Discovery of Lead Molecules from Natural Sources Targeting Pro-inflammatory Cytokines

THESIS

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by

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CERTIFICATE

This is to certify that the thesis entitled "**Discovery of Lead Molecules from Natural Sources Targeting Pro-inflammatory Cytokines**" and submitted by **KHAN RUKAIYYA** ID No. **2010PHXF428H** for award of Ph.D. of the Institute embodies original work done by her under my supervision.

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ABSTRACT

Dysregulation of key pro-inflammatory cytokines like tumour necrosis factor-α, interleukin-1β and interleukin-6 causes chronic inflammation resulting in fibrosis and tissue necrosis. The persistent chronic inflammation increases the development of the degenerative diseases such as rheumatoid arthritis, atherosclerosis, heart disease, alzheimer, asthma, acquired immunodeficiency disorder, cancer, congestive heart failure, multiple sclerosis, diabetes, infections (bacteria, fungi, parasites), gout, inflammatory bowel disease, aging and other neurodegenerative central nervous system depression, all of which are associated with immunopathological conditions that play a key role in the onset of the condition. Therefore these cytokines were targeted in the development of anti-inflammatory therapies. Several extensive research reports had revealed that blocking of TNF-α, IL-1β and / or IL-6 were found to be efficacious for many patients. Thus, agents that suppress the expression of these cytokines will have the potential for alleviating inflammatory processes leading to diseases.

In the present research work, attempts were made to discover low molecular weight cytokine inhibitory leads from two different natural sources targeting microorganism and plants. Based on literature review, *Pseudomonas* and *Abutilon indicum* were selected for the study. A *Pseudomonas* bacteria identified as *P. aeruginosa* 16S ribosomal RNA gene was isolated from the soil and the ethyl acetate extract of its culture broth (**EEPA**) was prepared. The chromatographic purification of **EEPA** yielded four cyclic dipeptides characterized as cyclo(L-Proline-Glycine) (**PA-1**), cyclo(L-Proline-L-Phenylalanine) (**PA-2**), cyclo(*trans*-4-hydroxy-L-Proline-L-Phenylalanine) (**PA-3**), cyclo(*trans*-4-hydroxy-L-Proline-L-Leucine) (**PA-4**). Isolation of these 2,5-dioxopiperazine derivatives from the culture broth of *P. aeruginosa* is reported for

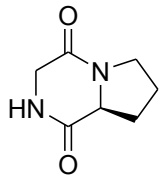
the first time. The chemical investigation on aerial parts of *Abutilon indicum* lead to the isolation of five aromatic compounds, characterized as methyl *trans-p*-coumarate (**AI-1**), methyl caffeate (**AI-2**), syringic acid (**AI-3**), vanillic acid (**AI-4**) and pinellic acid (**AI-5**). Existence of these secondary metabolites in *A. indicum* is also reported for the first time except vanillic acid.

The mother extract and all isolated pure compounds were subjected for in-vitro LPS-induced production of TNF-α assay in RAW 264.7 cells using ELISA kit. **EEPA**, **PA-1**, **PA-2**, **PA-3**, **PA-4** and **AI-2** showed promising results on inhibition of TNF-α production levels during LPS challenge. **EEPA** exhibited 74.2% suppression (100 μg/ml) of TNF-α (IC_{50} 38.79 μg/ml). Among the single compounds 60% inhibition of TNF-α was observed by **PA-1** (IC_{50} 4.48 μg/ml), 44.95% by **PA-2**, 53.75% by **PA-3** (IC_{50} 14.24 μg/ml) and 49.26% by **PA-4** at 100 μM. Out of all isolated compounds from *A. indicum* only **AI-2** showed 55% inhibition at 100 μM. In addition, **EEPA** and **PA** compounds exhibited marked attenuation of mRNA expression of TNF-α in LPS-stimulated mouse macrophages measured by quantitative RT-PCR. **EEPA** was significantly decreasing the mRNA expression ($p < 0.001$) when compared to single compounds, where **PA-1** showed significant reduction ($p < 0.001$). Further, in-vivo testing of **EEPA** at various dose levels and chemically synthesized **PA-4** and **AI-2** in acute rat endotoxemia model, validated significant TNF-α reduction ($p < 0.001$) with 79.3% (500 mg/kg) by **EEPA** and 51% (50 mg/kg) by **PA-4**, which indicated drug-like properties of proline-based cyclic dipeptides. However, **AI-2** did not show any inhibition under in-vivo model.

Besides, **EEPA** and all **PA** samples were tested for other cytokines, IL-1β and IL-6 and were found to significantly diminish their cellular mRNA expressions when tested by quantitative RT-PCR technique. Likewise, **EEPA** and all **PA** samples controlled nitric oxide (NO) production at 25 and 100 μg/ml. Less toxicity to RAW 264.7 cells were demonstrated by

EEPA and **PA** compounds, when analyzed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay.

In summary, EEPA and its metabolites cyclo(Glycine-L-Proline) (**PA-1**) and cyclo(*trans*-4-hydroxy-L-Proline-L-Phenylalanine) (**PA-3**) emerged as potent small lead molecules inhibiting TNF-α production (IC_{50} 38.79 μg/ml, 4.48 μg/ml and 14.24 μg/ml, respectively) as well as IL-1β and IL-6 mRNA expressions, NO production and possessing weak cytotoxicity.



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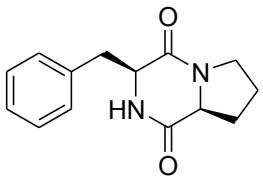

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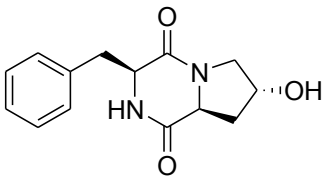

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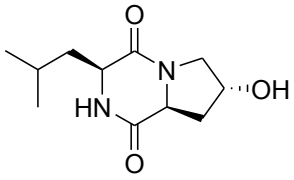

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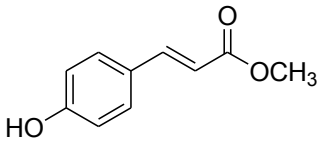

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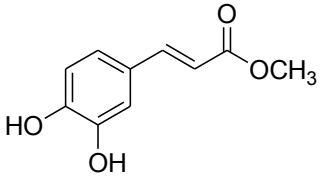

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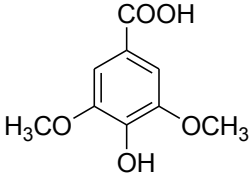

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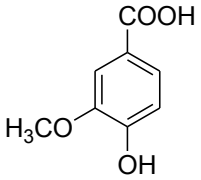

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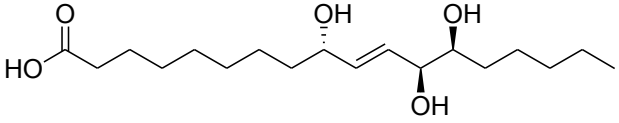

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ABBREVIATIONS

μg	:	Micro gram
μl	:	Micro liter
μM	:	Micro mole
α	:	Alpha
β	:	Beta
δ	:	Delta
γ	:	Gamma
κ	:	Kappa
λ	:	Lamda
μ	:	Micro
%	:	Percentage
°C	:	Degree Celsius
1-HP	:	1-hydroxyphenazine
3-HB	:	3-hydroxybutyric acid
3-HHD	:	3- hydroxyhexadecanoic acid
3-HOD	:	3-hydroxyoctadecanoic acid
3-HV	:	3-hydroxyvaleric acid
$CaCl_2$	:	Anhydrous calcium chloride
CH_2Cl_2	:	Dichloro methane
$CHCl_3$	:	Chloroform
CH_3CN	:	Acetonitrile
CO_2	:	Carbon di-oxide
$FeCl_3$	:	Ferric chloride

HCl	:	Hydrochloric acid
H_2SO_4	:	Sulphuric acid
$MgCl_2$	:	Magnesium chloride
$NaHCO_3$	:	Sodium bi-carbonate
Na_2SO_4	:	Anhydrous sodium sulphate
SiO_2	:	Silicon dioxide (Silica gel)
AHL	:	N-acylhomoserine lactones
AIDS	:	Acquired Immunodeficiency Disorders
AMs	:	Adhesion Molecules
CAM	:	Cellular Adhesion Molecules
CC	:	Column Chromatography
cDNA	:	Complementary DNA
CHF	:	Congestive Heart Failure
CF	:	Cystic Fibrosis
CNS	:	Central Nervous System
COX-1	:	Cyclo-oxygenase-1
COX-2	:	Cyclo-oxygenase-2
DC	:	Dendritic Cells
DNA	:	Deoxyribose Nucleic Acid
DMSO	:	Dimethyl Sulfoxide
DMEM	:	Dulbecco's Modified Eagle's Medium
DRA	:	Diffuse Reflectance Attachment
ESI	:	Electron Spray Ionization
EtOAc	:	Ethyl acetate
EEPA	:	Ethyl acetate extract of *P. aeruginosa*
ELISA	:	Enzyme Linked Immunosorbent Assay
FAB-MS	:	Fast Atom Bombardment-Mass Spectroscopy

FBS	:	Fetal Bovine Serum
g	:	Gram
GC-MS	:	Gas Chromatography-Mass Spectroscopy
GM-CSF	:	Granulocyte–Monocyte Colony-Stimulating Factor
h	:	Hour
HAAs	:	3-(3-hydroxyalkanoyloxy)alkanoic acids
HIV	:	Human Immunodeficiency Virus
HPLC	:	High Performance Liquid Chromatography
IACUC	:	Institutional Animal Care & Use Committee
IBD	:	Inflammatory Bowel Disease
ICAM-1	:	Intercellular Adhesion Molecule-1
IC_{50}	:	Inhibitory Concentration 50%
IFN-γ	:	Interferon-γ
IL-1	:	Interleukin-1
IL-1β	:	Interleukin-1β
IL-2	:	Interleukin-2
IL-6	:	Interleukin-6
IL-8	:	Interleukin-8
IL-10	:	Interleukin-10
IL-17	:	Interleukin-17
iNOS	:	Inducible Nitric Oxide Synthase
IR	:	Infrared
Kg	:	Kilo gram
LK	:	Leukotrienes
LPS	:	Lipopolysaccharide
LT	:	Lymphotoxin

mg	:	Milligram
MS	:	Mass Spectroscopy
MS	:	Multiple Sclerosis
MMP-9	:	Matrix Metalloproteinase-9
MeOH	:	Methanol
mM	:	Milli mole
min	:	Minute
ml	:	Milli liter
mAb	:	Monoclonal antibody
MNL	:	Mononuclear Leukocytes
MTT	:	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
ng	:	Nano gram
nm	:	Nano meter
nM	:	Nano Molar
NF- κB	:	Nuclear Factor kappa B-cells
NJ	:	Neighbour-Joining
NO	:	Nitric oxide
NSAIDs	:	Non-Steroidal Anti-Inflammatory Drugs
NMR	:	Nuclear Magnetic Resonance
ppm	:	Parts per million
PBMCs	:	Peripheral Blood Mononuclear cells
PCA	:	Phenazine-1-carboxylic acid
PBS	:	Phosphate Buffer Saline
PDA	:	Photo Diode Array
PCR	:	Polymerase Chain Reaction
PG	:	Prostaglandins
PML	:	Progressive Multifocal Leukoencephalopathy
QSSM	:	Quorum Sensing Signal Molecules

ROS	:	Reactive Oxygen Species
rpm	:	Rotation per minute
R_f	:	Retention factor
R_t	:	Retention time
RT-PCR	:	Real time-PCR
RNA	:	Ribose Nucleic Acid
sec	:	Seconds
SCL-LCL	:	Short-chain-length- long-chain-length
TLC	:	Thin layer chromatography
TLR4	:	Toll like receptor-4
TFA	:	Trifluoroacetic acid
TNF-α	:	Tumor Necrosis Factor-α
UV	:	Ultraviolet

CHAPTER 1
INTRODUCTION

Inflammation is a complex process initiated by bacterial infection, chemical injury and environmental pollution that result in cell injury or death (Dalgleish & O'Byrne, 2002; O'Byrne & Dalgleish, 2001) and release of inflammatory mediators including the cytokines and tumor necrosis factor (TNF-α), interleukin-1 (IL-1) from leukocytes, monocytes and macrophages (Paterson *et al*., 2003). Saklatvala *et al*., in 2003 reported that the cytokines further up-regulate the release of other pro-inflammatory cytokines and chemokines, immunoglobulins, as well as increase the expression of many cellular adhesion molecules (CAMs).

1.1. INFLAMMATORY PROCESS

The localized protective reaction of cells/tissues of the body to allergic or chemical irritation, injury and/or infections causes inflammation. The symptoms of inflammation are characterized by pain, heat, redness, swelling and loss of function that result from dilation of the blood vessels leading to an increased blood supply. Increased intercellular space results in the movement of leukocytes, protein and fluids into the inflamed regions (Parham, 2000). These mediators i.e. nitric oxide (NO), prostaglandins (PG), leukotrienes (LK), vasoactive amines (histamine, serotonin), and cytokines (TNF-α and interleukins–1, 12) are termed as pro-inflammatory mediators that bind to specific target receptors on the cells and may increase vascular permeability, promote neutrophil chemotaxis, stimulate smooth muscle contraction, increase direct enzymatic activity, induce pain and/or mediate oxidative damage (Coleman, 2002). Inflammation is mainly classified into 2 types i.e. acute and chronic inflammation.

1. Acute inflammation is characterized by rapid onset, short duration, exudation of fluids, plasma proteins and migration of leukocytes, most notably neutrophils into the injured area. This acute inflammatory response is a defense mechanism aimed at

killing of bacteria, virus and parasites while still facilitating wound repairs.

2. Chronic inflammation is of a more prolonged duration and manifests histologically by the presence of lymphocytes and macrophages, resulting in fibrosis and tissue necrosis. The persistent chronic inflammation increases the development of the degenerative diseases such as rheumatoid arthritis, atherosclerosis, heart disease, alzheimer, asthma, acquired immunodeficiency disorder (AIDS), cancer, congestive heart failure (CHF), multiple sclerosis (MS), diabetes, infections (bacteria, fungi, parasites), gout, inflammatory bowel disease (IBD), aging and other neurodegenerative central nervous system (CNS) depression, all of which are associated with immunopathological conditions that play a key role in the onset of the condition (O'Byrne & Dalgleish 2001; Dalgleish & O'Byrne 2002).

These various diseases and disorders have been linked to increased expression of pro-inflammatory mediators which activates inflammatory cells by increasing the expression of pro-inflammatory cytokines, up-regulating genes that produce NF-κB, NADPH oxidase, phospholipase A_2, cyclo-oxygenase (COX) -1 and -2, 5-LOX, myeloperoxidase, inducible nitric oxide synthase (iNOS), increasing oxygen consumption and producing many oxygen-free radicals that can finally lead to certain degenerative diseases (Iwalewa *et al*., 2007). NO is an example of reactive species that participates in normal physiological processes such as vasodilation and neurotransmission; however, overexpression may result in diseases like asthma, cardio-vascular disorders and organ transplant rejection (Coleman, 2002). Many other factors such as chronic lung and liver inflammation caused by tobacco smoking and alcohol consumption may lead to lung cancer and liver cirrhosis respectively, while the persistent inflammation of the stomach is caused by the bacterium, *Helicobacter pylori* which may lead to ulcers and ultimately to stomach cancer (Iwalewa *et al*., 2007).

Table 1.1: The role of pro-inflammatory cytokines as expressed in disease (Iwalewa *et al*., 2007)

Diverse disorder	Pro-inflammatory cytokines expression
AIDS	Increased secretion of TNF-α, IL-1 and IL-6 by macrophages and monocytes because of viral load and increased capacity of dendritic cells (DC) exposed to HIV-1 to produce TNF-α and IL-1β, IL-10. Overexpression contributes to B-cell hyperactivity and risk of AIDS.
Acute infection	Elevated myeloperoxidase, reactive oxygen species (ROS) and IL-6 in severe infections.
Parasitic infections	Increased TNF-α concentrations in patients with *Plasmodium falciparum*.
Asthma	Promotion of eosinophilia and cytokines that regulate allergic states and production of IL-4, IL-5, IL-10 and IL-13; administration of IL-12, IFN-α/γ are suggested to alleviate asthma disease; increased NO in exhaled air reflected airway inflammation in asthma patients.
Atherosclerosis	Increased endothelium concentrations of IL-1 and TNF-inducible adhesion molecules and intracellular adhesion molecule-1 in atherosclerotic tissue.
Congestive heart failure	Increased concentrations of TNF-α and IL-6 were associated with progression from asymptomatic to symptomatic left ventricular dysfunction and excessive TNF-α levels associated with mortality; IL-6 is a strong predictor of disease progression; patients without cachexia that experience acute decompensation have increased levels of TNF-α.
Fever	In periphery and brain increased concentrations of IL-1α, 1β, TNF-α and IL-6; post-myocardial infarction patients with prolonged fever had increased inflammatory activity.
Crohn's disease	High IL-1 and 12 activities increase pro-inflammatory state.

Peptic ulcer	High ulcerogenic potential of *Helicobacter pylori* increases the activity of IL-8 and TNF-α; *Helicobacter pylori* and non-steroidal anti-inflammatory drugs (NSAIDs) cause ulcer recurrence through production of IL-1 and TNF-α by macrophages accumulated at the ulcer scar.
Alzheimer's disease	Neuroinflammation due to inflammatory mediator overexpression is associated with behavioral disturbances; increased IL-1 expression in alzheimer brain is directly related to plaque formation and progression and neuronal overexpression of acetylcholinesterase; TNF-α, IL-1β and IL-6 overexpression stimulated production of amyloid-β, which is crucial for neurodegeneration in alzheimer's patients.
Down's syndrome	Overexpression of IL-1 in middle-aged individuals that have concurrent alzheimer-type changes.
Multiple sclerosis	Elevated TNF-α concentrations in serum and cerebral spinal fluid; brain endothelium and astrocytes increased expression of intercellular adhesion molecule (ICAM)-1.
Diabetes	Th1 and Th2 cells and their respective mediators participate and cooperate in inducing and sustaining pancreatic islet cell β-cell destruction in insulin dependent diabetes; inflammation important factor in pathogenesis of diabetes and metabolic disorders in women; obesity and diabetes inflammatory states in which mediators of inflammation contribute to insulin resistance.
Pain	TNF-α, IL-1 and IL-6 pathway is associated with altered pain perception; hyperalgesia induced by TNF-α via stimulating release of IL-1; hyperalgesia induced by peripheral inflammation is associated with IL-1 overexpression; spinal cord glia derived pro-inflammatory cytokines suggested to be powerful modulators of pain; IL-1β mediated induction of COX-2 in neurons of the CNS contributes to inflammatory pain hypersensitivity; bradykinin-B_2 receptors are suggested to be involved with the acute phase of the inflammatory and pain response;

	TNF-α expression is suggested to be up-regulated in Schwann cells influencing central in painful neuropathies.
Depression	Increased expression of IL-1β, IL-6 and IFN-γ, IL-1ra, sIL-6r and TNF-α; increased IL-1β concentrations in cerebrospinal fluid; increased concentrations of IL-6, sIL-6r, sIL-2r and transferrin receptor in major depression.
Schizophrenia	Increased concentrations of IL-6 and TNF-α increased IL-1β polymorphism; drug-naive schizophernic patients had increased IL-2 and IFN-γ production compared to controls.
Sleep disorders	TNF-α and IL-6 suggested to play an important role in mediating sleepiness and fatigue in disorders of excessive daytime sleepiness; systemic inflammatory response and reduced plasma availability of tryptophan was related to primary sleep disorders and major depression.
Stress	Psychological stress is associated with increased production of TNF-α, IL- 1, IL-1ra, IFN-γ and lower production of IL-4 and IL-10; increased expression of neutrophils, monocytes, $CD8^+$, $CD2^+CD26^+$ and $CD2^+$HLA-DR^+T cells and $CD19^+$ B cells; post traumatic stress disorder was associated with increased IL-6 signaling.
Rheumatoid arthritis	Increased concentrations of TNF-α as a central pro-inflammatory mediator increased concentrations of IL-1, IL-6, TNF-α, Granulocyte–Monocyte Colony-Stimulating Factor (GM-CSF), and chemokines IL-8.
Sepsis	Systemic inflammatory response syndrome due to pro-inflammatory mediator excess is associated with severe inflammatory responses then excessive anti-inflammatory responses possibly leading to increased susceptibility to infection; septic shock is caused by excessive or deregulated host inflammatory responses.

1.2. SOLUBLE MEDIATORS OF INFLAMMATION

Numerous cytokines (small soluble proteins) including TNF-α, IL-1β and IL-6 are released on stimulation of monocytes, macrophages, fibroblasts, and T cells to influence the behavior of other cells, specifically including the regulation of cellular immunity and the inflammatory response (Murphy *et al*., 2008; Steinke *et al*., 2006). IL-1β, IL-6, and TNF-α commonly being induced together are often viewed as pro-inflammatory cytokines. These cytokines are produced both peripherally and in the CNS, typically by microglia (Brough *et al*., 2011) where they mediate neuro-inflammation and response to injury (Brough *et al*., 2011; Konuk *et al*., 2007). Because of their role in the CNS, these cytokines have been the subject of much investigation in psychiatric disease (Konuk *et al*., 2007).

1.2.1. Tumor Necrosis Factor-α

TNF-α has shown a diverse effect by stimulating a variety of cells and hence has been found to be a potent cytokine. It is a soluble 17-kd protein composed of three identical subunits. Mainly monocytes and macrophages, but also by B cells, T cells, and fibroblasts produce TNF-α. The newly synthesized TNF-α is inserted into the cell membrane and subsequently released through the cleavage of its membrane-anchoring domain by a serine metalloproteinase. Thus, inhibitors of this enzyme might suppress TNF-α secretion (Paul *et al*., 2006).

