00
Sample Weight:	0.00
Sample Volume (ul):	0.00
ISTD Amount:	0.00
Dil Factor:	1.00

RT: 0.00 - 0.76 SM: 7G

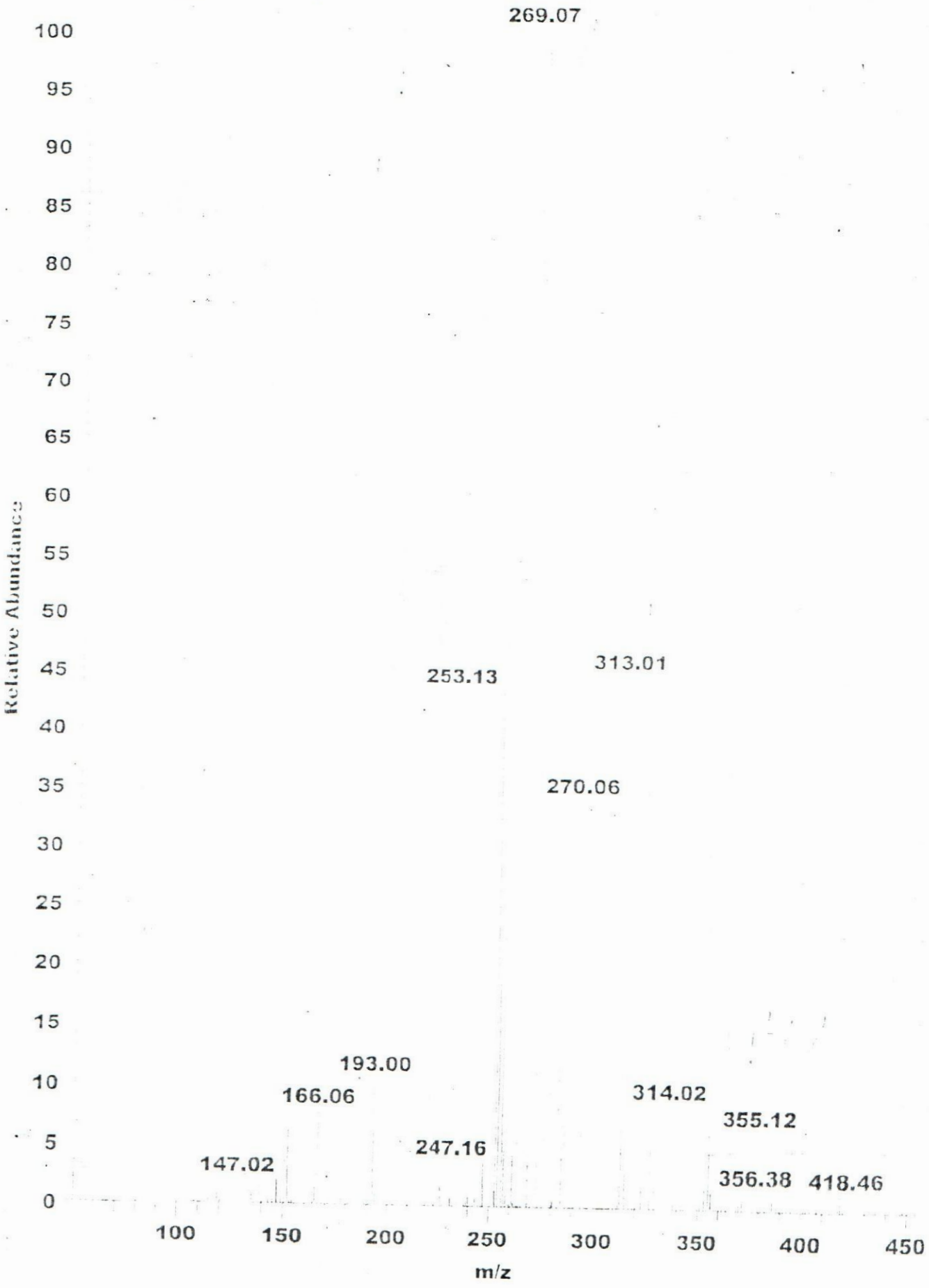


em12030701 #35-54 RT: 0.24-0.35 AV: 20 SB: 10 0.13-0.18 NL: 5.16E5
T: - c Full ms [50.00-650.00]