TNF-α is best known for its ability to promote inflammation. It is an autocrine stimulator as well as a potent paracrine inducer of other inflammatory cytokines, including IL-1β, IL-6, IL-8, and GM-CSF. TNF-α also promotes inflammation by stimulating fibroblasts to express adhesion molecules, such as ICAM-1. Interaction of these adhesion molecules, with their respective ligands on the surface of leukocytes, resulting in increased transport of leukocytes into inflammatory sites. Though TNF-α is protective in local infection, systemic release of TNF-α by activated macrophages of the liver and spleen in

response to sepsis causes shock, disseminated intravascular coagulation, and ultimately multi-organ failure. In response to injury, TNF-α production is increased in the brain, where it may react with NO to regulate the blood–brain barrier (McCoy & Tansey 2008). Besides its role in endothelial activation and phagocytosis, TNF-α mediates activation of the adaptive immune system by stimulating DC migration to regional lymph nodes, where DCs mature and present antigen to lymphocytes (Murphy *et al*., 2008). Like other proinflammatory cytokines, TNF-α stimulates its own production and that of IL-1β (Cairns *et al*., 2000).

1.2.2. Interleukin-1β

IL-1β is a 153–amino acid cytokine produced by activated blood monocytes and tissue macrophages such as microglia that affects most cell types and promotes inflammation by indirectly promoting lymphocyte function and activating macrophages (Dinarello, 2009). IL-1β up-regulates endothelial cell expression of the adhesion molecules, which bind to leukocyte integrins and promote infiltration of inflammatory cells into tissue. It also induces expression of inflammatory mediators such as COX-2 and iNOS, increases circulating neutrophils through the IL-17 driven T-helper type 17 (Th17) response, induces fever, and is a potent stimulator of IL-6 production by endothelial cells (Dinarello, 2009; Gray & Bloch, 2012). IL-1β is also important for T-cell dependent antibody production and may contribute to stimulating Th2 cell–mediated immune responses (Wang, 2006; Nakae, 2001). These diverse pro-inflammatory functions of IL-1β demonstrate its critical role in the inflammatory response and host defense against pathogens. However, similar to TNF-α, IL-1β has an important role in mediating the anti-inflammatory response, most notably through stimulation of the acute-phase reaction (Gray & Bloch, 2012).

1.2.3. Interleukin-6

IL-6, discovered in the 1980s as a lymphocyte-derived signal for B-cell maturation, functions as both a pro- and anti-inflammatory cytokine and has been implicated in a variety of inflammatory and autoimmune disorders (Spooren, 2011). IL-6 is a 184–amino acid cytokine produced by both immune cells, including macrophages, B cells, and T cells, and non-immune cells, such as endothelial cells and fibroblasts, in response to homeostatic disturbances such as infection and injury. IL-6 is a critical cytokine for the differentiation and growth of T and B cells (Gray & Bloch, 2012). As a pro-inflammatory cytokine, IL-6 is important in neuro-inflammation, the brain-specific activation of microglia and astrocytes that accompanies neurodegenerative disorders (Spooren, 2011). Like other pro-inflammatory cytokines, including TNF-α and IL-1β, IL-6 induces the acute-phase reaction, the body's systemic anti-inflammatory response to local inflammation. The acute-phase reaction consists of fever, the production of acute-phase proteins such as protease inhibitors by the liver, and systemic release of corticosterone (Gray & Bloch, 2012). Excess IL-6 also causes anaemia, a condition in which the body lacks haemoglobin (Hashizume *et al*., 2010). This lowers the capacity of the blood to carry oxygen around the body, which can result in tiredness and fatigue. High IL-6 levels may also cause permanent damage of bone and cartilage, as it encourages the body to increase bone resorption and blocks new bone tissue formation (Katagiri *et al*., 2002).

1.3. ANTI-INFLAMMATORY AGENTS

A number of ''biological agents'' have been developed to treat inflammation (Table 1.2), including agents that reduce the activity of specific cytokines or their receptors, block lymphocyte trafficking into tissues, prevent the binding of monocyte-lymphocyte co-stimulatory molecules, or deplete B lymphocytes.

Table 1.2: Biological agents in the treatment of chronic autoimmune and inflammatory diseases (Dinarello 2010)

Drugs	Function	Disease target
Anti-CD3 (eplizumab); anti-IL-2 receptor mAb (daclizumab)	Targeting T cells	Transplant rejection; Type 1 diabetes
Anti-CD20 (rituximab, crelizumab, ofatumumab); anti-CD22 (epratuzumab); anti-Blys mAb IgG1 (belimumab)	Targeting B cells	Type 1 diabetes; rheumatoid arthritis; multiple sclerosis
Anti-TNF-α mAb (infliximab, adulimumab, golimumab); anti-TNF-α pegylated Fab' (certolizumab); soluble TNF-α p75 receptor Fc fusion (etanercept)	Reducing TNF-α activties	Rheumatoid arthritis; Crohn's disease; Psoriasis
Anti-IL-6 mAb (MEDI5117); Anti-IL-6 receptor (toculizumab)	Reducing IL-6 activties	Rheumatoid arthritis; juvenile arthritis
Anti-IL-12/23 (ustekinumab); Anti-IL-17 mAb (AIN457/LY24398)	Neutralization of IL-12, IL-23 and IL-17	Rheumatoid arthritis; Crohn's disease; Psoriasis
IL-1 receptor antagonist (anakinra); soluble IL-1 receptor (rilonacept); anti-IL-1b (IgG1) (canakinumab); anti-IL-1b (IgG2) (Xoma 052); anti-IL-1R mAb IgG1 (AMG 108)	Reducing IL-1β activties	Anti-inflammatory disease
Anti-a4 integrins mAb (natalizumab); anti-LFA-1 MoAb (efalizumab)	Blocking cell adhesion and migration	Multiple sclerosis; Crohn's disease; Psoriasis
CTLA-4 Ig fusion protein (abatacept)	Blocking T cell co-receptor	Type 1 diabetes; rheumatoid arthritis

mAb: Mono-clonal antibody

1.4. SIDE EFFECTS OF BIOLOGICAL AGENTS

The major side effect of biological agent is the reduction in host defense against infections, which can be treated effectively with antibiotics if detected early. Progressive multifocal leukoencephalopathy (PML) is a rapidly demyelinating and potentially fatal disease that is caused by a virus and is often observed in patients treated with immunosuppressive drugs or in patients with AIDS. PML has been associated with patients with multiple sclerosis or Crohn's disease treated with the monoclonal antibody natalizumab (Major, 2010). PML is also developed in patients treated with the B cell-depleting antibody rituximab and in psoriasis patients treated with the monoclonal antibody efalizumab. Drug like, natalizumab and efalizumab prevent the migration of T cells into tissues, whereas rituximab lyses CD20-bearing B cells and does not affect T cell migration.

Orally active small-molecule inhibitors of intracellular signaling pathways will likely be the new frontier of anti-inflammatory drug development. However, because many intracellular signaling molecules are involved in normal cellular functions, the effective concentration that does not elicit organ toxicity will need to be carefully determined. Statins, a safe class of drugs used for lowering serum cholesterol, also have anti-inflammatory properties. Orally active inhibitors of histone deacetylases, which are also safe and used clinically, are effective drugs with anti-inflammatory properties that also block cell proliferation. Naturally occurring resolvins are also being developed as anti-inflammatory agents (Dinarello, 2010).

1.5. NATURAL PRODUCTS

Natural products have long been recognized as anti-cancer supportive remedies (Diwanay *et al*., 2004), and anti-infectious agents including human immunodeficiency virus (HIV) (Asres *et al*., 2005). Their beneficial effects may be at least partially mediated by multiple interventions with cytokine expression (Spelman *et al*., 2006). Herbal and other natural products thus represent a rich source of potential drugs including immunomodulatory agents. A huge effort is being done to reconcile the healing experience of traditional medicines with Western medical practice and research (Patwardhan & Gautam, 2005). The search for new therapeutic means is greatly facilitated by recent extensive progress in phytochemistry, analytical biochemistry, biochemistry and bio-analytical methods allowing isolation and identification of the bioactive principles in botanicals (Gullo *et al*., 2006).

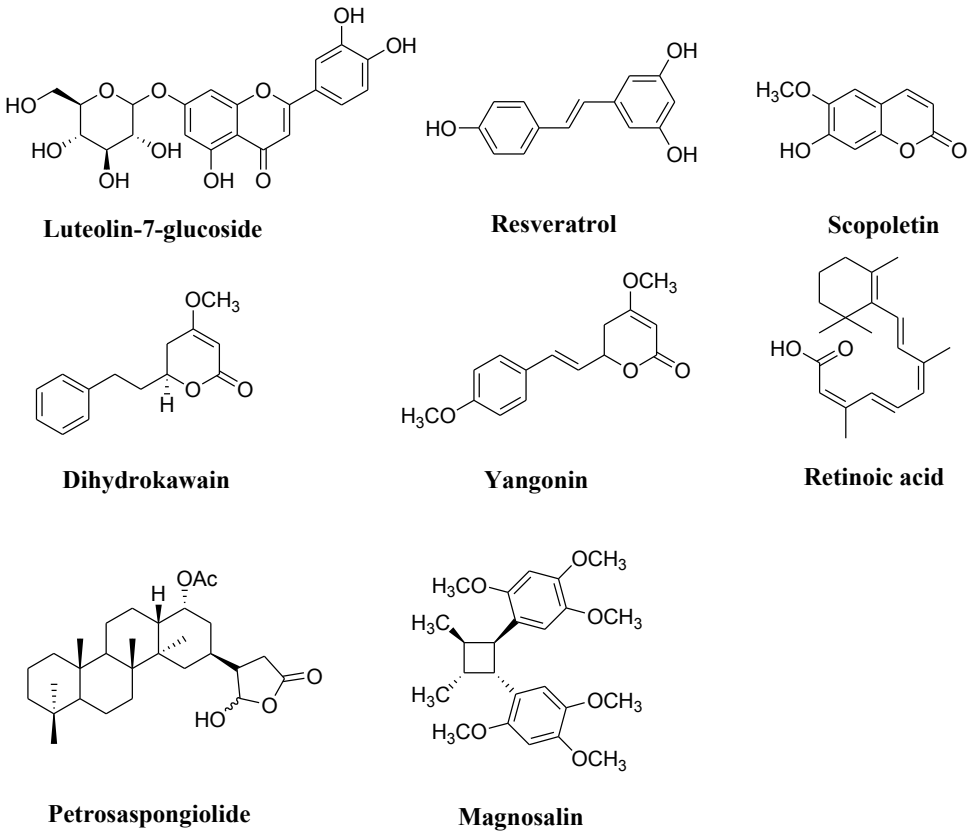


Figure 1.1: Representative natural compounds interfering with release of pro-inflammatory mediators (Paul *et al*., 2006)

A number of natural compounds, including polyphenols (e.g. resveratrol, quercetin, luteolin, hesperetin, kaempferol, scopoletin, aucubin, nardostachin, honokiol), alkaloids (lycorine), terpenes (acanthoic acid, tanshinone), sterols (guggulsterol) and other chemical classes, have been revealed as inhibitors of production of TNF-α, IL-1β and IL-6 (Paul *et al*., 2006). These pro-inflammatory cytokines also are down-regulated by a polyphenol curcumin from *Curcuma longa* (Gonzales & Orlando, 2008). The mechanism of action is obviously inhibition of the Toll like receptor-4 (TLR-4) signalling induced by lipopolysaccharide (Youn *et al*., 2006).

Fungal metabolites isolated from mycelia of *Verticimonosporium ellipticus*, *bis*-thiodiketopiperazines (emestrins) and cytochalasins are potent antagonists of chemokine receptor CCR2. The compounds are thus interesting agents for treatment of inflammatory processes associated with rheumatoid arthritis, multiple sclerosis and atherosclerosis (Herath *et al*., 2005). The plant cannabinoids, cannabidiol, cannabigerol, cannabichromene, cannabidiol acid have been reported to suppress tumour growth, the most effective being cannabidiol (Ligresti *et al*., 2006). Caffeine possesses prominent cytokine-inhibitory effects. It suppresses production of all Th1 (IL-2, IFN-g), Th2 (IL-4, IL-5) and T-regulatory (IL-10) cytokines (Horrigan *et al*., 2006).

Table 1.3: Molecular targets of some natural products that exhibit anti-inflamamtory potential (Khanna *et al*., 2007)

Compounds	Source	Molecular targets
Boswellic acid	*Boswellia 13urmeri* (Salai guggul)	NF-κB, COX-2, MMP-9, ICAM-1
Berberine	*Berberis vulgaris* (barberry)	NF-κB, COX-2, TNF-α, IL-1β, IL-6
Celastrol	*Tripterygium wilfordii*	NF-κB, COX-2, MMP-9, TNF-α, Ams
Curcubitacin R	*Cayaponia tayuya*	NF-κB, COX-2, TNF-α
Curcumin	*Curcuma longa* (13urmeric)	NF-κB, COX-2, TNF-α, IL-1β, IL-6, IL-8, MMPs, Ams
Eugenol	*Syzygium aromaticum* (cloves)	NF-κB, COX-2, TNF-α, IL-1β
Guggulsterone	*Commiphora mukul* (guggul)	NF-κB, COX-2, MMP-9
Genistein	*Glycine max* (soybeans)	NF-κB, TNF-α, IL-1β, IL-6
Luteolin	*Thymus vulgaris* (thyme)	NF-κB, COX-2, TNF-α
Morin	*Chlorophora tinctoria* (fustic)	NF-κB, COX-2, MMP-9, TNF-α, IL-1β, IL-6
Quercetin	*Allium cepa* (onions)	NF-κB, COX-2, TNF-α, IL-1β, Ams
Resveratol	*Vitis vinifera* (red grapes)	NF-κB, COX-2, TNF-α, Ams
Rosmarinic acid	*Rosmarinus officinalis* (rosemary)	NF-κB, COX-2, TNF-α, Ams

Silymarin	*Silybum marianum* (milk thistle)	NF-κB, COX-2, TNF-α, Ams
Statins	*Aspergillus terreus* (yeast)	NF-κB, COX-2, MMP-9, Ams
Tea polyphenols	*Camellia sinensis* (black tea)	NF-κB, COX-2, TNF-α, Ams, MMPs
Ursolic acid	*Ocimum sanctum* (holy basil)	NF-κB, COX-2, MMP-9
Withanolides	*Withania somnifera* (Ashwagandha)	NF-κB, COX-2, MMP-9, ICAM-1

(NF-κB, nuclear factor kappa B-cells; COX-2, cyclo-oxygenase-2; MMP-9, matrix metalloproteinase-9; ICAM-1, intercellular adhesion molecule-1; Ams, adhesion molecules)

Natural products have been the source of most of the active ingredients of medicines currently available and down the line identification of newer metabolites from natural sources will be the core of pharmaceutical discovery efforts. The chemical properties of the several natural molecules had been analysed (Ganesan, 2008) and half of them were found to be closely compliant with Lipinski's Rule of Five for orally available compounds, but the remainder had higher molecular weights, more rotatable bonds and more stereogenic centres, although they retained relatively low log P values which clears that on average, natural products are more readily absorbed than synthetic drugs. Over a 100 natural product derived compounds are currently undergoing clinical trials and at least a 100 similar projects are in preclinical development. Although projects based on natural products are predominantly being studied for use in cancer or as anti-infective, there are many other therapeutic areas where there is a huge scope for (Table 1.4) the development of bio-active leads from plants and microbes (Butler, 2008).

Table 1.4: Therapeutic categories of natural product-derived drugs at different stages of development (Harvey, 2008)

Therapeutic areas	Pre-clinical	Clinical Phase I	Clinical Phase II	Clinical Phase III	Pre-registration	Total
Cancer	34	15	26	9	2	86
Anti-infective	25	4	7	2	2	40
Neuropharmacolgical	6	3	9	4	0	22
Cardiovascular/gastro-intestinal	9	0	5	6	0	20
Inflammation	6	2	9	1	0	18
Metabolic	7	3	6	1	0	17
Skin	7	1	2	0	0	10
Hormonal	3	0	2	1	0	6
Immunosuppressant	2	2	0	2	0	6
Total	99	30	66	26	4	225

Source: Pharmaprojects database (March 2008)

There is, however, also a growing interest in the possibility of developing products that contain mixtures of natural compounds from traditionally used medicines (Charlish, P., 2008). A defined mixture of components extracted from green tea (Veregen™) has been approved by the FDA and has recently come on the market. Hence natural products are still major sources of innovative therapeutic agents for infectious diseases, cancer, lipid disorders and immunomodulation.

As many unresolved difficulties exist for people with inflammatory disorders, continuing introduction of innovative drugs can overcome many of them.

CHAPTER 2
LITERATURE REVIEW

2.1. SELECTION OF NATURAL SOURCES

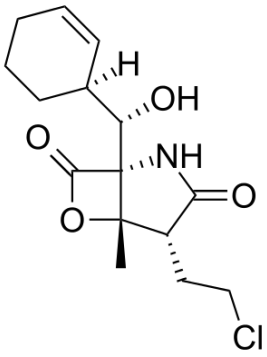
Natural products, including plants, animals and minerals have been the basis of treatment of several human diseases since the existence of human civilization. The current accepted modern medicine or allopathy has gradually developed over the years by scientific observations. However, the basis of its development remains rooted in traditional medicine and therapies (Patwardhan *et al*., 2004). Natural products, or derivatives there-of, remain the single most important source of new medicines (Newman *et al*., 2003) and most of the leads from natural products that are currently in development have come from either plant or microbial sources. The number indicates relatively little of the world's plant biodiversity which has been extensively screened for bioactivity and that very little of the estimated microbial biodiversity has been available for screening (Baker *et al*., 2007; Harvey, 2000). Hence, more extensive collections of plants or further advances in the ability to culture microbes could provide many novel chemicals for use in drug discovery assays.

2.2. SELECTION OF MICROBIAL SOURCE

Microorganisms inhabiting the world's oceans have largely been overlooked though microbial natural products remain an important resource for drug discovery. Pharmaceutical research expanded after the Second World War to include massive screening of microorganisms for new antibiotics because of the discovery of penicillin (Li *et al*., 2009). Among the potential sources of natural products, bacteria with a small group of taxa have proven to be a particularly abundant resource for the vast majority of compounds discovered (Lam, 2006; Fenical *et al*., 2006; Bull *et al*., 2007). For example, of the 53 known bacterial phyla, only five are reported to produce anti-infective agents (Keller *et al*., 2004).

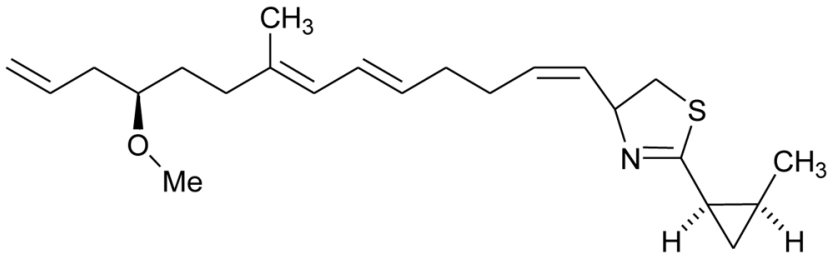
The recent discovery of novel secondary metabolites from taxonomically unique populations of marine actinomycetes added an important new dimension to microbial natural

product research (Jenesen *et al*., 2005). For example, salinosporamide A from *Salinospora tropica* potently inhibits the 20S proteasome and has anti-cancer activity in experimental models (Fenical *et al*., 2006); it is undergoing clinical trials.

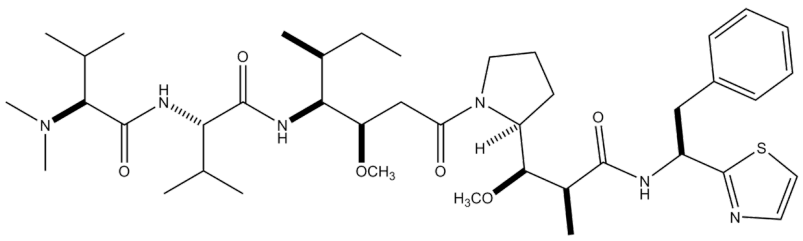


Salinosporamide

Another source of bioactive compounds that is receiving increasing attention is cyanobacteria (Tan, 2007). These have yielded curacin A and dolastatin 10, which are being, evaluated as anti-cancer agents. Over 120 cyanobacterial alkaloids were published between 2001 and 2006, and they have wide structural diversity and a variety of biological actions, such as cytotoxicity, sodium channel modulation, anti-fungal and inhibition of proteases.



Curacin A



Dolastatin 10

2.2.1. *PSEUDOMONAS*

Pseudomonas is a diverse genus of Gammaproteobacteria with more than 60 species exhibiting varied life styles in a wide range of environments, including soil, water, plant surfaces, and animals. They are well known for their ubiquity in the natural world, capacity to utilize a variety of organic compounds as energy sources, resistance to a wide range of medically and agriculturally important antimicrobial compounds, and the production of a remarkable array of secondary metabolites (Gross *et al*., 2009).

2.2.1.1. An overview of *Pseudomonas* Spp.

Since the entry of *Pseudomonas* spp. in the genomics era, research is accelerated in virtually all aspects of *Pseudomonas* biology, including secondary metabolism. The complete genomes of at least 22 strains representing seven species have been sequenced, and many more genomic sequences of *Pseudomonas* spp. will soon become available due, in part, to the application of new rapid and affordable sequencing technologies (Reinhardt *et al*., 2009; Almeida *et al*., 2009). Genomes of *Pseudomonas* spp. reflected remarkable ecological and metabolic diversity of these bacteria.

Table 2.1: Strains of *Pseudomonas* spp. (Gross *et al*., 2009; Tvrzova *et al*., 2006)

Strain	Source of isolation
P. aeruginosa 2192, *P. aeruginosa* C3719, *P. aeruginosa* LESB58	Chronically infected cystic fibrosis
P. aeruginosa PA14	Wound (culture collection)
P. aeruginosa PAO1	Burn wound
P. entomophila	Fruit or fruit fly
P. fluorescens	Soil and Leaf of sugar beat
P. putida	Soil, Poplar
P. stutzeri	Rice paddy soils
P. syringae	Rice, Bean, Leaf of bean and Tomato
P. moraviensis	Soil
P. vranovensis	Soil
P. aeruginosa PA96, *P. aeruginosa* UCBPP-PA14, *P. aeruginosa* PSE305	Soil

Pseudomonas species produces varieties of natural products representing varied metabolic origins and biological activities (Mavrodi *et al*., 2006; Raaijmakers *et al*., 2006). For decades *Pseudomonads* were known only for the production of siderophores, phenazines and small molecular weight antibiotics or phytoxins (Gross *et al*., 2009). But then it became evident that most *Pseudomonads* are capable of producing, in addition to pyoverdines, an additional siderophore (i.e. pyochelin, pseudomonine, achromobactin, or yersiniabactin) (Buell *et al*., 2003; Paulsen *et al*., 2005; Feil *et al*., 2005) lipopeptides, (Bruijn *et al*., 2007)

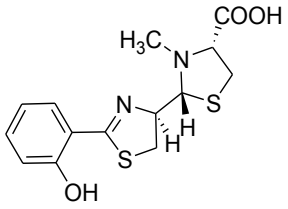
and numerous non-ribosomally derived peptides, polyketides, or hybrids thereof, many of unknown structure (Table 2.2) (Lindeberg *et al*., 2008; Paulsen *et al*., 2005; Donadio *et al*., 2007).

Table 2.2: Secondary metabolites from *Pseudomonas* spp. (Gross *et al*., 2009; Chung *et al*., 2008; Kobayashi *et al*., 2004; Kamal *et al*., 2012)

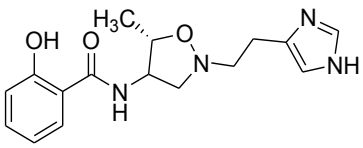
Compound	Producer	Biological activity
Pyochelin, Paerucumarin, Pseudoverdine	*P. aeruginosa*	-
Pseudomonine	*P. fluorescens* AH2, *P. luorescens* WCS374, *P. entomophila* L48	-
Cyclic lipopetptides: Syringomycin, Syringopeptin Arthrofactin Massetolides Putisolvin	 *P. syringae* Pseudomonas sp. MIS38 *P. fluorescens* SS101, *Pseudomonas sp*. MF-30 *P. putida* PCL1445	-
Orfamides	*P. fluorescens*	-
Syringofactins	*P. syringae*	-
Safracin	*P. fluorescens* A2-2	Antitumor
Tabtoxin (Dipeptide)	*P. syringae*	-
Pyrrolnitrin	*P. fluorescens, P. aurantiaca* BL915, *Pseudomonas sp*.	Antifungal / Antimycotic
Indole-3-acetic acid (IAA)	*Pseudomonas spp*.	-

Polyketides and fatty acid: Mupirocin (Pseudomonic acid A) 2,4-Diacetylphloroglucinol (DAPG) 2, 5-Dialkylresorcinols	 *P. fluorescens* NCIMB 10586 *P. fluorescens* *P. aurantiaca* BL915	 Antibacterial Antibacterial / Antihelmentic Antifungal / Antibacterial
Cyclic peptide-polyketides: Syringolin, Coronatine Pyoluteorin Pederin	 *P. syringae* *P. aeruginosa, P. fluorescens* Pf-5, *Pseudomonas sp.* M18 *Pseudomonas sp.*	 - Cytotoxic
Rhizoxins	*P. fluorescens* Pf-5	-
Phenazines: Pyocyanin (5-N-methyl-1-hydroxyphenazine) Phenazine-1-carboxylic acid, 2-hydroxyphenazine-1-carboxylic acid, and phenazine-1-carboxamide 1-Hydroxyphenazine, Phenazine-1-carboxylic acid	*P. chlororaphis*, *P. fluorescens, P. aeruginosa* *P. aeruginosa* *P. fluorescens*, *P. chlororaphis* *Pseudomonas sp.* strain ICTB-745	Antibiotic / Antitumor / Antiparasitic Antifungal Insecticidal
Quinolone	*P. aeruginosa*	Antibacterial alkaloids

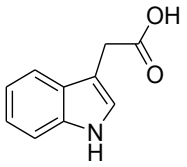
Hydrogen cyanide (HCN) / Prussic acid	*Pseudomonas spp*.	Inhibitior of cytochrome c oxidase and other metalloproteins
2,4,6-trihydroxyacetophenone (THA)	*P. brassicacearum*	Antifungal
Aldoxime dehydratase	*P. chlororaphis*	Involve in carbon-nitrogen triple bond synthesis
Rhamnolipids-1, Rhamnolipids-2	*Pseudomonas sp*. strain ICTB-745	Antifeedant, Insecticidal



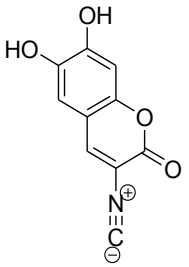
Phyochelin

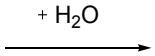


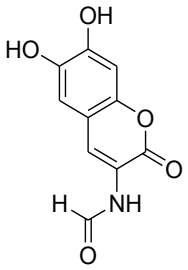
Pseudomonine



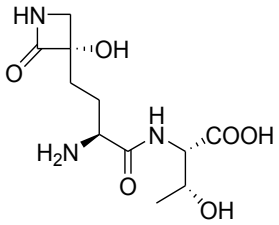
Indole-3-acetic acid



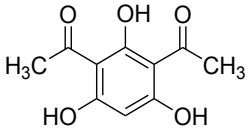




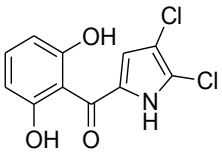
Paerucumarin **Pseudoverdine**



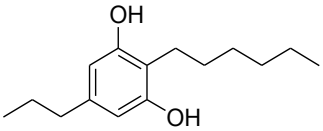
Tabtoxin



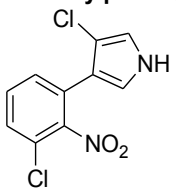
2,4-Diacetylphloroglucinol



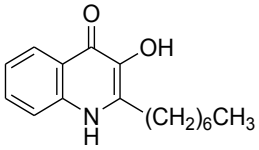
Pyoluterin



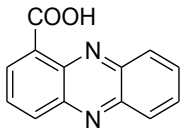
2,5-Dialkylresorcinol



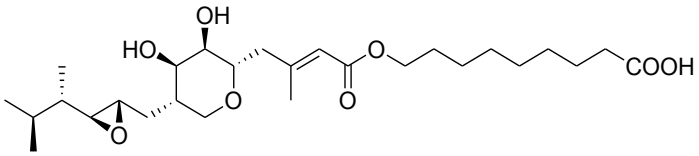
Pyrrolnitrin



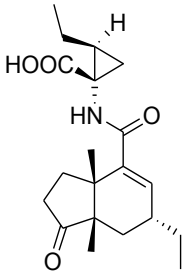
Quinolone



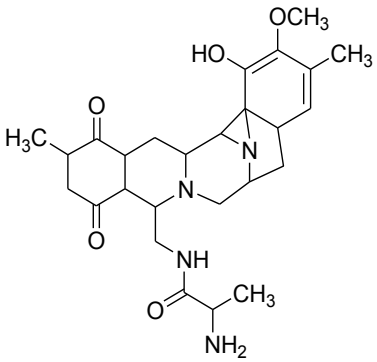
Phenazine-1-carboxylic acid



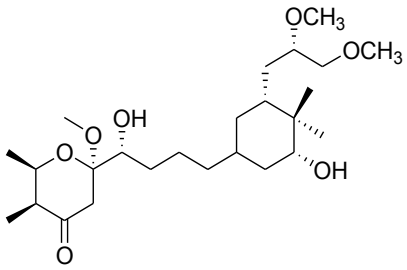
Mupirocin



Coronatine



Safracin



Pederin

From above literature it is clear that *Pseudomonas* spp. exhibit enormous metabolic capabilities and versatile biochemistry through their production of structurally diverse, bioactive chemical structures.

2.2.2. *PSEUDOMONAS AERUGINOSA*

P. aeruginosa is a gram-negative, rod-shaped (1-5 μm long and 0.5-1.0 μm wide), asporogenous, and monoflagellated bacterium that has an incredible nutritional versatility. *P. aeruginosa* uses aerobic respiration (with oxygen) as its optimal metabolism and anaerobically respires on nitrate or other alternative electron acceptors. *P. aeruginosa* catabolizes a wide range of organic molecules such as benzoate. *P. aeruginosa* has been found in environments such as soil, water, humans, animals, plants, sewage, and hospitals, making it ubiquitous in nature. *P. aeruginosa* is the most abundant organism on earth because it is predominant inhabitant in all oligotropic aquatic ecosystems, which contain high-dissolved oxygen content but low plant nutrients throughout. *P. aeruginosa* is a soil bacterium and capable of breaking down polycyclic aromatic hydrocarbons and making rhamnolipids, quinolones, hydrogen cyanide, phenazines, and lectins (Lederberg, 2000).

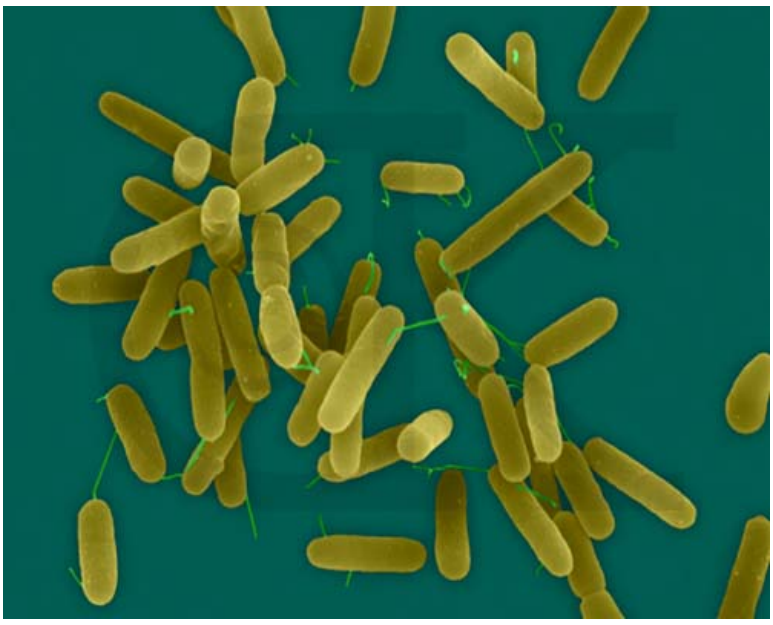


Figure 2.1: ***Pseuodomonas aeruginosa*** (Image courtesy: *Pseuodomonas* Genome Database)

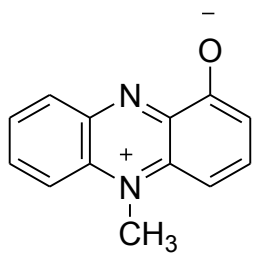
2.2.2.1. Classification

Domain	Bacteria
Phylum	Protobacteria
Class	Gamma protobacteria
Order	Pseudomonadales
Family	Pseudomonadaceae
Genus	*Pseudomonas*
Genus species	*Pseudomonas aeruginosa*

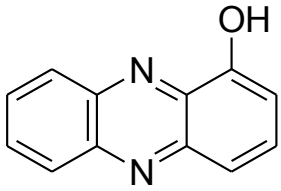
2.2.2.2. Secondary metabolites of *Pseudomonas aeruginosa* and their biological activity

P. aeruginosa secretes numerous proteins and secondary metabolites, which have been shown to affect the specific and non-specific defense mechanisms of the host like heat-killed *P. aeruginosa* or a lyophilizate of this bacteria inhibits immune responses in-vivo as well as in-vitro; alkaline proteases and elastase are exoproteins of the bacteria which inhibit T-lymphocyte and natural killer cell functions in-vitro (Ulmer *et al*.,1990).

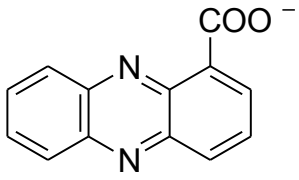
Pyocyanine (N-methyl-1-hydroxyphenazine) a bacterial product that is released by most clinical *P. aeruginosa* isolates; is a blue phenazine pigment with a molecular weight of 210.23. Pyocyanine has been found to inhibit the T-lymphocyte response to antigens and mitogens in-vitro and superoxide production by neutrophils. Ulmer *et al*., in 1990 found that the influence of pyocyanine on mitogen-induced production of immunoglobulins and the polypeptide hormones (cytokines) IL-1, TNF-α, and IL-2 was both stimulatory and inhibitory effects on cellular responses. In this way pyocyanine may be involved in the inhibition of specific immune responses but also enhances non-specific inflammatory reactions and therefore concluded that pyocyanine may contribute to the immunosuppressive action of *P. aeruginosa*.



Pyocyanin



1-hydroxyphenazine



Phenazine-1-carboxylic acid

Other reported phenazines from *P. aeruginosa* are 1-hydroxyphenazine (1-HP) and phenazine-1-carboxylic acid (PCA), other phenazine derivatives like redox-active compounds that exert many of their effects by altering oxidative metabolism (Denning *et al*., 2003).

P. aeruginosa produces a number of quorum sensing signal molecules (QSSM), which have been chemically characterized as *N*-acylhomoserine lactones (AHL) and a quinolone i.e. two major AHL [*N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butanoyl-L-homoserine lactone (C_4-HSL)] and two minor AHL [*N*-(3-oxohexanoyl)-L- homoserine lactone (3-oxo-C_6-HSL) and *N*-hexanoyl-L-homo- serine lactone (C_6-HSL)], together with 2-heptyl-3-hydroxy-4 (1*H*)-quinolone (PQS) (Hooi *et al*., 2004).

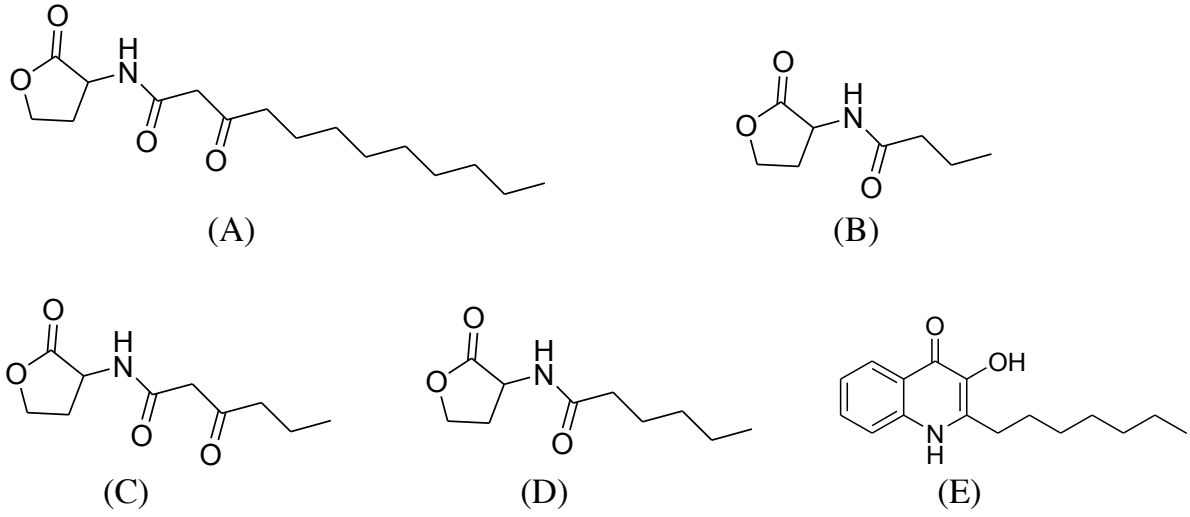


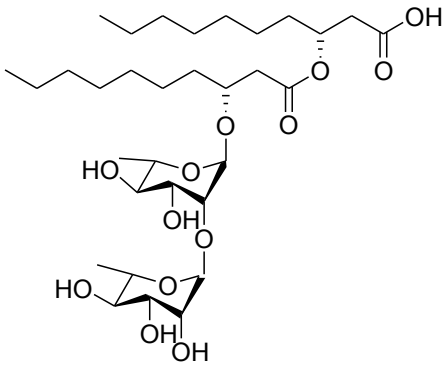
Figure 2.2: Chemical structures of PA QSSM (A) 3-oxo-C_{12}-HSL (B) C_4-HSL (C) 3-oxo-C_6-HSL (D) C_6- HSL (E) PQS

QSSM mediate cell-to-cell communication among bacteria. 3-oxo-C_{12}-HSL secreted and sensed by *P. aeruginosa* directly modulates host defense systems (Teplitski *et al*., 2010; Pritchard, 2006) via TLR-4 independent mechanisms. It has shown to modulate diverse activities of macrophages, fibroblasts, epithelial cells, mast cells, T-lymphocytes, B-lymphocytes, and neutrophils. Multiple studies showed that lipopolysaccharide (LPS) induced production of the key pro-inflammatory cytokine TNF-α is suppressed in the presence of 3-oxo-C_{12}-HSL (Kravchenko *et al*., 2008). In 2013 Glucksam-Galnoy *et al*., assessed activity of 3-oxo-C_{12}-HSL (50 μM) in RAW264.7 macrophages where TNF-α levels were measured following stimulation with LPS which was found to decline by 40% and LPS induced production of IL-10 which is an anti-inflammatory cytokine was substantially increased without any effect on cell viability and reported it to be immune suppressive with an IC_{50} of 18.24 μM. These results suggest that 3-oxo-C_{12}-HSL can manifest its immunosuppressive activity through facilitating the expression of the anti-inflammatory cytokine IL-10 in stimulated immune cells which proves QSSM may serve a dual purpose promoting bacterial communication while suppressing the immune system.

Chronic bronchial infection is believed to be a major factor in the pathophysiology of cystic fibrosis (CF) and *P. aeruginosa* is the dominant bronchial pathogen in the majority of CF patients. In 1992 Staugas *et al*., studied the interaction of *P. aeruginosa* with human mononuclear leukocytes (MNL) to understand the role of *P. aeruginosa* in mechanism of lung inflammation in CF and found that *P. aeruginosa* stimulates MNL and induces the release of TNF-α and IL-1. However exotoxin-A, a major extracellular product of *P. aeruginosa* at 100 ng/ml caused a 90% depression in the production of TNF-α, it not only failed to induce TNF-α, IL-1 and lymphotoxin (LT) production but inhibited the ability of cells to produce these cytokines, which suggested that exotoxin-A mediates the virulence of *P. aeruginosa* by inducing immunosuppression.

Rupesh *et al*., in 2012 isolated a marine bacteria from 1000 m deep sea water column in the Bay of Bengal near Andaman and Nicobar Island which they later identified as *P. aeruginosa* and found that *P. aeruginosa* exhibited greater inhibition of proliferation of LPS induced Peripheral Blood Mononuclear cells (PBMCs) as evidenced by [^{3}H] thymidine assay. The crude ethyl acetate extract of *P. aeruginosa* showed dose dependent inhibition of lymphocyte proliferation (IC_{50} 12 μg/ml). Upon chemical investigation of bacteria a cyclic dipeptide was isolated and characterized as (3*S*, 8a*S*)-3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione and the same was subjected for LPS induced PBMCs model. At IC_{50} value of 84 μM, the compound was found to inhibit the expression levels of pro-inflammatory cytokines, TNF-α and IL-1β with significant reduction of NO production. Also the compound at IC_{50} of 60 μM effectively inhibited COX-1.

In 1949 Jarvis and Johnson shown that *P. aeruginosa* produces the bio-surfactant rhamnolipids, which are amphiphilic molecules composed of a hydrophobic fatty acid moiety and a hydrophilic portion composed of one or two rhamnose. They have been mostly studied for their ability to solubilize and promote the uptake of hydrophobic substrates, especially hydrocarbons such as n-alkanes. Another mechanism through which rhamnolipids enhance the biodegradation of poorly soluble molecules is by causing the cell surface to become more hydrophobic (Soberon-Chavez *et al*., 2005).



Rhamnolipid

Anabolic precursors of rhamnolipid i.e. without the sugar moiety, 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), are also released by the bacteria and display tensioactive properties (Deziel *et al*., 2003).

Recently a novel short-chain-length- long-chain-length (SCL-LCL) co-polymer with 3-hydroxybutyric acid (3HB), 3-hydroxyvaleric acid (3HV), 3-hydroxyhexadecanoic acid (3HHD) and 3-hydroxyoctadecanoic acid (3HOD) were isolated from *P. aeruginosa* MTCC 7925, a sludge isolate (Kumar Singh *et al*., 2013).

Thus, the thorough literature survey on *P. aeruginosa* indicated that the bacteria have diversity in the production of secondary metabolites other than antibiotics. *P. aeruginosa* might become an interesting source for immunosuppressive compounds and hence need to be investigated for the same.

2.3. SELECTION OF PLANT SOURCE

Just over 200 years ago, a 21-year-old pharmacist's apprentice named Friedrich Serturner isolated the first pharmacologically active pure compound morphine from opium plant produced by cut seed pods of the poppy, *Papaver somniferum* (Li *et al*., 2009). An era was initiated wherein drugs from plants could be purified, studied, and administered in precise dosage form that did not vary with the source or age of the material. By 1990, about 80% of drugs were either natural products or their analogs. Antibiotics (e.g., penicillin, tetracycline, erythromycin), antiparasitics (e.g., avermectin), antimalarials (e.g., quinine, artemisinin), lipid control agents (e.g., lovastatin and analogs), immunosuppressants for organ transplants (e.g., cyclosporine, rapamycins) and anticancer drugs (e.g., taxol, doxorubicin) revolutionized medicine and the life expectancy in much of the world increased from about 40 years early in the 20th century to more than 77 years today (Harvey *et al*., 2008).