MS spectra

em12030701 #35-54 RT: 0.24-0.35 AV: 20 SB: 10 0.13-0.18 NL: 5.16E5
T: - c Full ms [50.00-650.00]

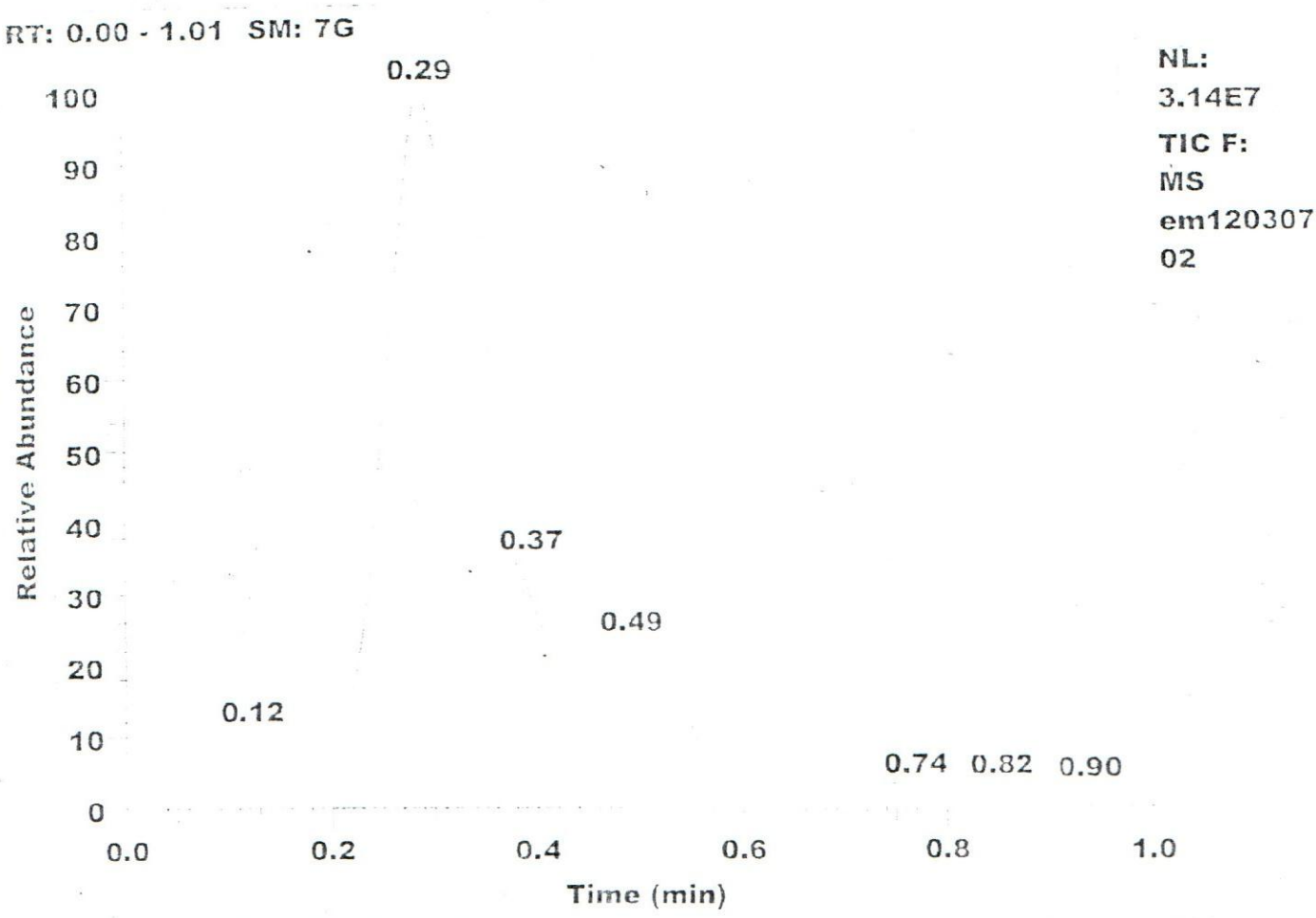


MS spectra

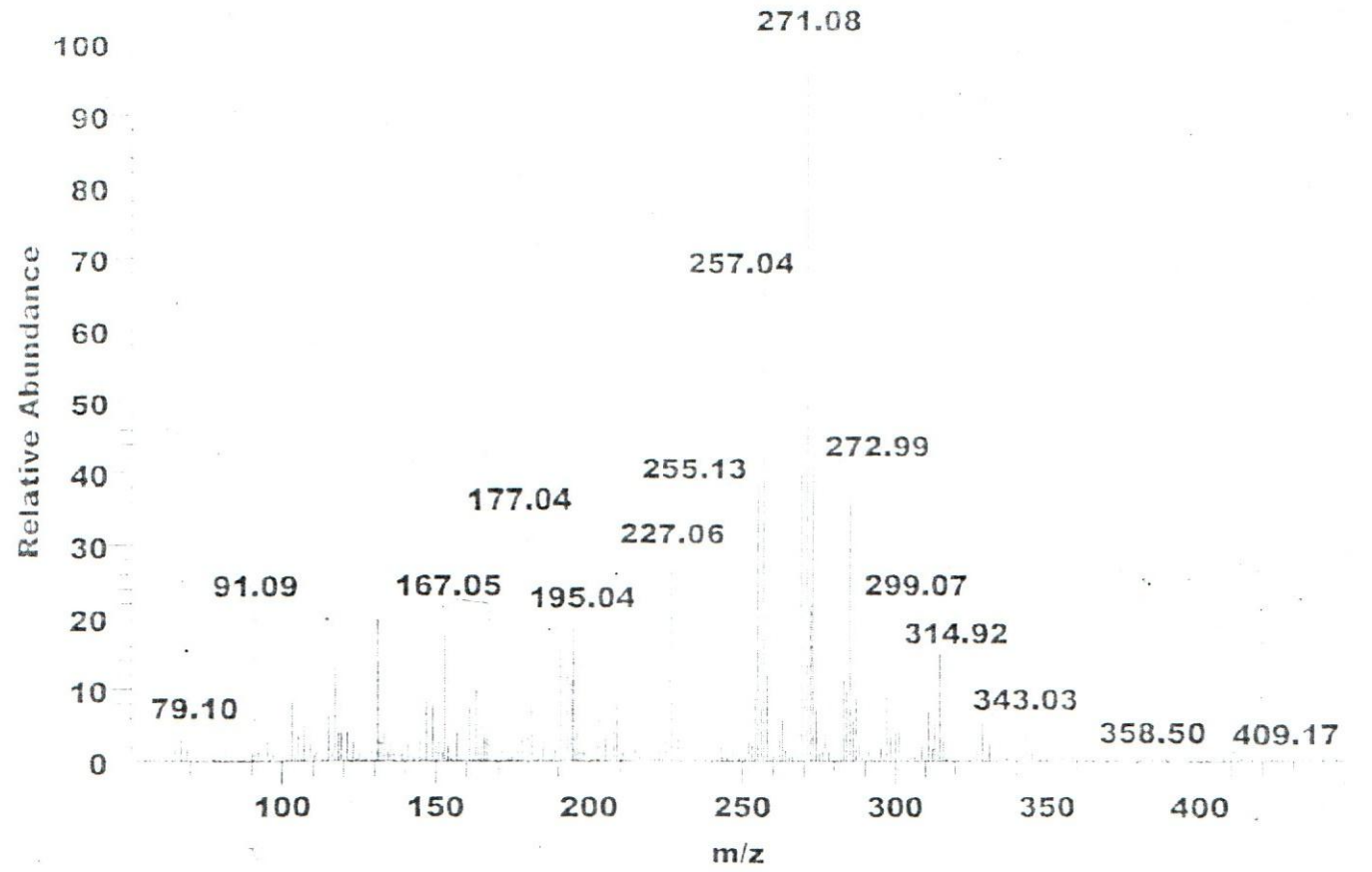
em12030707
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Sample Name:	EMK Fraction 1
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Client:	E.Mutanibi
Pol/Mode:	+CI-CH4 Probe
Column:	N/A
Operator:	B.Tattam
Instrument Method:	C:\Xcalibur\methods\Proces\ProbeMeth50to650.meth
Processing Method:	
Vial:	1
Injection Volume (µl):	1.00
Sample Weight:	0.00
Sample Volume (µl):	0.00
ISTD Amount:	0.00
Dil Factor:	1.00