2.3.1. *Abutilon indicum*

A. indicum belonging to family Malvaceae is a perennial shrub, upto 3 m in height. The leaves are evergreen, base-cordate, stipulate, ovate, acuminate, toothed, and 1.9-2.5 cm long. Petiole 1.5-1.70 cm long, cylindrical, yellowish in colour, stellate and hairy. The flowers are yellow in colour, peduncle jointed above the middle (Prajapati *et al*., 2003). *A. indicum* has been used as anthelmentic, antiemetic, anti-inflammatory, in urinary or uterine discharge, piles, and antidote. It is used in treatment of fever, dry cough, bronchitis, gonorrhea and leprosy (Mohite *et al*., 2012).

It is distributed throughout plains and wetlands. All the parts of plant have medicinal uses. In folk medicine the plant is used in treating fever, cough, lung disease, urine output. They are also used in the treatment of deafness, ringing in the ears, high fever, mumps, cough, pulmonary tuberculosis. The whole herb is used in Ayurveda preparations to treat hemorrhoids, diabetes, and menorrhoea (Saraswathi *et al*., 2011).

Figure 2.3: ***Abutilon indicum*** (Shivhare *et al*., 2010)

2.3.1.1. Regional names (Singh *et al*., 2005)

The plant is known by various names in different languages as follows.

Hindi name	Atibala, Kangahi, Kanghi, Kanghani
Sanskrit name	Kotibala, Kankatika
English name	Country-mallow
Tamil name	Tutti, Thuththi, Peruntutti
Telugu name	Tutiri-chettu, Thuteribenda

2.3.1.2. Scientific classification (Mohite *et al*., 2012)

Kingdom	Plantae
Order	Malvales
Family	Malvaceae
Genus	*Abutilon*
Species	*Abutilon indicum*
Botanical name	*Abutilon indica*

2.3.1.3. Habit and habitat

The species occurs in number of tropical and subtropical areas and subtropical zones. The plant is found in India, Sri Lanka, topical regions of America and Malaysia. It is found as a weed in sub-Himalayan tracts, hills upto 1200 m in hotter parts of India (Mohite *et al*., 2012) and extending through the whole of northern and central India (Rajurkar *et al*., 2009).

2.3.1.4. Traditional uses

Almost all the parts of Atibala are of medicinal importance and traditionally used for the treatment of various ailments. Root and bark are used as aphrodisiac, anti-diabetic, nervine tonic, diuretic, anthelmintic, pulmonary sedative in fever. According to the Chinese in Hong Kong, the seeds are employed as an emollient and demulcent and are used in urinary disorders (Kashmiri *et al*., 2009). Leaves of the plant used as demulcent, aphrodisiac,

laxative, diuretic, pulmonary and sedative also for the treatment of ulcers, headaches, and gonorrhea. In addition, it is used in cleaning wounds, treating vaginal infections, diabetes, and hemorrhoids and can also be used as an enema. The decoction of the leaves is used in toothache, tender gums and internally for inflammation of bladder. The bark is astringent and diuretic, febrifuge, anthelmintic and astringent. Whole plant is used as laxative and tonic, anti-inflammatory and anthelmintic, analgesic (Rajakaruna *et al*., 2002). The roots of the plant are considered as demulcent, diuretic, in chest infection and urethritis. The infusion of the root is prescribed in fevers as a cooling medicine and is considered useful in hematuria and in leprosy. The seeds are used in piles, laxatives, expectorants, in chronic cysticis, gonorrhea, aphrodisiac, urinary disorders, chronic dysentery, and fever (Mohite *et al*., 2012; Khadabadi *et al*., 2010).

2.3.1.5. Ethnobotanical uses

Almost all the parts of *A. indicum* are documented to be useful in ethanobotanical surveys conducted by ethanobotanists. It has been documented that natives of India, Malaya, Philippine Islands and Indo-china use its parts for the medicinal purposes such as febrifuge, anthelmentic, antiemetic, anti-inflammatory, in urinary or uterine discharge, piles and lumbago (Jain *et al*., 2004; Jain *et al*., 2005). The leaves are used in eye wash, mouthwash, in cataract and diarrhea. A leaf paste is taken orally to cure piles and to relieve leg pains (Kumar *et al*., 2006). The bread prepared from the mixture of leaf powder and wheat flour is taken daily during night for about one month to cure uterus displacement (Ganesan *et al*., 2007).

The leaf juice is used for the treatment of snakebite as antidote (Mohapatra *et al*., 2008). The fruit is used to treat piles, gonorrhea, and cough (Samy *et al*., 2008). Fruit decoction mixed with ammonium chloride is given orally with water to treat hemorrhagic septicemia (Ignacimuthu *et al*., 2008). Seed powder is used orally with water as aphrodisiac and laxative. The root of the plant is used to treat gonorrhea and leprosy. Root infusion is

given to cure fever, dry cough and bronchitis (Mohite *et al*., 2012).

2.3.1.6. Pharmacological studies

Phytochemical studies have detected presence of linoleic, oleic, stearic, palmitic, myristic, lauric, capric and caprylic acids as fixed oil from petroleum ether extract of *A. indicum* as analgesic whereas root extract, obtained from different solvents exhibited antibacterial and antifungal activity (Matlawska *et al*., 2002). A methanol extract of *A. indicum* had some antimicrobial properties (Parekh *et al*., 2006). A chemical compound, β-sitosterol, which has been identified as the active ingredient in many medicinal plants, is present in *A. indicum* isolated from petroleum ether extract provided larvicidal properties against the mosquito larvae *Culex quinquefasciatus* (Rahuman *et al*., 2008).

A. indicum leaf is also used in cosmetics reason being its high quantity of essential oils like 5-oxatricyclo(dodecane 12-trimethyl, 9-methylene) 4.35%, cubenol 4.45%, hinesol 12.04%, acetic acid 4.70%, palmitic acid 43.18%, phytol 31.27%, all-trans-squalene 13.66%, n-tetracosane 5.68%, α-Tocopherol 15.79%, γ-sitosterol 6.62%, lupeol 5.23%. Phytol is essential in activating enzymes for the production of insulin. It can also decrease the blood cholesterol and is useful for healthy skin and hair. α-Tocopherol (vitamin E) protects the body against tissue damage by oxidation and also protect against heart diseases (Shanthi *et al*., 2011). One of the essential oil i.e. eugenol has been reported to possess antioxidant and anti-ulcer potential (Ponnudurai *et al*., 2011).

Table 2.3: Pharmacological profile of *Abutilon indicum*

Formulation / Extract / Fraction	Biological Activity	Brief detail of activity	Reference
Abutilon indicum silver nanoparticles (AIAgNPs) from leaf extract	Free radical scavenging, bacteriostatic and anti-proliferative	Human colon cancer (COLO 205) and normal cells (MDCK) with an IC_{50} of 3 and 4 µg/ml and 100 and 75 µg/ml, resp. after 24 and 48 h	Sudha *et al*., 2015
Whole plant as composition of Chinese medicine	Goiter	-	Cui *et al*., 2015
Whole plant as composition of Chinese medicine	Chronic renal failure	-	Lv *et al*., 2014
$CHCl_3$ fraction of aerial parts	Cytotoxic against U87MG	IC_{50} 42.6 µg/ml	Khan *et al*., 2014
Ethanol extract of leaf parts	Anti-inflammatory and anti-proliferative	Anti-inflammatory (IC_{50} 8.89 µg/ml) and on human Caucasian lung carcinoma i.e. A549 cell line (IC_{50} 85.2 µg/ml)	DSVGK *et al*., 2014
Petroleum ether extract of root parts	Hypouricaemic and Uricosuric	200 and 400 mg/kg	Kousalya *et al*., 2014

Roots as a composition of herbal formulation	Bone fracture	-	Maity *et al*., 2014
Silver nanoparticles (AgNPs) of leaf extract	Antibacterial	-	Ashok kumar *et al*., 2015
Whole plant as composition of Chinese medicine	Abdominal migraine	-	Yu *et al*., 2014
Methanol extract of whole plant	Anti-inflammatory and analgesic	100, 200 and 400 mg/kg	Venkatakrishnan *et al*., 2014
Whole plant as composition of Chinese medicinal tablet	Bladder cancer	-	Lin *et al*., 2014
Whole plant as composition of Chinese medicine	Swelling and pain in throat	-	Yu *et al*., 2014
Crude extract of roots	Anti-inflammatory and Xanthine oxidase inhibitors	1000 µg/ml	Kousalya *et al*., 2013
Alcoholic extract of whole plant	Antioxidant	500 mg/kg	Harini *et al*., 2012; Yasmin *et al*., 2010; Kaushik *et al*., 2011; Javed *et al*., 2012
Methanol extract of whole plant	Antibacterial	-	Muhamed Mubarack *et al*., 2012

Ethanol extract of whole plant	Anti-inflammatory	250, 500 and 750 mg/kg	Tripathi *et al*., 2012
Methanol extract of whole plant	Antioxidant and anticancer	Human Melanoma cancer cell lines (SK-MEL-28) and Lung Adeno Carcinoma (NCI-H23) IC_{50} was found to be 4.71 and 15.8 mg/ml respectively	Srikanth *et al*., 2012
Methanol extract of whole plant	Anti-Arthritic	400 mg/kg	Bhajipale, 2012
Alcoholic extract of whole plant	Antifungal	-	Ramasubramania, 2012; Vairavasundaram *et al*., 2009
Petroleum ether, chloroform, ethanol and aqueous extract of whole plant	Analgesic and anti-inflammatory	400 mg/kg	Saraswathi *et al*., 2011
Petroleum ether and methanol extract of leaf	Antimicrobial	-	Gurumurthy *et al*., 2011; Suresh *et al*., 2012
Aqueous, ethanol and chloroform extract of leaves	Antibacterial	-	Ramasubraramaniaraja *et al*., 2011; Poonkothai, 2006; Prabahar *et al*., 2009
Ethyl acetate and Aqueous extract of	Anthelminic	-	Venkatachalam *et al*., 2010

leaves			
Chloroform fraction from alcoholic extract of whole plant	Antioxidant and antimicrobial	-	Kaushik *et al*., 2010
Whole plant as Herbal composition	HIV/AIDS infection	-	Haswani *et al*., 2010
Whole plant as Synergistic composition	Metabolic dysfunction in Cancer treatment	-	Shankar *et al*., 2010
Aqueous and Organic extract of whole plant	Free radical scavenging	-	Yasmin *et al*., 2010; Chakraborthy *et al*., 2010
Seed extract	Diuretic	200 and 400 mg/kg	Balamurugan *et al*., 2010
Aqueous and ethanol extract of leaf	Anti-convulsant	100 and 400 mg/kg	Golwala *et al*., 2010
Ethanol and aqueous extract of leaves	Immunomodulatory	200 and 400 mg/kg	Dashputre *et al*., 2010
Methanol extract of leaves	Anti-inflammatory	200 and 400 mg/kg	Devi *et al*., 2010
Methanol extract of leaves	Antifungal	-	Vairavasundaram *et al*., 2009
Alcoholic and aqueous extract of leaf	Anti-ulcer	400 mg/kg	Malgi *et al*., 2009; Dashputre *et al*., 2011

Petroleum ether and ethanol extract of roots	Analgesic	-	Goyal *et al*., 2009
Water soluble extract of whole plant	In-vitro anti arthritic	100 and 250 μg/ml	Deshpande *et al*., 2009
Petroleum ether, benzene, water and methanol extract of leaf	Hypoglycemic	400 and 500 mg/kg	Adisakwattana *et al*., 2009; Seetharam *et al*., 2002; Nelluri *et al*., 2003
Aqueous extract of whole plant	Antidiabetic	500 and 1000 mg/kg	Krisanapun *et al*., 2009
Aqueous extract of leaves	Antinociceptive	200 and 400 mg/kg	Gunasekaran *et al*., 2009
Ethanol and water extract of leaf	Lipid lowering	-	Giri *et al*., 2009
Aqueous, ethanol and acetone extract of whole plant	Antibacterial activity against UTI causing pathogen	-	Sharma *et al*., 2009
Aqueous, ethanol and chloroform extract of leaves and roots	Anti-inflammatory	-	Rajurkar *et al*., 2009; Sharma *et al*., 2008
Ethanol extract of leaves	Hepatic antioxidant status and lipid peroxidation against alcohol induced liver damage	100 and 200 mg/kg	Singh *et al*., 2008

Petroleum ether extract of whole plant	Larvicidal	-	Rahuman *et al*., 2008
Whole plant	Immunomodulatory	500 mg/kg	Singh *et al*., 2008
Ethanol extract of whole plant	Wound healing	400 mg/kg	Roshan *et al*., 2008
Methanol extract of aerial part	Anti-inflammatory and Anti-asthmatic	250 and 500 mg/kg	Paranjape *et al*., 2008
Methanol extract of whole plant	Acetyl cholinesterase inhibitory	-	Mukherjee *et al*., 2007
Aqueous extract and butanol fraction whole plant Petroleum ether and benzene extract of leaves	Antidiabetic Analgesic	 400 mg/kg	Sarkar *et al*., 2006; Krisanapun *et al*., 2011; Nelluri *et al*., 2003
Aqueous extract of whole plant	Hepatoprotective	-	Porchezhian *et al*., 2005
Petroleum ether and benzene extract of leaves	CNS depressant	400 mg/kg	Nelluri *et al*., 2003
Methanol and aqueous extract of leaves	Anti-diarrheal	-	Chandrashekhar *et al*., 2004

2.3.1.7. Chemical constituents of *Abutilon indicum*

Phytochemical reports of *A. indicum* leaves showed the presence of amino acids, glucose, fructose and galactose. From the roots, non-drying oil consisting of various fatty acids e.g. linoleic, oleic, stearic, palmitic, lauric, myristic, caprylic, capric and unusual fatty acid having C_{17} carbon skeleton, sitosterol, and amyrin from unsaponifiable matter were reported (Rajurkar *et al*., 2009). Extract of the whole plant decreases peroxidative damage in liver through free radical scavenging activity due to its flavonoids (Singh *et al*., 2008). Seven flavonoid compounds including quercetin and its glycosides have been isolated from flowers of *A. indicum* (Matlawska *et al*., 2002). Alkaloids, flavonoids, steroids, terpenoids and saponins have been isolated and characterized from genus *Abutilon*. The analysis of phenolic compounds in plants is of considerable commercial importance, since it is known that they contribute to the flavour (Kashmiri *et al*., 2009). The essential oils like Phytol, n-hexadecanoic acid, 2-pentadecanone, 6,10,14-trimethyl,9-octadecenamide, tetracosane and the pentacosane are reported from the whole plant of *A. indicum* (Chen *et al*., 2013).

Gas chromatography mass spectroscopy (GC-MS) analysis of ethanolic leave extract revealed the presence of 6 bioactive chemical compounds Such as 5-oxatricyclo (dodecane-12-trimethyl, 9-methylene) 4.35%; cubenol 4.45%; hinesol 12.04%; acetic acid 4.70%; palmitic acid 43.18%; phytol 31.27% whereas the hexane extract of leaves showed 18 compounds in which palmitic acid 5.47%; phytol 17.12%; all-trans-squalene 13.66%; n-tetracosane 5.68%; n-tetra cosine 7.35%; α-Tocopherol 15.79%; γ-sitosterol 6.62%, lupeol 5.23% are in high proportion (Shanthi *et al*., 2011).

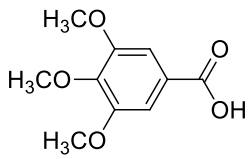
Table 2.4: Chemical constituents of *Abutilon indicum* and reported biological activities

Formulation / Extract / Fraction	Chemical constituents	Biological activity	Reference
Chloroform fraction of leaves	Methyl *trans-p*-coumarate; Methyl caffeate; Syringic acid, C-18 trihydroxy octadecenoic acid and Pinellic acid	Methyl caffeate cytotoxicity against human glioblastoma (U87MG) cells (IC_{50} 8.2 μg/ml)	Khan *et al*., 2014
Ethyl acetate extract of whole plant	β-carotene, stigmasterol-3-O-β-D-glucopyranoside; n-pentatriacontano; Benzoic acid; Glyceryl behenate and Oleanolic acid	-	Chen *et al*., 2012
Methanol extract of Leaves	Eudesmic acid, Ferulic acid and Caffeic acid.	Antibacterial and antifungal	Rajput *et al*., 2012
Stems	16-Hydroxyhentetracontan-17-one-1; 20,23-Dimethylcholesta-6,22-dien-3-β-ol-3; Hexatriacontanoic acid-2; Octacosan-8-one; 7-Methylpentatriacontan-7, 24-diol; 3β-acetoxy-urs-20(30)-ene; β-sitosterol and Betulin	-	Singh *et al*., 2011; Singh *et al*., 2010

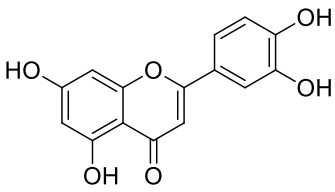
Leaves and seeds	Gums and Mucilage i.e. Acacia; HPMC E15 LV and Sodium Carboxy Methyl Cellulose	-	Shekhar *et al*., 2011
Leaf extract	Linoleic acid	Anti-convulsant	Golwala *et al*., 2010
Seeds	Oil (Neutral lipids, polar lipids); Fatty acids like linoleic acid	Anti-oxidant and antibacterial	Kashmiri *et al*., 2009
Whole plant	β-sitosterol	Larvicidal	Rahuman *et al*., 2008
Methanolic extract of Whole plant	Abutilin A (biphenyl ether); (R)-N-(1'-Methoxycarbonyl-2'-phenylethyl)-4-hydroxybenzamide; Vanillin; Methylcoumarate; 4-hydroxyacetophenone; *p*-hydroxybenzaldehyde, Aurantiamide acetate; Methyl indole-3-carboxylate; 3,7-dihydroxychromen-2-one; Methylparaben; Scoparone; Scopoletin; Syringaldehyde; 1-methoxycarbonyl-β-carboline; 4-hydroxy-3-methoxy-*trans*-cinnamic acid methyl ester; *trans*-	Aurantiamide acetate inhibit DNA polymerase	Kuo *et al*., 2008

	p-coumaric acid; Thymine; Adenine; Methyl 4-hydroxyphenylacetate; Riboflavin; 1-lycoperodine; 3-hydroxy-*β*-damascone, adenosine; *p*-hydroxybenzoic acid; 3-hydroxy-*β*-ionol; N-feruloyl tyrosine; Vanillic acid, and benzoic acid		
Leaves	Hypolaetin 8-O-β-glucuronopyranoside 3''-O-sulfate; Isoscutellarein 8-O-β-glucuronopyranoside 3''-O- sulfate; Hypolaetin 8-O-β-glucuronopyranoside; kaempferol 3-O-α-rhamnopyranosyl-β-glucopyranoside and quercetin 3-O-α-rhamnopyranosyl-β-glucopyranoside	Anti-microbial	Matlawska *et al*., 2007
Whole plant	Dotriacontanol, lacceroic acid; Stigmasterol; β-sitosterol; Stigmasterol-3-O-β-D-	-	Liu *et al*., 2009; Chen *et al*., 2010; Pandey *et al*., 2011; Sharma *et al*., 1989

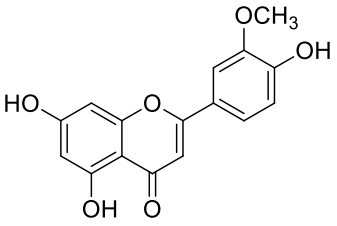
	glucopyranoside; *p*-β-D-Glucosyloxybenzoic acid; *p*-Hydroxybenzoic; Caffeic acid; Oleanic acid; (24R)-5α-stigmastane-3,6-dione; daucosterol; 2, 6-dimethoxy-1,4-benzoquinone; Vanillic acid; Sesquiterpenes lactones i.e. alantolactone and isoalantolactone		
Flowers	Luteolin; Chrysoeriol; Luteolin 7-O-β-glucopyranoside; Chrysoeriol 7-O-β-glucopyranoside; Apigenin 7-O-β-glucopyranoside; Quercetin 3-O-β-glucopyranoside and Quercetin 3-O-α-rhamnopyranosyl-β-glucopyranoside	-	Matlawska *et al*., 2002
Whole plant	Eugenol [4-allyl-2-methoxyphenol]	Analgesic at 10, 30 and 50 mg/kg	Ahmed *et al*., 2000
Seeds	Water soluble galactomannan	-	Singh *et al*., 1997



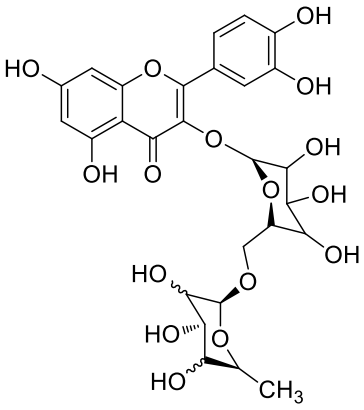
Eudesmic acid



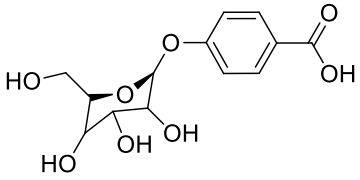
Luteolin



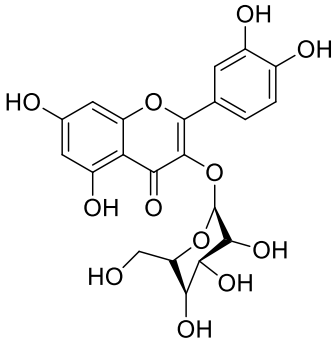
Chrysoeriol



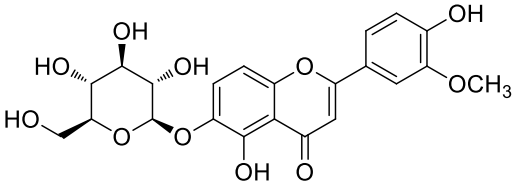
Rutin



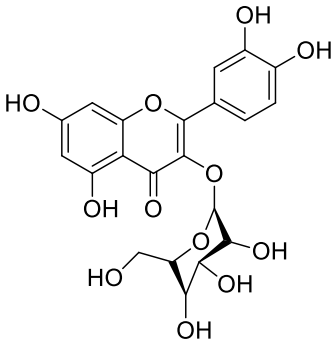
***p*-beta-D-glucosylbenzoic acid**



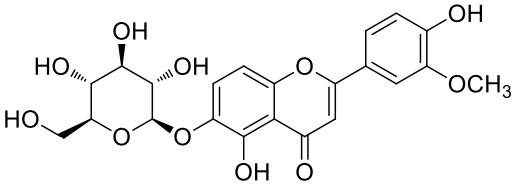
Quercetin-3-*O*-beta-glucopyranoside



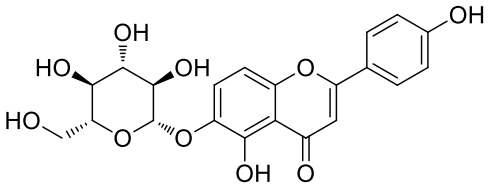
***p*-beta-D-glucosylbenzoic acid**



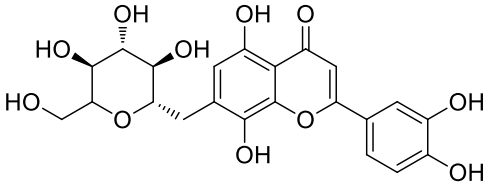
Quercetin-3-*O*-beta-glucopyranoside



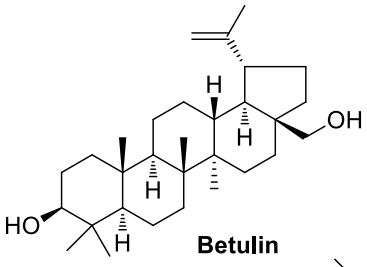
Chrysoeriol-7-*O*-beta-glucopyranoside



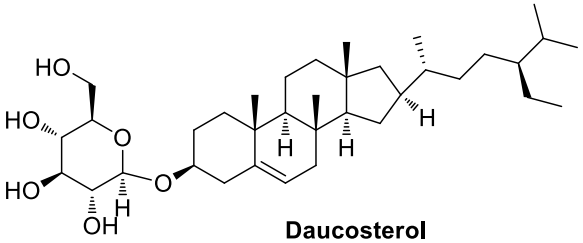
Apigenin-7-*O*-beta-glucopyranoside



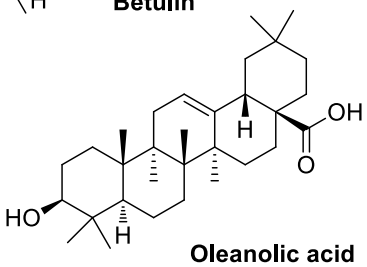
Luteolin-7-*O*-beta-glucopyranoside



Betulin



Daucosterol



Oleanolic acid

The extensive literature review of *A. indicum* revealed that it is an important medicinal plant with diverse pharmacological spectrum. A severe pharmacological study has been carried out with extract of the different parts of the plant. The plant is widely used in traditional medicinal system of India and has been reported to possess hepatoprotective, anti-inflammatory, lipid lowering, antifungal, wound healing and antibacterial properties. The whole plant contains mucilaginous substances and asparagines, saponins, flavonoids, alkaloids, hexoses, n-alkane mixtures (C_{22-34}), alkanol. Based on the important pharmacological and phytochemical findings and isolated principles, the plant can be researched further to achieve anti-inflammatory lead molecules in the search of novel natural drugs. Also due to diverse medicinal properties there is an enormous scope for future research on *A. indicum* and further chemical cum pharmacological investigation will disclose the unexploited potential of this plant.

CHAPTER 3

OBJECTIVES AND PLAN OF WORK

3.1. Objectives

The immune response occurs when immunologically competent cells are activated in response to foreign organisms or antigenic substances liberated during the acute or chronic inflammatory response. Chronic inflammation involves the release of a number of mediators that are not prominent in the acute response. The mediators such as macrophages and lymphocytes produce pro-inflammatory cytokines e.g. TNF-α, various interleukins like IL-1β, IL-6, GM-CSF, IFN-γ etc., in the systemic circulation and tissues. Earlier studies had revealed that release of pro-inflammatory mediators majorly contributes to the systemic inflammation leading to various disease conditions.

Conventional therapy for inflammation usually begins with NSAIDs, and biological agents like mAbs are used in chronic inflammatory conditions. These antibodies are complicated peptides produced by recombinant DNA technology using mammalian cell expression system involving expensive manufacturing processes (Tracey *et al*., 2008). These are found to cause injection site and infusion reactions. Also, there are certain issues concerning the safety and side effects of mAbs such as acute anaphylaxis, serum sickness and generation of antibodies alongside some organ-specific adverse events like cardio-toxicity, etc. (Hansel *et al*., 2010). Hence search for potential small molecules is highly indispensable.

Based on the complete literature review on pathogenesis of inflammation and the existing drug treatments, following objectives were framed.

I. Selection of natural sources for identifying pro-inflammatory cytokine inhibitors
II. Isolation and characterization of low molecular weight natural molecules from selected sources
III. Identification of lead compounds through in-vitro and in-vivo screening against pro-inflammatory cytokines

3.2. Plan of Work

Work plan was divided into following phases

Phase-1: Selection of natural sources based on literature review

Phase-2: Isolation of low molecular weight natural compounds using chromatographic techniques

Phase-3: Identification and characterization of isolated compounds by spectral analysis

Phase-4: In-vitro screening of characterized compounds against major pro-inflammatory cytokines to identify lead compounds

Phase-5: In-vivo screening of identified lead compounds against key pro-inflammatory cytokine

CHAPTER 4
RESULTS AND DISCUSSION

Microbes and higher plants have been the important source of several widely used drug molecules since ages. Extreme knowledge on the bioactive constituent of these promising natural sources will definitely yield newer lead compounds for the development of modern therapeutics. Keeping this in mind, the medicinally and chemically important sources like *Pseudomonas* bacteria and *Abutilon indicum* plant were selected for the investigation. The results of our research outcome are incorporated in this chapter.

4.1. ISOLATION AND IDENTIFICATION OF BACTERIA

Bacteria was isolated from the rizhosphere (soil) of groundnut crop, which is commonly found to contain *Pseudomonas* bacteria. The bacterial colony was identified through the fluorescence shown under UV transilluminator when grown in King's B medium. The bacteria was determined as *Pseudomonas* based on yellow fluorescence (due to fluorescein) observed under King's B media. Also the bacteria exhibited blue-green pigmentation when grown under King's A media. Production of blue-green zone is a characteristic property of pyocyanin producing *Pseudomonas aeruginosa* (King agar A, product information, Sigma-Aldrich). Thus the isolated bacteria was identified as *P. aeruginosa* and further molecular characterization was carried out for the strain confirmation.

4.1.1. Molecular identification of the isolate

Molecular characterization by 16S rDNA sequencing was conducted to confirm the identified environmental isolates. These small subunits ribosomal RNA gene of isolated strain *P. aeruginosa* was amplified from bulk genomic DNA by PCR. The isolated small ribosomal subunit DNA sequence was 1500 nucleotides long as shown in Figure 4.1. The strain showed 3% variation from the other reported species of database and was closest to *P. aeruginosa* (16S ribosomal RNA gene) (97% similarity, Accession number: HM067869.1) as shown in Figure 4.2. Further, the phylogenetic tree was drawn to analyze evolutionary

relationships among sequences of isolated microorganism and nearest neighbors (Figure 4.3).

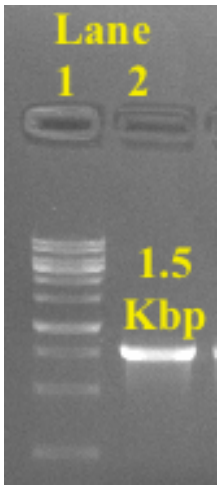


Figure 4.1: Lane 1, 1 Kbp DNA ladder (New England BioLabs Cat # N3232S: 1000 bp, 2000 bp, 3000 bp, 4000 bp, 5000 bp, 6000 bp, 7000 bp, 8000 bp, 10000 bp.), **lane 2 *P. aeruginosa* 16S rDNA PCR amplified product**

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident
Pseudomonas aeruginosa strain GIM 32 16S ribosomal RNA gene, partial sequence	1973	1973	97%	0.0	97%
Pseudomonas aeruginosa strain SUB 10 16S ribosomal RNA gene, partial sequence	1971	1971	97%	0.0	97%
Pseudomonas sp. G6 16S ribosomal RNA gene, partial sequence	1971	1971	97%	0.0	97%
Streptomyces sp. 2011 16S ribosomal RNA gene, partial sequence	1971	1971	97%	0.0	97%
Pseudomonas aeruginosa strain DPS 16S ribosomal RNA gene, partial sequence	1969	1969	97%	0.0	97%
Pseudomonas aeruginosa strain HMR1 16S ribosomal RNA gene, partial sequence	1969	1969	97%	0.0	97%
Pseudomonas sp. YR2 16S ribosomal RNA gene, partial sequence	1969	1969	96%	0.0	97%

Figure 4.2: BLAST hits generated after database search of test strain

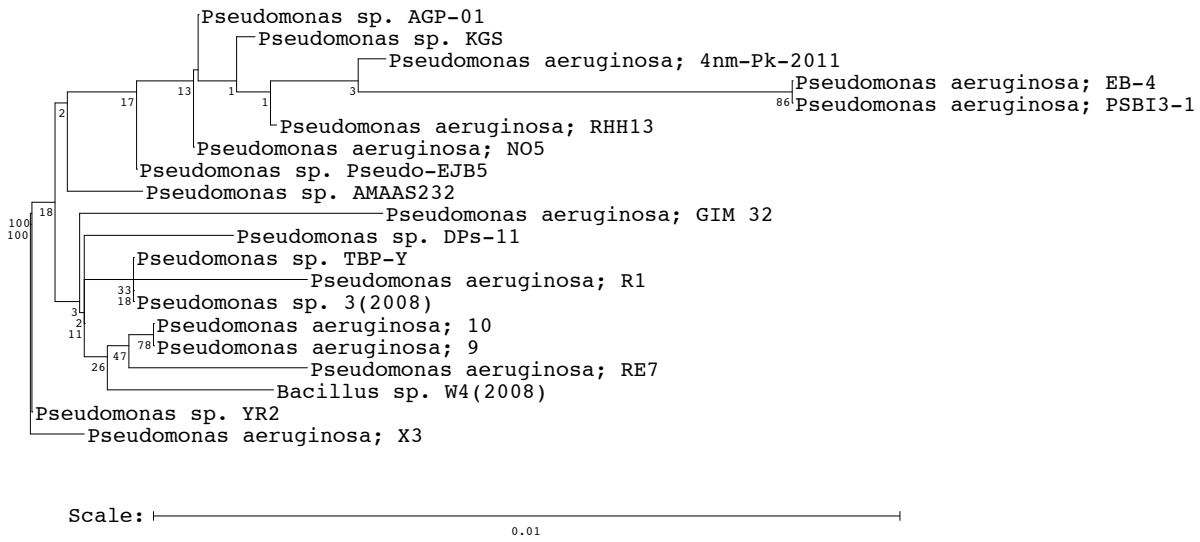


Figure 4.3: Phylogenetic tree based on the 16S rDNA sequences of bacteria using MEGA version 5

4.2. EXTRACTION AND LC-MS ANALYSIS OF *PSEUDOMONAS AERUGINOSA* CULTURE BROTH

The carefully prepared ethyl acetate (EtOAc) extract of culture broth of soil derived *P. aeruginosa* was evaporated to get a dry residue (**EEPA**). Before beginning the isolation of chemical metabolites of **EEPA**, it was subjected for LC-MS analysis, whose fingerprint under PDA detection (200-400 nm) displayed around 12 prominent peaks of having *m/z* values in the range of 154 - 311 amu (Figure 4.4). Ascertaining the presence of small molecules in **EEPA**, it was realized to be worthy to carry out chemical investigation on **EEPA**.

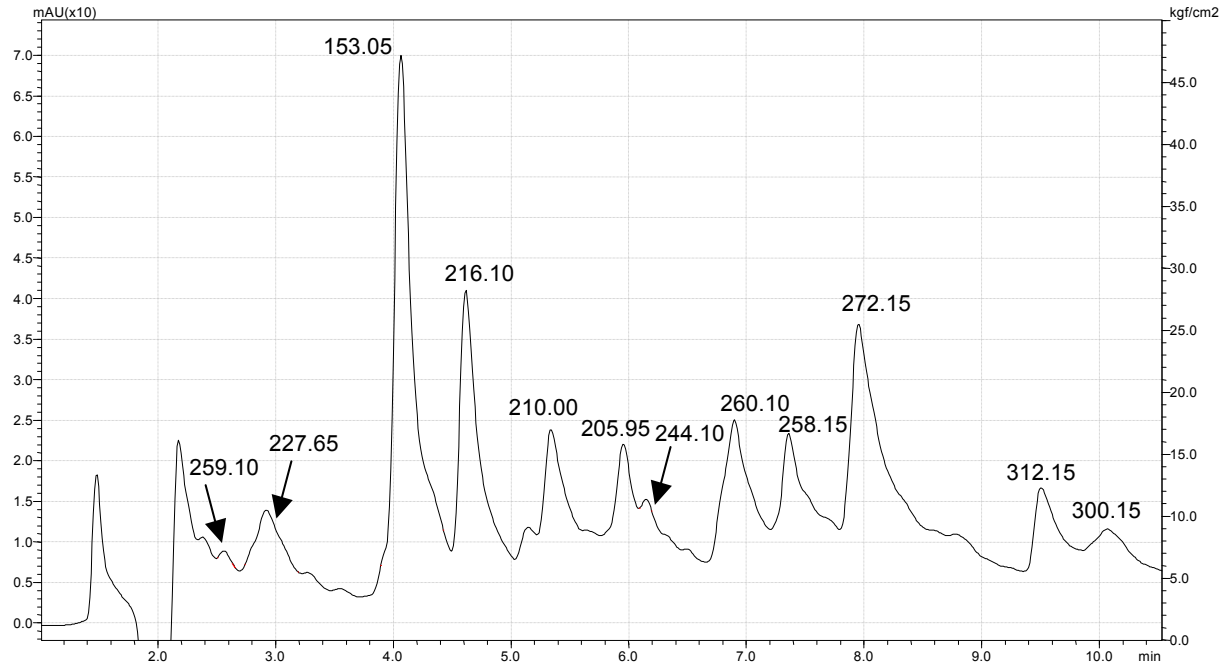
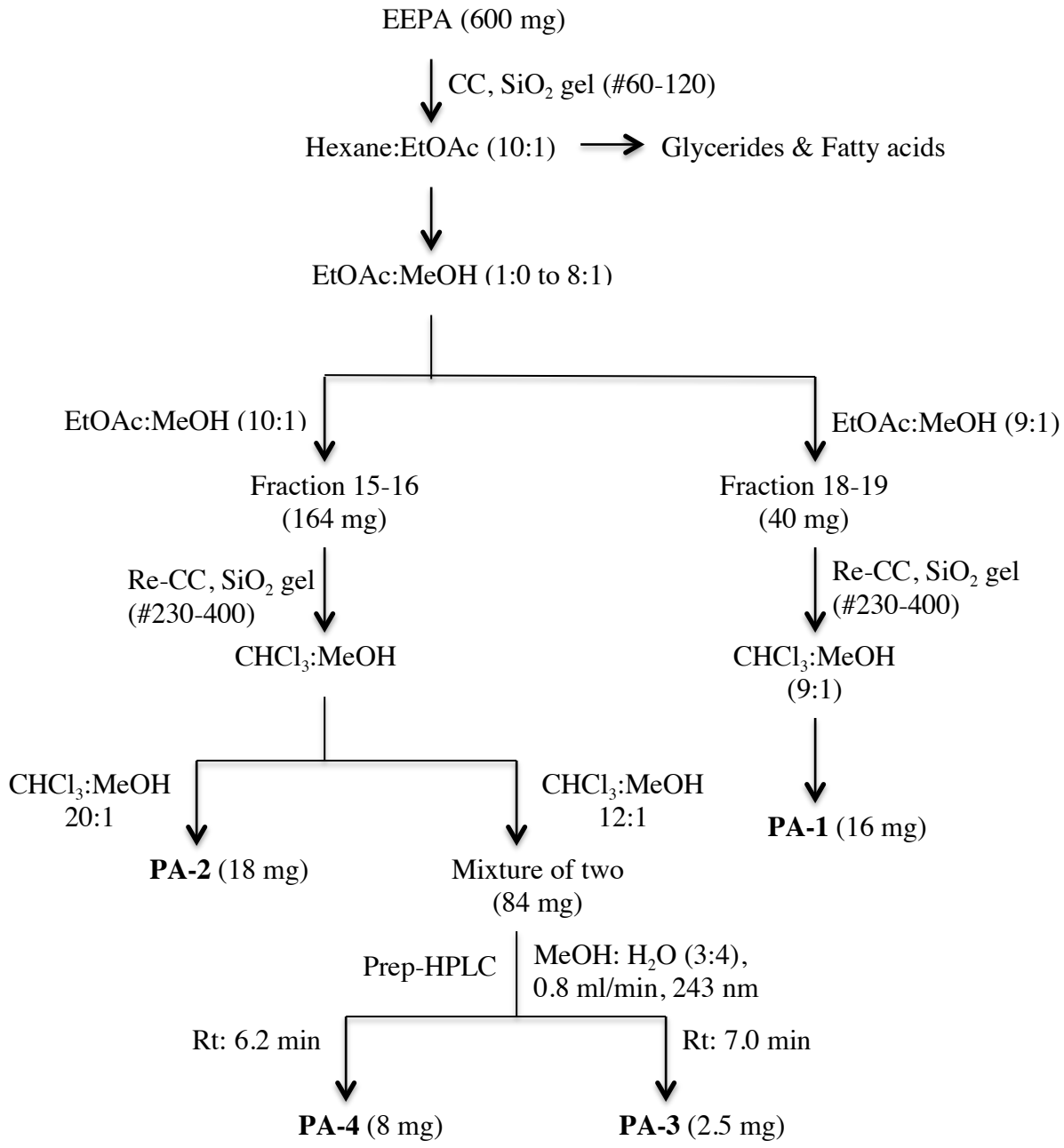


Figure 4.4: LC-MS fingerprint of EEPA

4.3. ISOLATION OF CHEMICAL CONSTITUENTS OF EEPA

The **EEPA** on repeated column chromatographic purification using solvents of increasing polarity yielded four compounds. They were designated as **PA-1**, **PA-2**, **PA-3** and **PA-4**. The isolation procedure is outlined in scheme 1.

Scheme 1

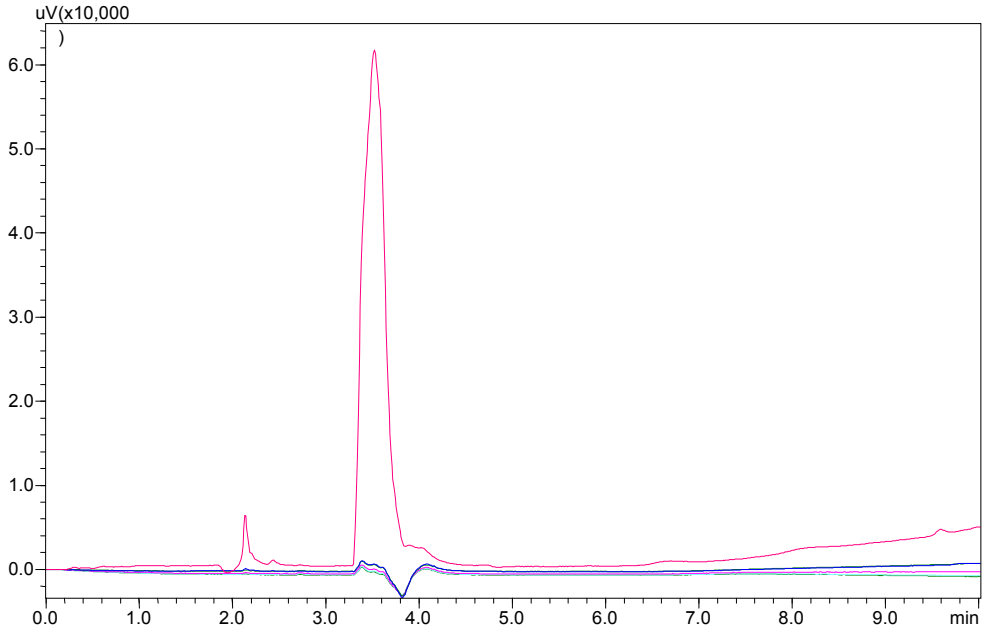


CC: Column chromatography, SiO_2: Silica gel

4.4. CHARACTERIZATION OF ISOLATED CONSTITUENTS OF *PSEUDOMONAS AERUGINOSA*

4.4.1. Characterization of PA-1

PA-1 was obtained as colourless crystals showing melting point of 177-180 °C. The homogenous nature of **PA-1** was assessed through TLC studies using different mobile phase systems (Hexane:EtOAc, 9:1, R_f 0.45 and $CHCl_3$:MeOH, 8:2, R_f 0.55). Single spot due to **PA-1** was observed when the plates were sprayed with 10% methanolic sulphuric acid and heated at 110 °C for 10 min. The compound was found to be soluble in methanol, chloroform and ethyl acetate. It was found to be insoluble in hexane and sparingly soluble in water. HPLC analysis, considered to be a more authentic method of confirming the purity of compounds was performed using reverse phase technique (C_{18} 100A, 250 x 4.60 mm) under gradient mode (water and acetonitrile) as **PA-1** was found to be polar in nature. A single peak chromatogram with **PA-1** eluting at R_t 3.51 min was observed under photo diode array (PDA) detection (214 nm) as shown in figure 4.5.