MS spectra

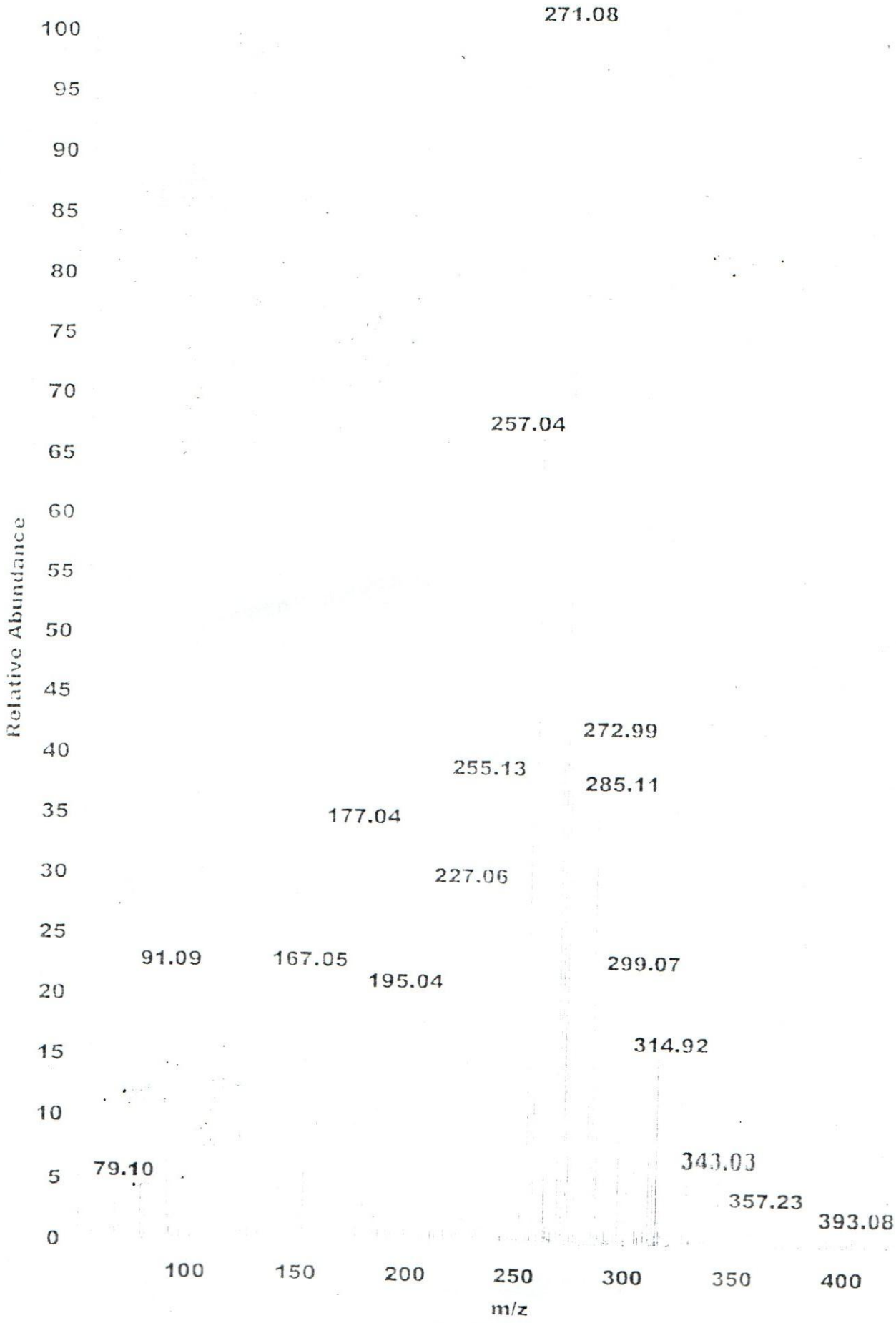


em12030702 #34-54 RT: 0.24-0.36 AV: 21 SB: 22 0.13-0.18 , 0.61-0.67 NL: 1.62E6
T: + c Full ms [50.00-650.00]