(Detection at 214, 250, 280, 300, 320 and 350 nm)

Figure 4.5: The HPLC chromatogram of PA-1

The UV spectral analysis of PA-1 measured using methanol showed λ_{max} at 248, 300, 320 and 368 nm (Figure 4.6). The IR spectrum of **PA-1** recorded using Diffuse reflectance attachment (DRA) technique showed absorption bands at 1680 cm^{-1}, 1648 cm^{-1} (CO stretch), 1460 cm^{-1} (NH-in plane vibration) and 1298 cm^{-1} (*cis*-CONH) pertinent to *cis*-amide I band, amide II band and amide III band, respectively (Figure 4.7). Also absorption bands at 1414 cm^{-1} (NH-bending), 1340 cm^{-1} (C-N stretch) and 3180 - 3100 cm^{-1} (N-H stretch) confirmed the presence of *cis*-amide group characteristic of diketopiperazines or cyclic peptides.

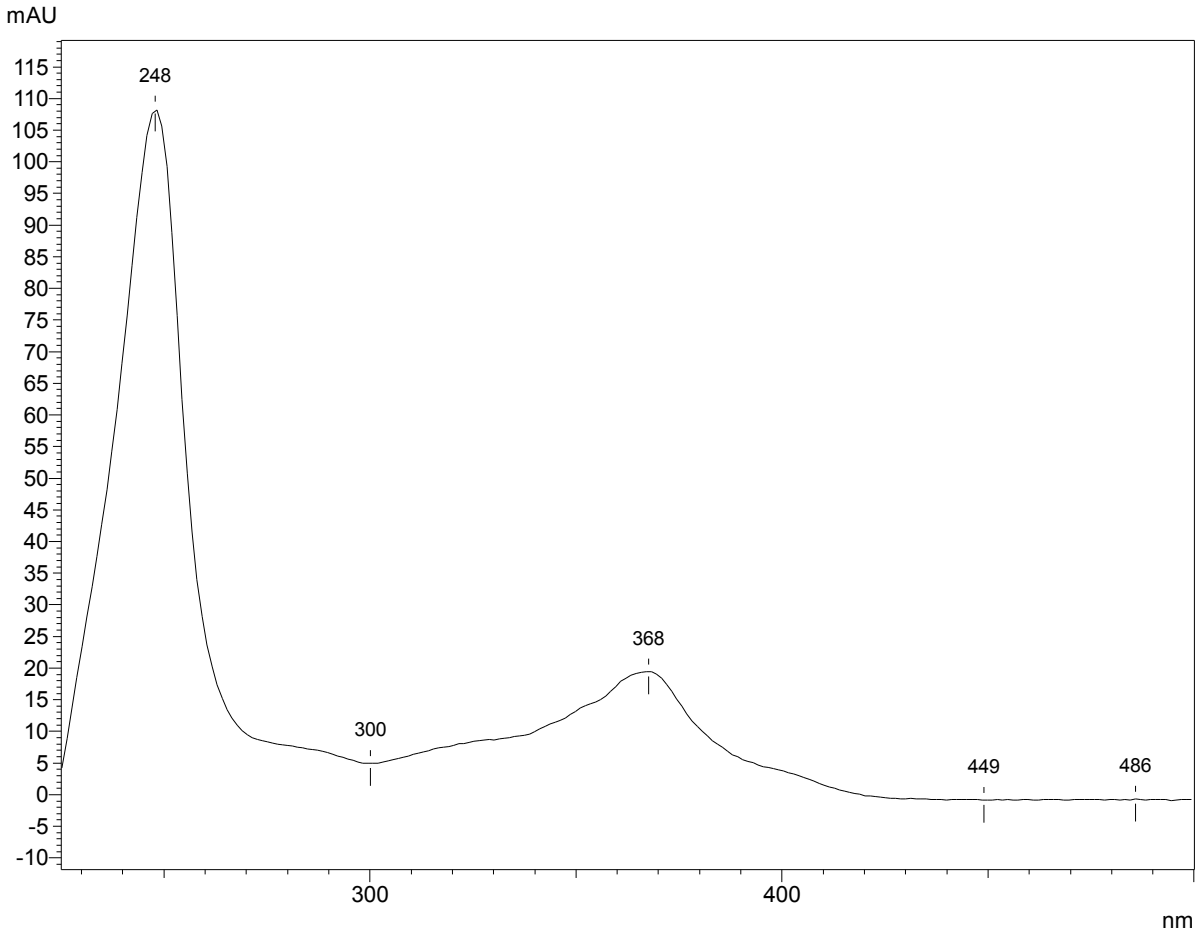


Figure 4.6: UV spectrum of PA-1

PA-1 showed $[M+H]^+$ ion peak at *m/z* 155 under FAB mass spectral analysis, which was indicative of presence of diketopiperazine-pyrrolidine fragment (Figure 4.8A). This

finding was further confirmed from the existence of characteristic peaks at *m/z* 113 and 95 discerned in the ESI mass spectrum of **PA-1** as shown in Figure 4.8B and 4.8C.

A careful analysis of 125 MHz ^{13}C NMR spectrum of **PA-1** measured using CD_3OD revealed seven distinct carbon signals constituting two amide carbonyl groups (CONH, δ_C 165.0 and 170.6 ppm), four methylene groups (δ_C 45.6, 44.9, 28.0 and 21.9 ppm) and one deshielded methine group (δ_C 58.5 ppm) confirming the presence of diketopiperazines having three extended carbon chain in cyclic form (Figure 4.9). The broad methylene multiplets between δ_H 1.7 - 3.7 ppm along with one proton integrated broad triplet at 4.20 - 4.24 ppm found in the 500 MHz ^1H NMR spectrum, strongly confirmed the diketopiperazine derivative as a proline and glycine based cyclic dipeptide (Figure 4.10). Finally, **PA-1** was identified as cyclo(Gly-Pro) ($C_7H_{10}N_2O_2$) by correlating the data congregated from MS, ^{13}C NMR and ^1H NMR analysis. The pragmatic δ_C and δ_H values as depicted around the derived chemical structure (Figure 4.11) was in complete agreement with the reported carbon and proton NMR data (Furtado *et al*., 2005) of cyclo(Gly-L-Pro), through which the stereochemistry of chiral center at C-9 was also identified as *S*. Further, **PA-1** showed $[\alpha]^{25}_D$ of -117.7 (*c* 1.55, MeOH) [reported -179.58 (*c* = 8.31mg/mL; EtOH)] and hence was confirmed as cyclo(Gly-L-Pro). The chemical structure of **PA-1** was explicitly identified as (9*S*)-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione (**1**).

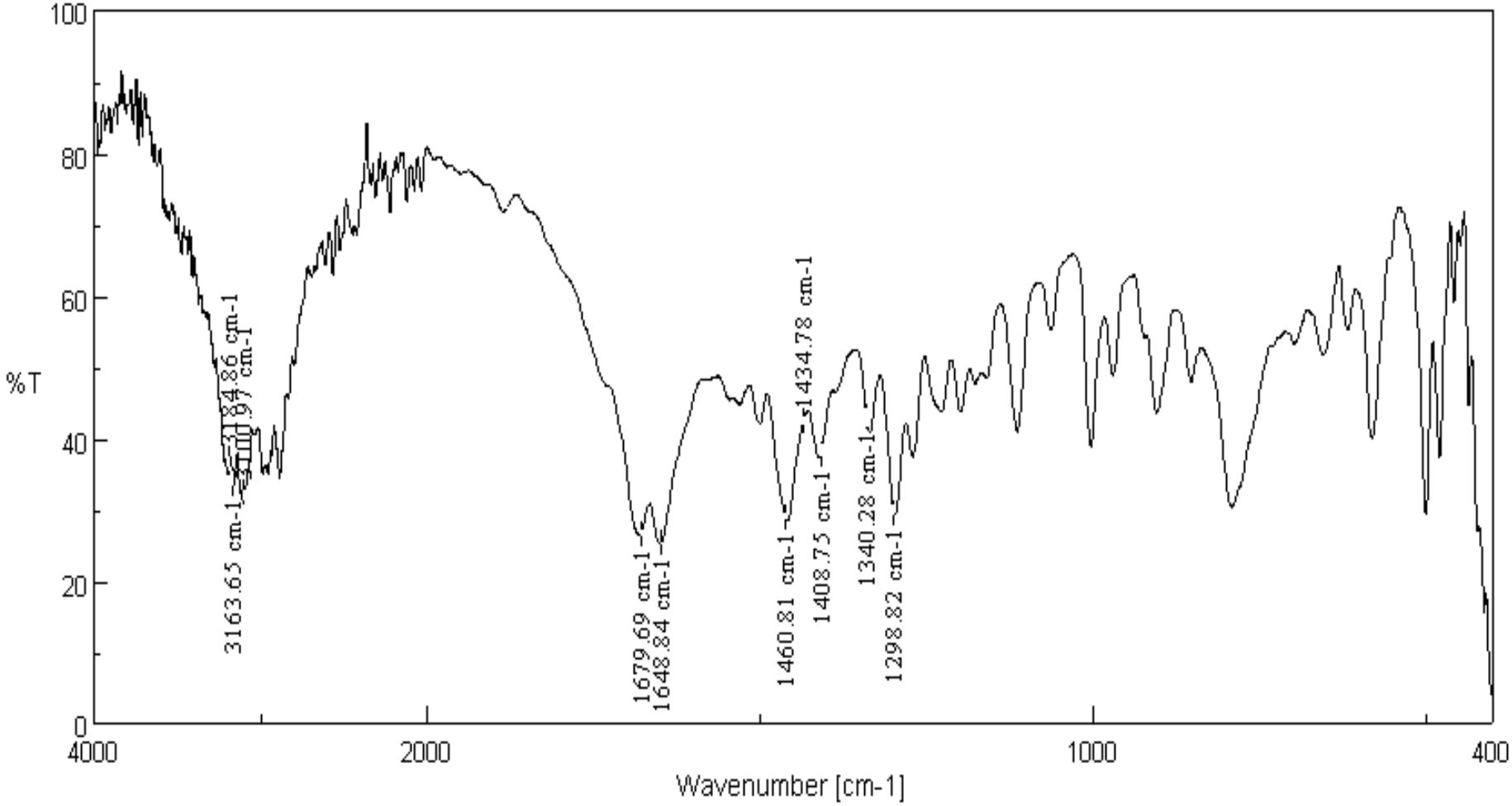


Figure 4.7: IR spectrum of PA-1

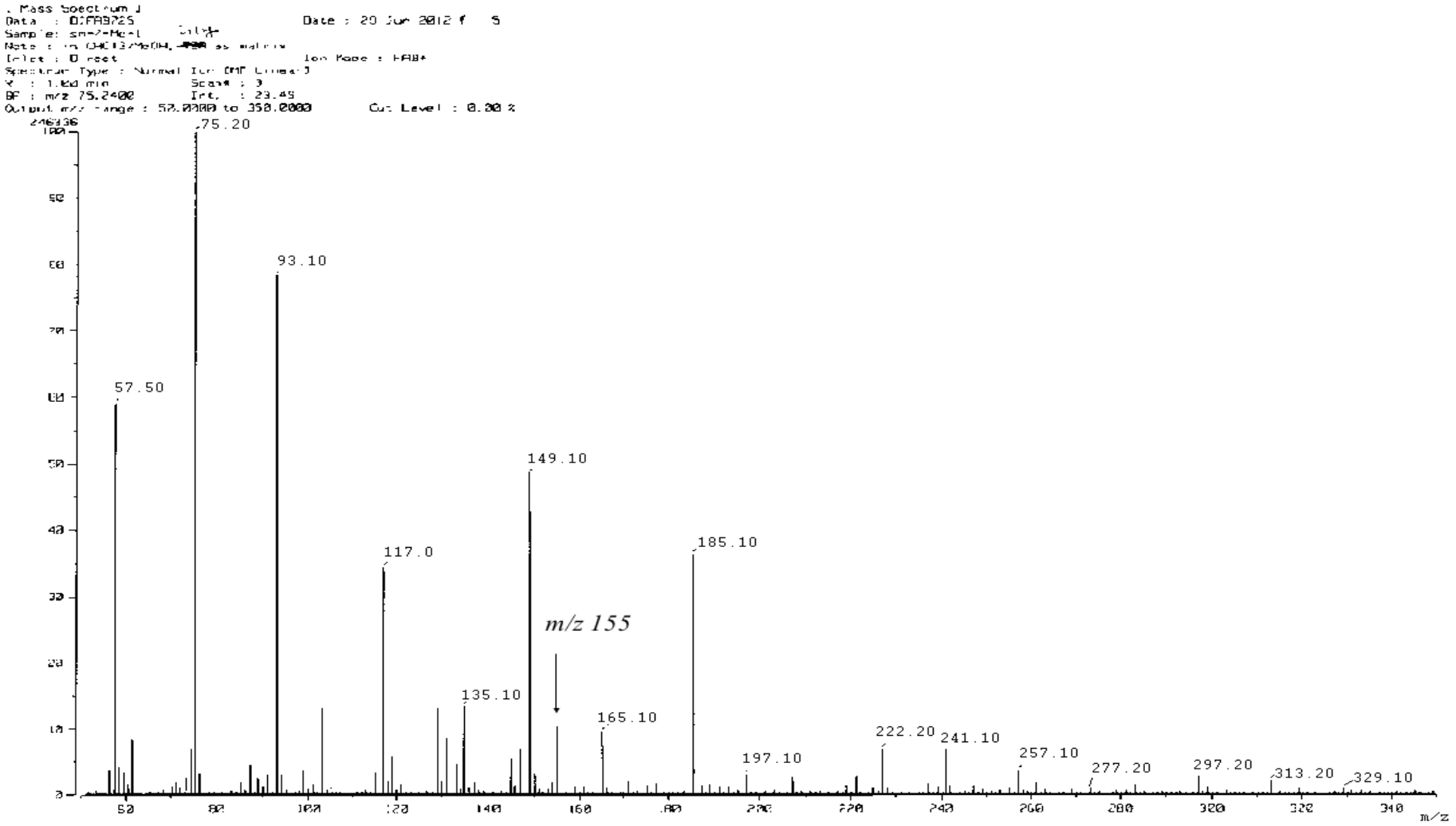


Figure 4.8 A: FAB mass spectrum of PA-1

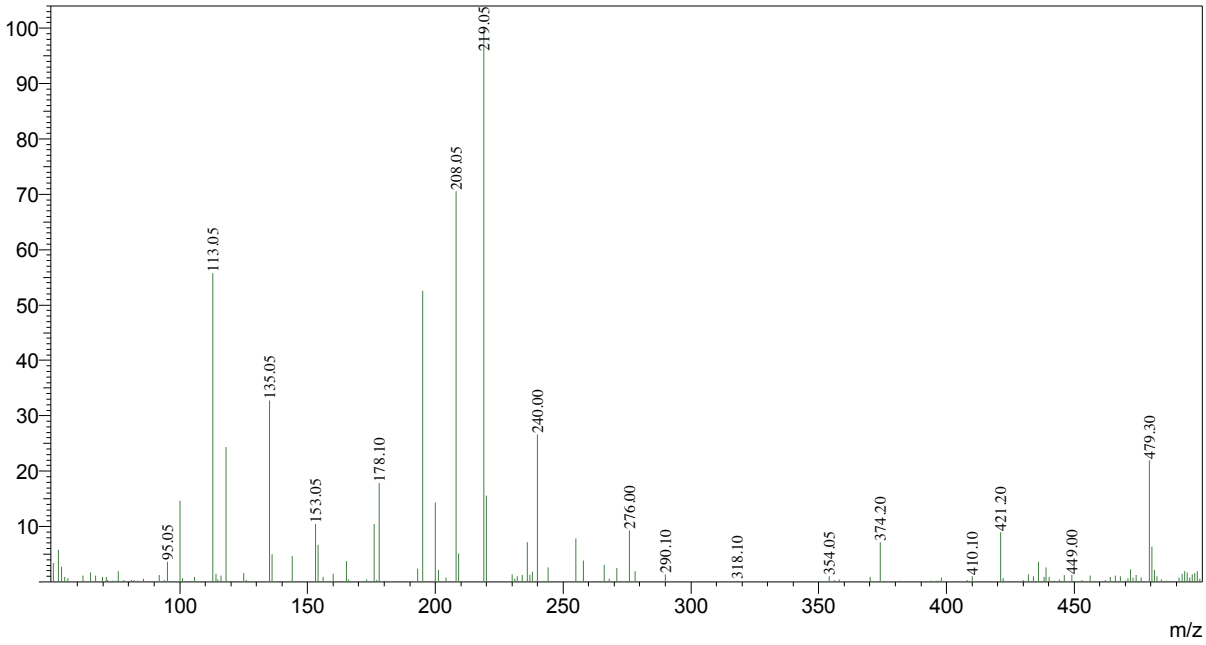


Figure 4.8 B: ESI mass spectrum of PA-1

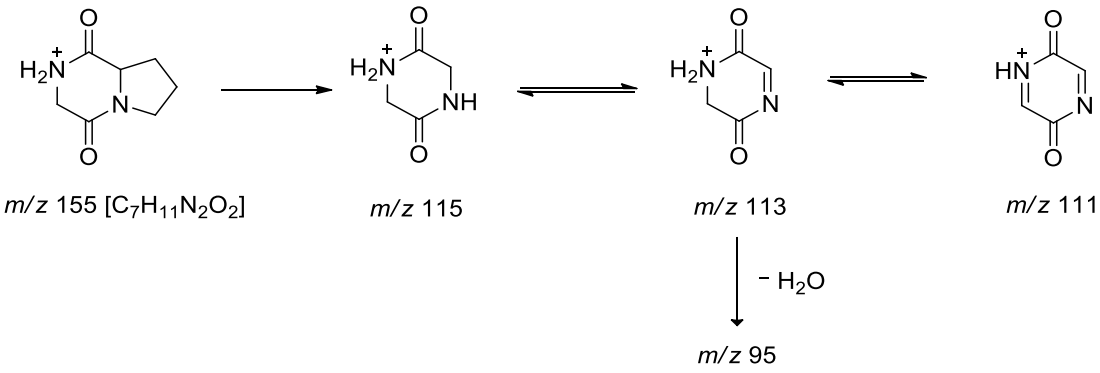


Figure 4.8 C: Mass spectral fragmentation of PA-1

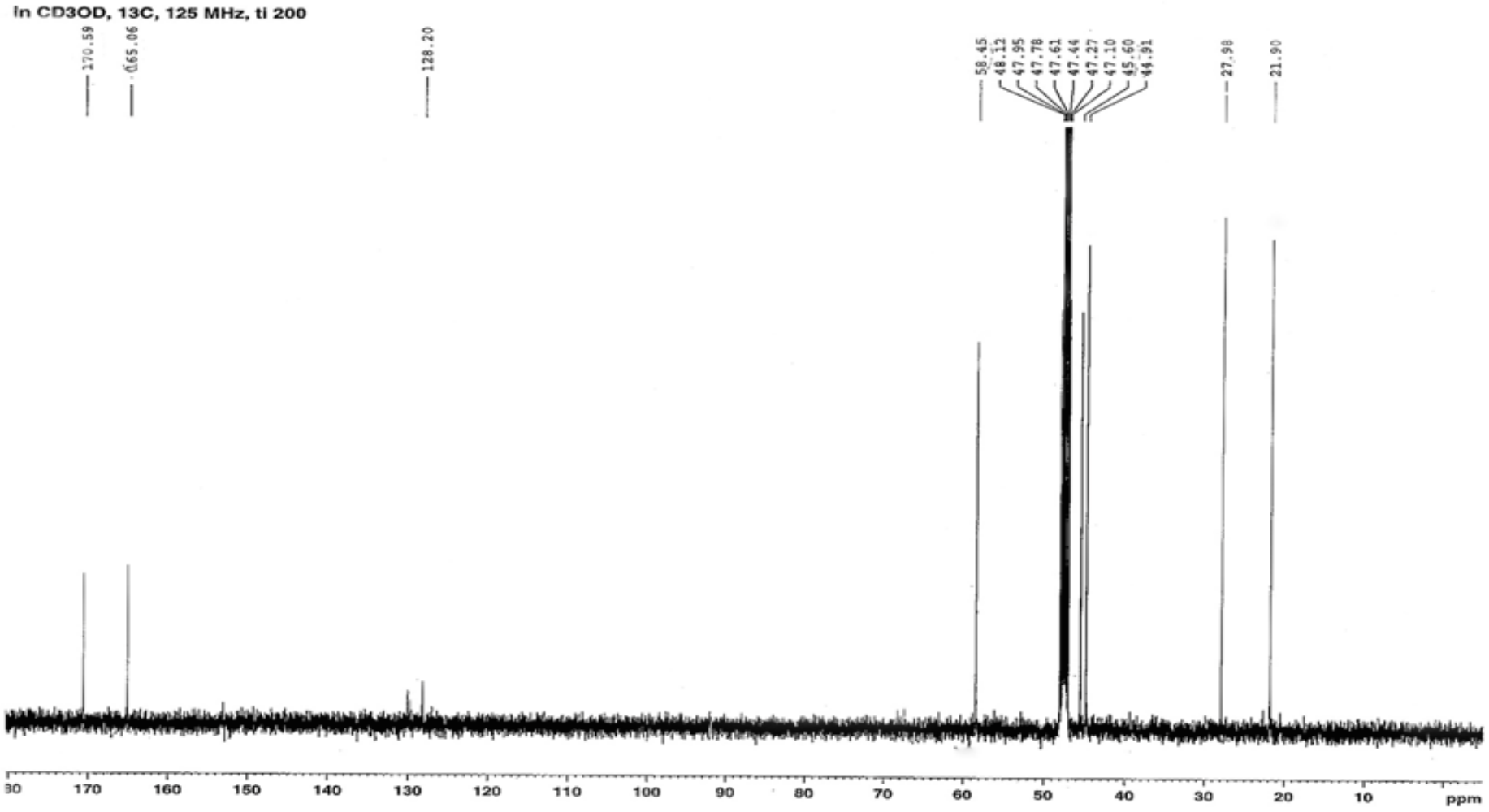


Figure 4.9: 125 MHz ^{13}C NMR spectrum of PA-1

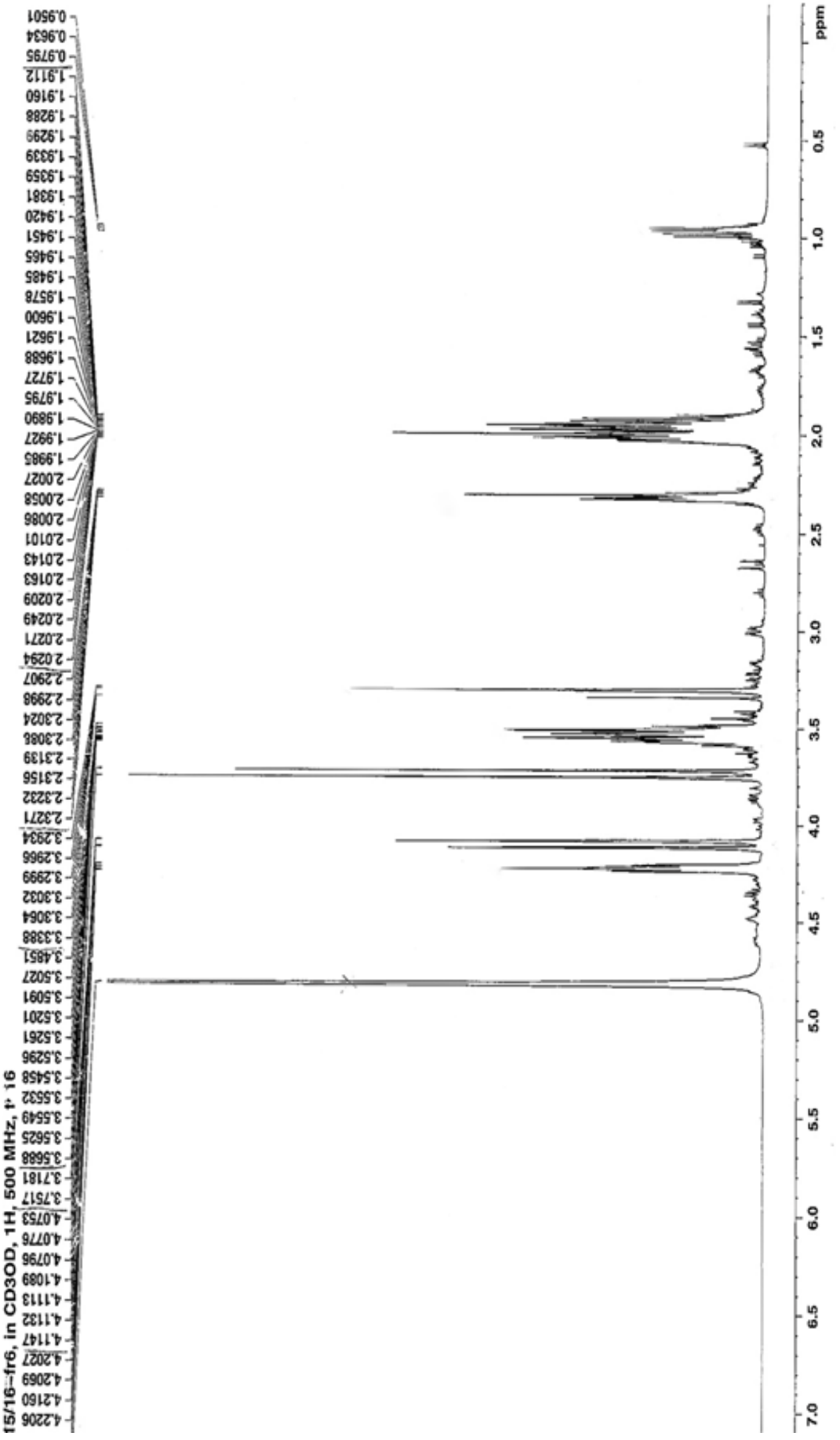
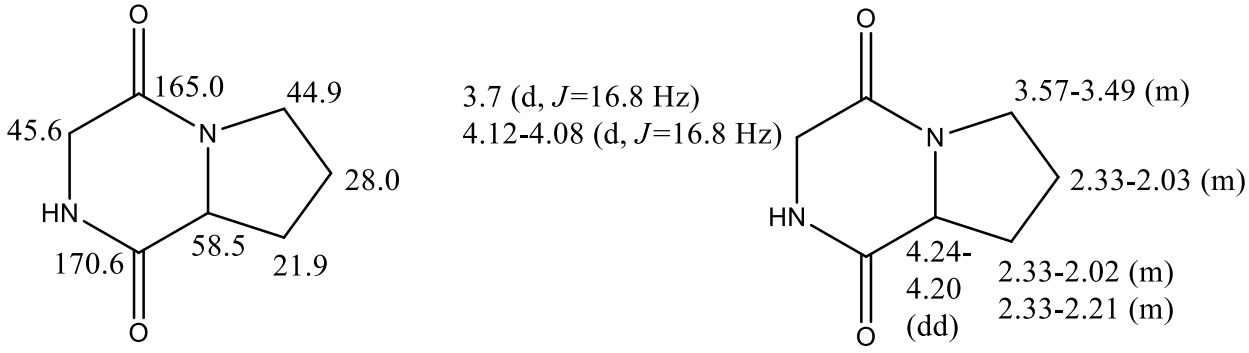
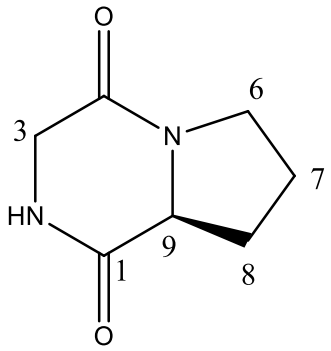


Figure 4.10: 500 MHz ^{1}H NMR spectrum of PA-1



(A) **(B)**

Figure 4.11: Structure of PA-1 depicted with δ_C (A) and δ_H (B) values



(1)

4.4.2. Characterization of PA-2

PA-2 was obtained as white amorphous solid. The homogenous nature of the compound was ascertained using TLC studies. The plates developed in different solvent systems exhibited single spot when sprayed with 10% methanolic sulphuric acid and heated at 110 °C for 10 min. **PA-2** was found to be sparingly soluble in water and freely soluble in MeOH, EtOAc, $CHCl_3$-MeOH mixture and DMSO. The UV spectrum (Figure 4.12) of **PA-2** showed absorption maximum at λ_{max} 235, 256, 288, 315 and 325 nm pointing the presence of benzenoid chromophore.

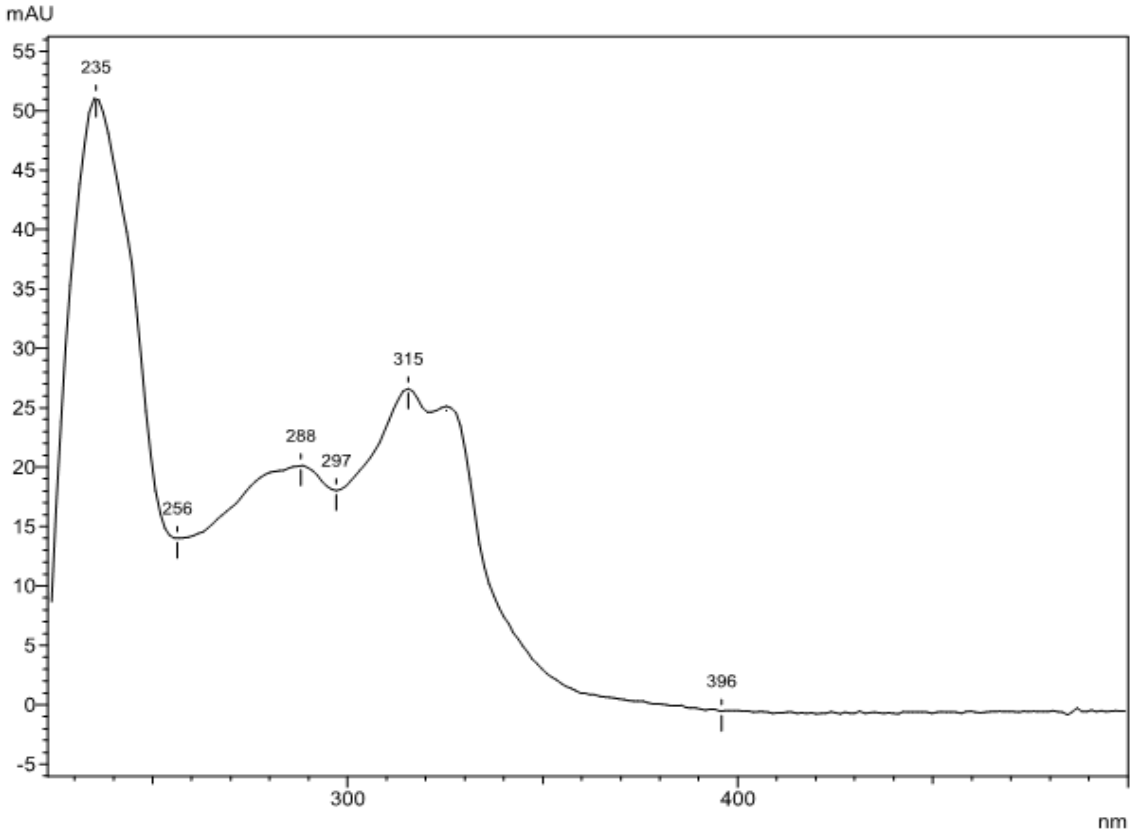
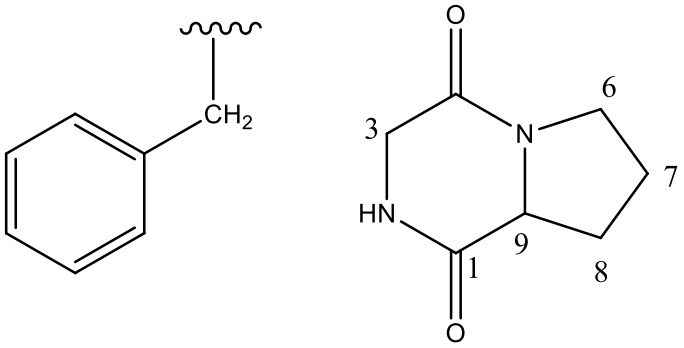


Figure 4.12: UV spectrum of PA-2

The molecular formula of **PA-2** was settled as $C_{14}H_{16}N_2O_2$ on the basis of congregated spectral and it was also found to be in good agreement with the mass spectrometrically derived molecular weight 244, based on the molecular ion peak $[M+H]^+$ found in the FAB

mass spectrum at *m/z* 245. The existence of base peak at *m/z* 154 corroborated the presence of 2,5-dioxopiperazine nucleus and additional peaks at *m/z* 107, 120 and 136 confirmed **PA-2** as a proline based cyclic peptide, like **PA-1**. However the difference in the molecular weight of 90 between **PA-1** and **PA-2** suggested the occurrence of benzyl fragment in **PA-2** (Figure 4.13, Figure 4.14). This assumption gained proof from ^{1}H NMR spectrum (Figure 4.15), which showed a five proton multiplet at 7.27 - 7.32 ppm and two non-equivalent single proton multiplets at 2.8 and 3.54 - 3.67 ppm due to benzyl group, accompanied by the methylene and methine protons signals of cyclo(Gly-L-Pro) (**PA-1**). The ^{13}C NMR spectrum measured using CD_3OD well explained the presence of benzylic group attached to cyclo(Gly-L-Pro) (**PA-1**), making fourteen carbons, classified as two amide carbonyls (δ_C 169.4 and 165.6 ppm), six aromatic carbons (δ_C 127.5, 129.2, 135.9, 127.5, 129.1 and 135.9 ppm), four methylene carbons and two methine carbons (Figure 4.16). Based on the above discussion, the part structure of **PA-2** was derived as A and B.



(A) **(B)**

There were four probable positions for the attachment of A with B, i.e. 3, 6, 7 and 8. The exact point of attachment was settled as C-3 through homonuclear 2D NMR spectroscopy. The ^{1}H-^{1}H COSY analysis of **PA-2** (Figure 4.17) showed correlation between C-3 proton and Ar-C**H$_2$** protons. Similarly correlation between C-9 (Pro-α), C-8 (Pro-β), C-7 (Pro-γ) protons were found and the structure of **PA-2** was clearly identified as **2a**. **PA-2** was

found to be levo rotatory showing $[\alpha]^{25}_{D}$ – 182.2 (*c* 0.09, MeOH) and the stereochemistry at C-3 and C-9 were settled as *S* and *S* by comparing the observed ^{13}C NMR and ^{1}H NMR data with the reported data of cyclo(L-Phe-L-Pro) (Tullberg *et al*., 2006) and cyclo(D-Phe-L-Pro) (Campbell *et al*., 2009) (Table 4.1 and 4.2). Thus **PA-2** was unambiguously identified as cyclo(L-Phe-L-Pro) or (3*S*,9*S*)-hexahydro-3-(phenylmethyl)pyrrolo[1,2-a]pyrazine-1,4-dione (**2**).