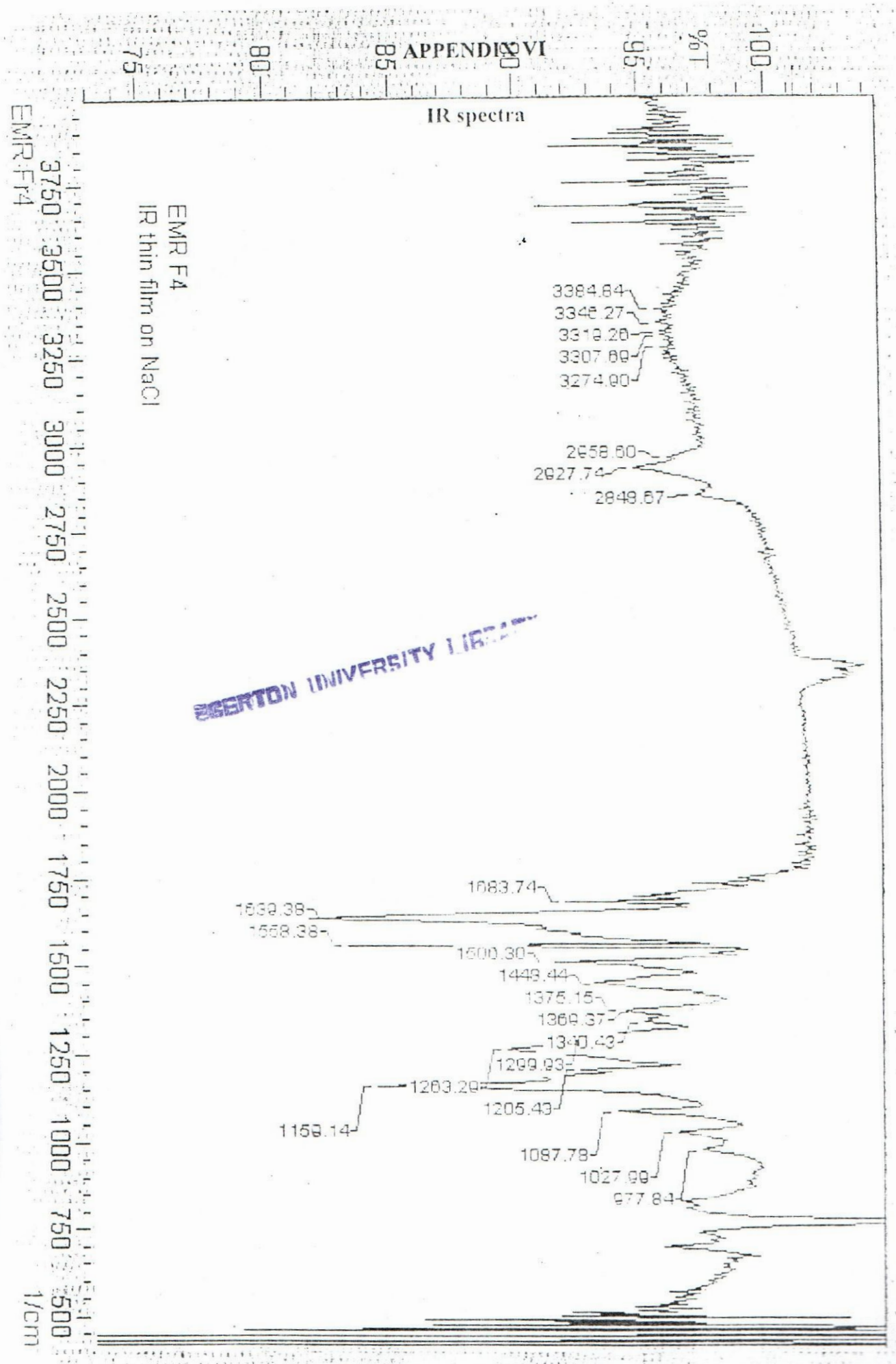


MS spectra

em12030702 #34-54 RT: 0.24-0.36 AV: 21 SB: 22 0.13-0.18 , 0.61-0.67 NL: 1.62E6
T: + c Full ms [50.00-650.00]



IR spectra



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