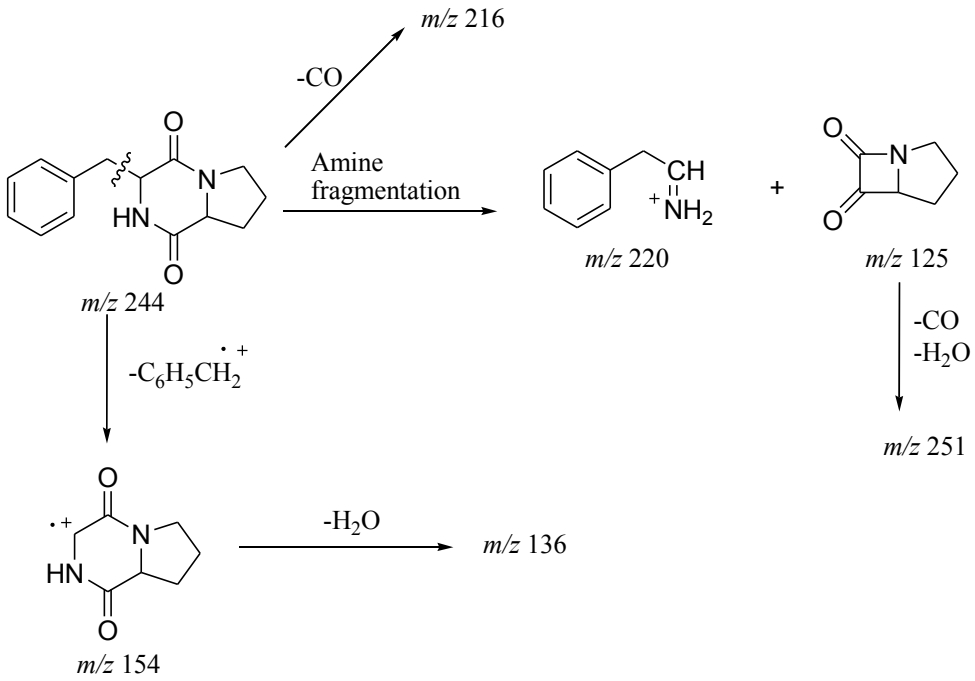


Figure 4.13: Mass spectral fragmentations of PA-2

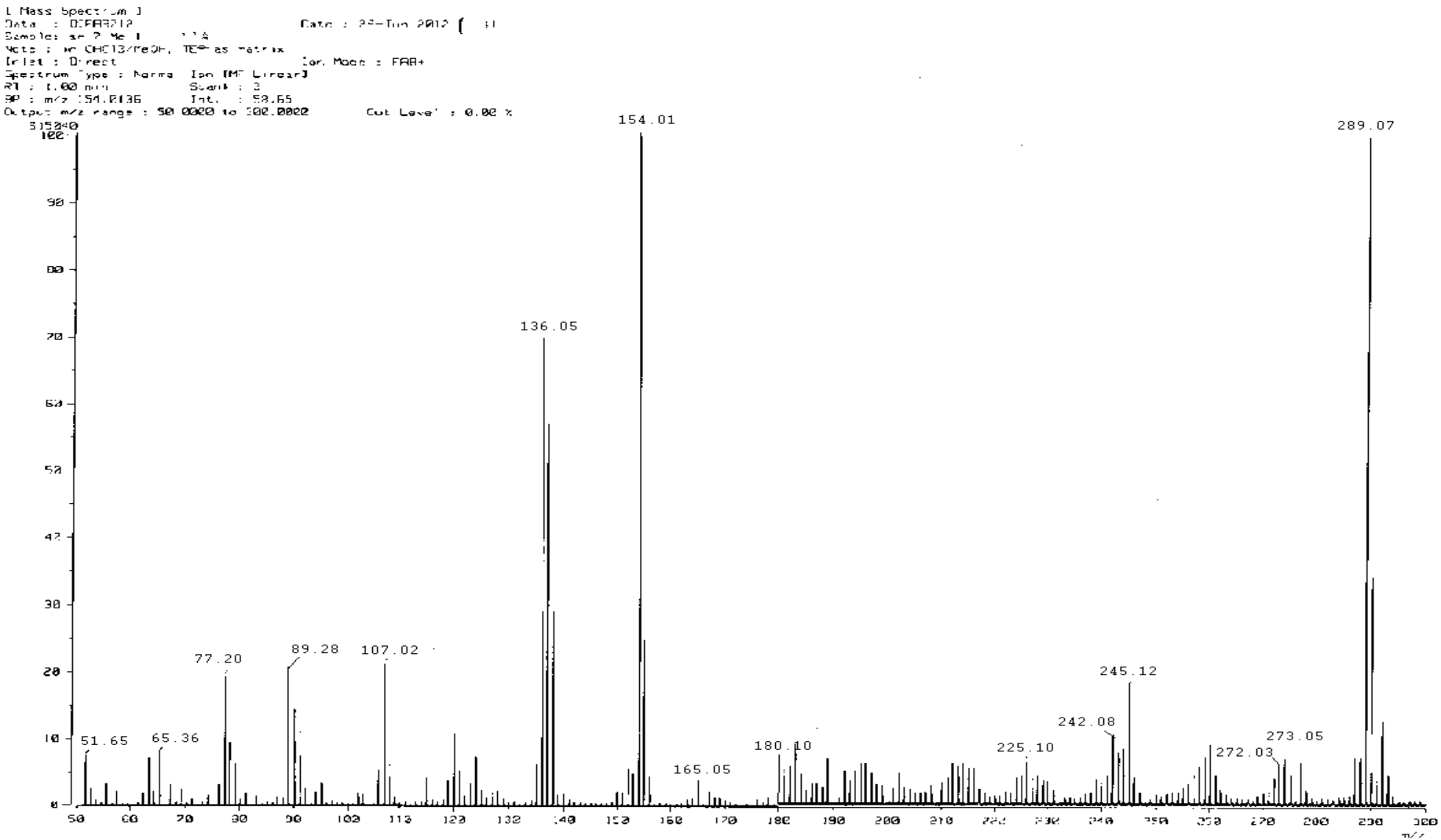


Figure 4.14: FAB mass spectrum of PA-2

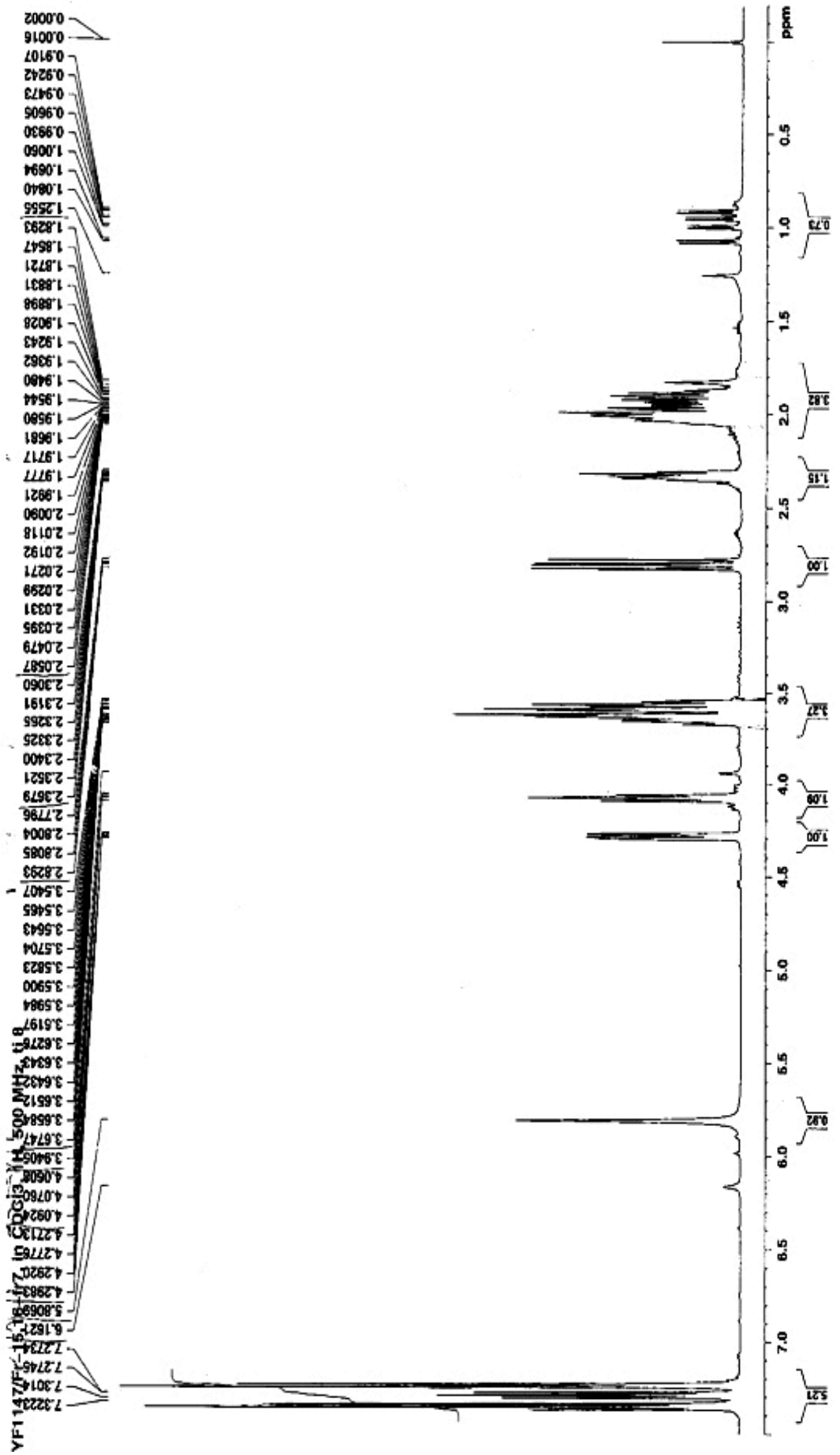


Figure 4.15: 500 MHz ^{1}H NMR Spectrum of PA-2

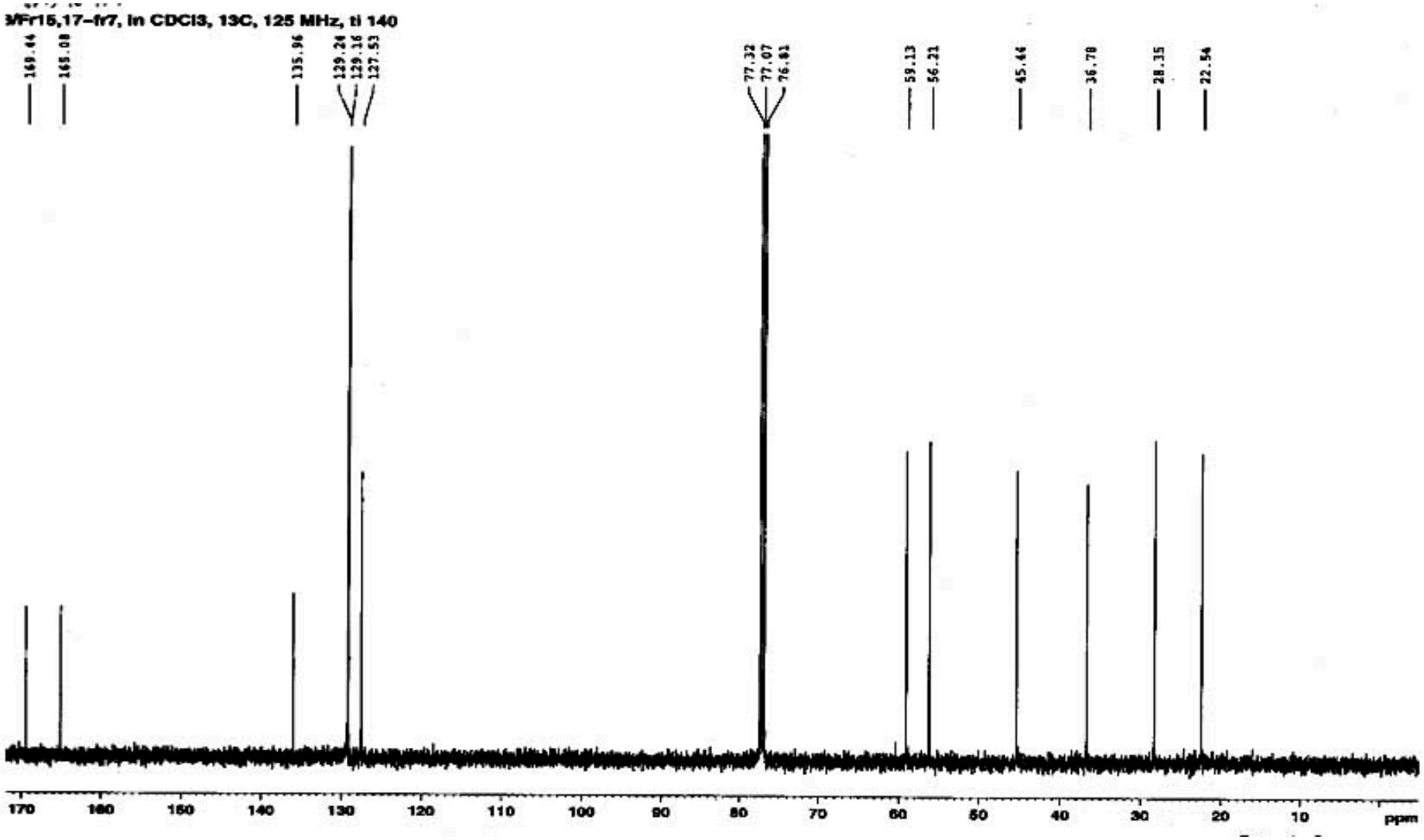


Figure 4.16: 125 MHz ^{13}C NMR Spectrum of PA-2

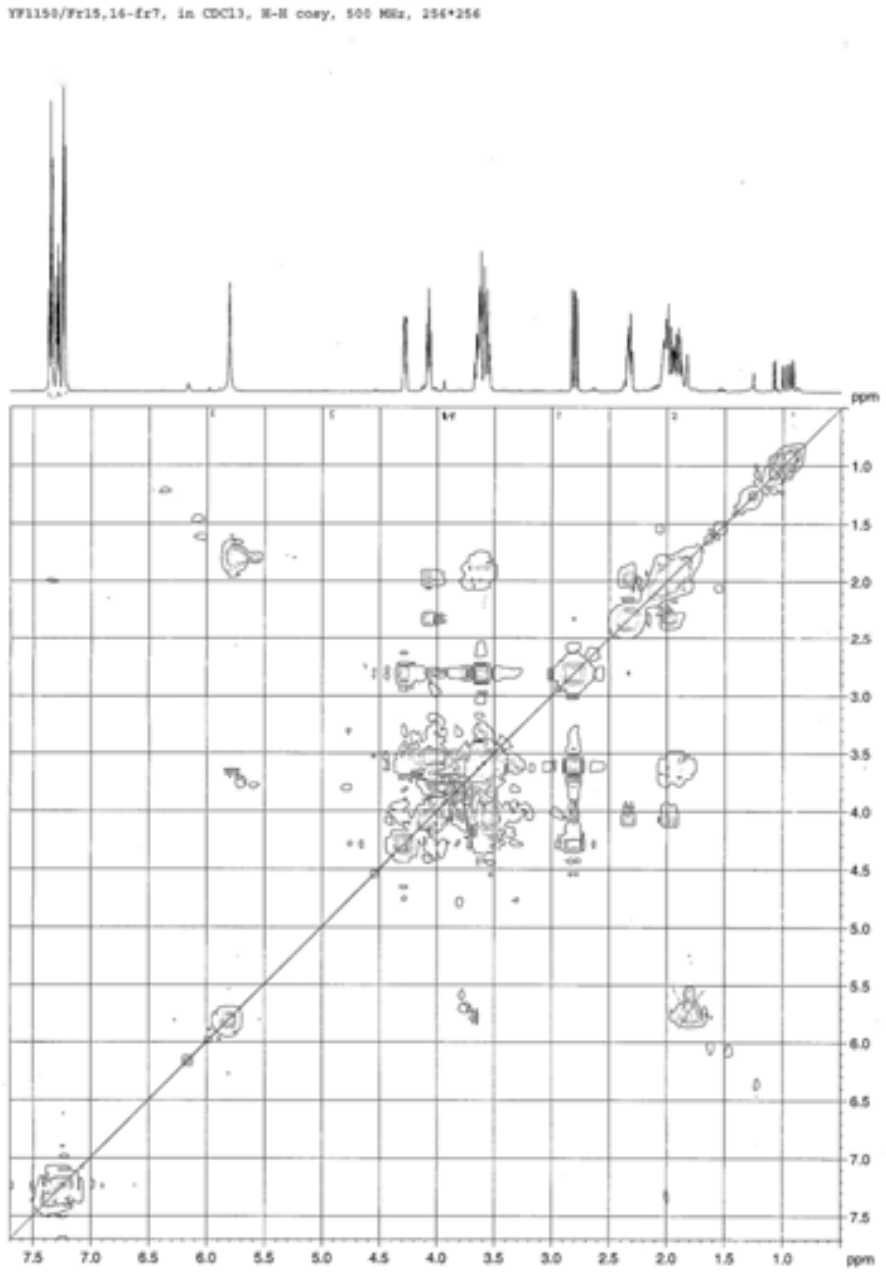
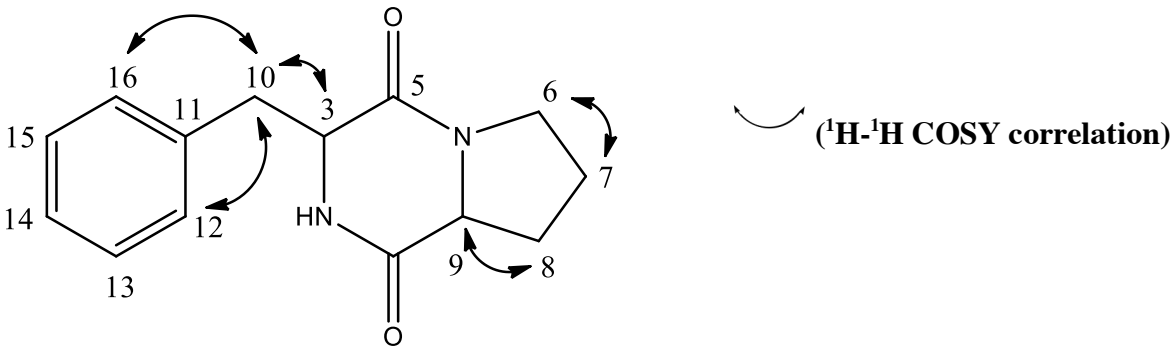


Figure 4.17: ^{1}H-^{1}H COSY spectrum of PA-2



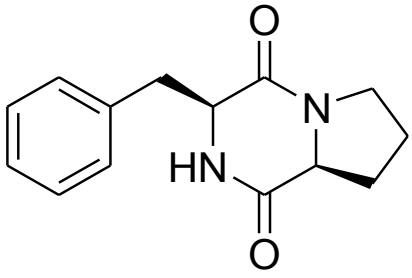
2a

Table 4.1: 125 MHz ^{13}C NMR spectral data of PA-2

Position	δ_C recorded ($CDCl_3$)	cyclo(L-Phe-L-Pro) ($CDCl_3$)	cyclo(D-Phe-L-Pro) ($CDCl_3$)
1	165.1 or 169.4	169.5 or 165.1	169.75 or 165.10
2	-	-	-
3	56.2 or 59.1	59.2 or 56.3	59.12 or 57.90
4	165.1 or 169.4	169.5 or 165.1	169.75 or 165.10
5	-	-	-
6	45.4	45.5	45.27
7	22.5	22.6	21.84
8	28.4	28.4	29.10
9	56.2 or 59.1	59.2 or 56.3	59.12 or 57.90
10	36.8	36.9	40.64
11	136.0	136.1	135.55
12	129.16 or 129.24	129.3 or 129.2	-
13	129.16 or 129.24	129.3 or 129.2	130.19, 128.88, 127.68
14	127.5	127.6	-
$[\alpha]^{25}_D$	-182.2 (*c* 0.09, MeOH)	-184 (*c* 0.3, CH_2Cl_2)	-79 (*c* 9.38 mg/ml, EtOH)
m.p.	130-132 °C	130-132 °C	153-157 °C

Table 4.2: 500 MHz ^{1}H NMR spectral data of PA-2

Position	δ_H recorded ($CDCl_3$)	cyclo(L-Phe-L-Pro) ($CDCl_3$)	cyclo(D-Phe-L-Pro) ($CDCl_3$)
1	-	-	-
2	5.81 (1H, brs)	5.80 (1H, brs)	7.02 (1H, brs)
3	4.28 (1H, dd, *J* = 3.2, 10.4 Hz)	4.26 (1H, dd, *J* = 3.5, 10.4 Hz)	4.25 (1H)
4	-	-	-
5	-	-	-
6	3.67-3.54 (2H, m)	3.66-3.51 (2H, dd, *J* = 10.3, 14.3 Hz)	3.18(1H, *J* = 13.8, 5.76 Hz), 3.06 (1H, *J* = 13.8, 4.15 Hz)
7	2.03-1.83 (2H, m)	2.03-1.83 (2H, m)	1.58-1.81 (2H, m)
8	2.06-1.83 (1H, m), 2.37-2.31 (1H, m)	2.03-1.83 (1H, m), 2.33-2.27 (1H, m)	1.84-1.78 (1H, m), 2.20-2.11 (1H, m)
9	4.07 (1H, t, *J* = 8.2 Hz)	4.07 (1H, t, *J* = 7.9 Hz)	3.66-3.57 (1H, m)
10	2.80 (1H, dd, *J* = 10.4, 14.5 Hz), 3.67-3.54 (1H, m)	2.78 (1H, dd, *J* = 10.3, 14.3 Hz), 3.66-3.51 (1H, m)	2.84 (1H, dd, *J* = 10.4, 6.5 Hz), 3.41-3.33 (1H, m)
11	-	-	-
12,16 13,15 14	7.32-7.27 (5H, m)	7.33-7.25 (5H, m)	7.38-7.19 (5H, m)



(2)

4.4.3. Characterization of PA-3

PA-3 was gained as amorphous solid showing $[\alpha]^{25}_{D}$ – 7.0 (*c* 0.09, MeOH). It was found to be freely soluble in MeOH, sparingly soluble in water and insoluble in hexane. The UV spectrum (Figure 4.18) revealed the presence of benzenoid chromophore (λ_{max} 223, 253 and 343 nm).

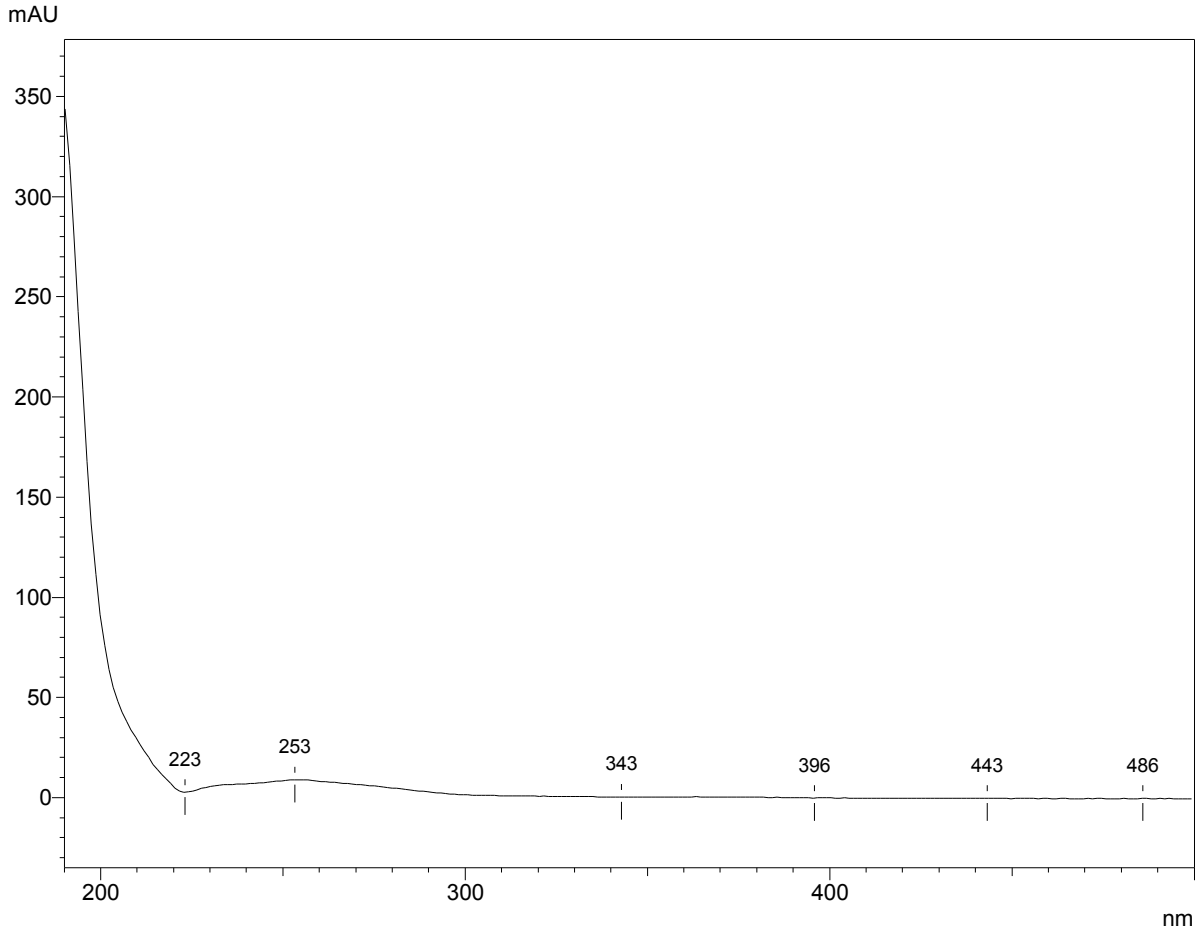


Figure 4.18: UV spectrum of PA-3

The molecular formula of **PA-3** was settled as $C_{14}H_{16}N_2O_3$ on the basis of protonated molecular ion peak at *m/z* 261.12 found in its FAB mass spectrum (Figure 4.19). The mass fragmentation of **PA-3** (base peak m/z 154) was found to be similar to that of **PA-2** suggesting it to be a proline based aromatic cyclic dipeptide having an additional mass unit of 16 amu.

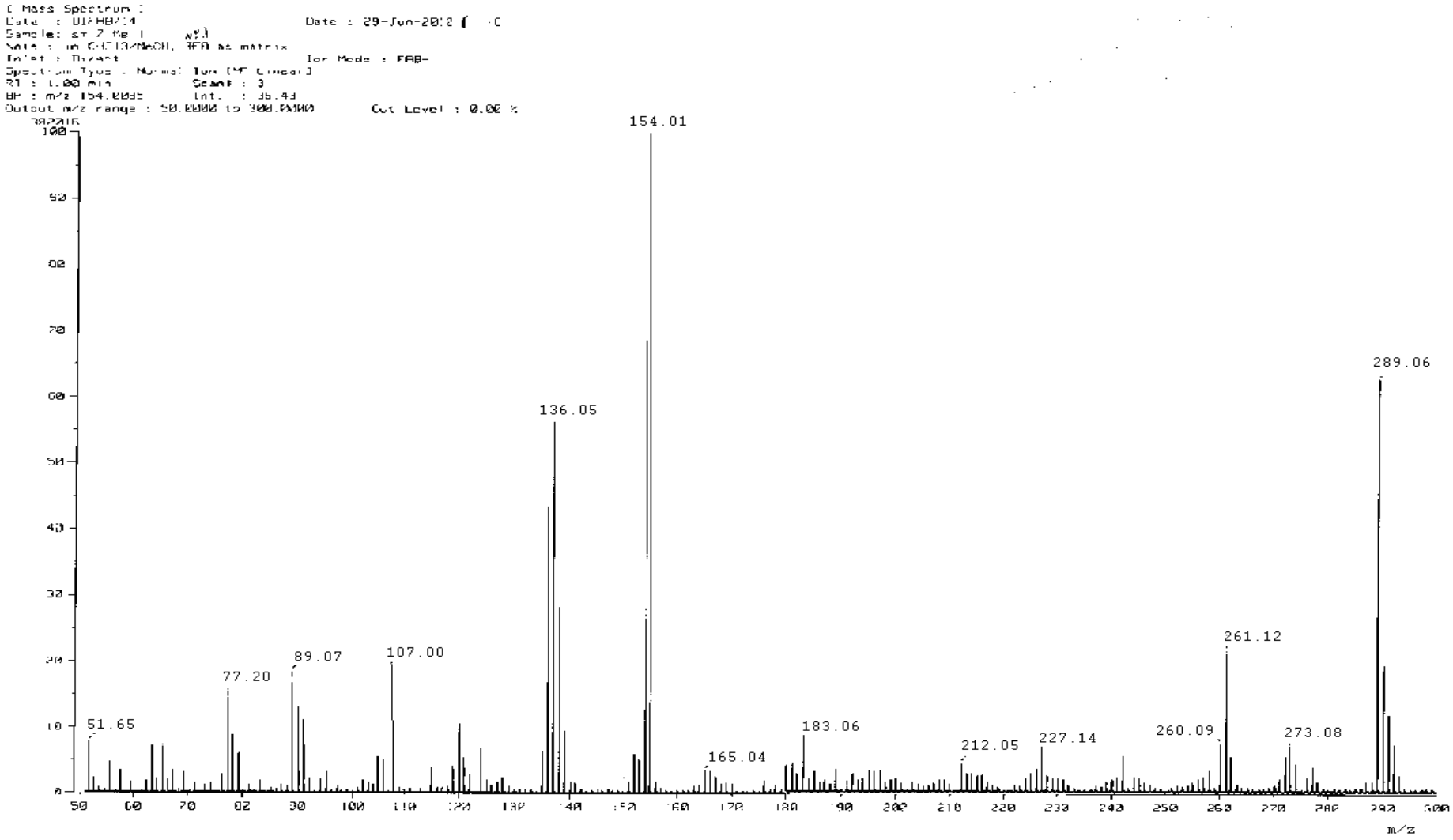


Figure 4.19: FAB mass spectrum of PA-3

On comparison of ^{1}H NMR spectrum (Figure 4.20) of **PA-3** with **PA-2**, difference was found only concerning proline signals. Non-equivalent chemical shift for protons of Pro-δ and Pro-β carbons suggested a chiral center at Pro-γ due to substitution. Also, a mass unit of 16 amu can be accommodated as a hydroxyl substituent at Pro-γ carbon. Literature search followed by comparison of observed ^{1}H NMR data with those of reported data (Strom *et al*., 2002) shaped the molecular structure (Table 4.3). Thus **PA-3** was identified as cyclo(*trans*-4-hydroxy-L-Pro-L-Phe), having absolute structure as (3*S*,7*R*,9*S*)-hexahydro-7-hydroxy-3-(phenylmethyl)pyrrolo[1,2-a]pyrazine-1,4-dione (**3**).

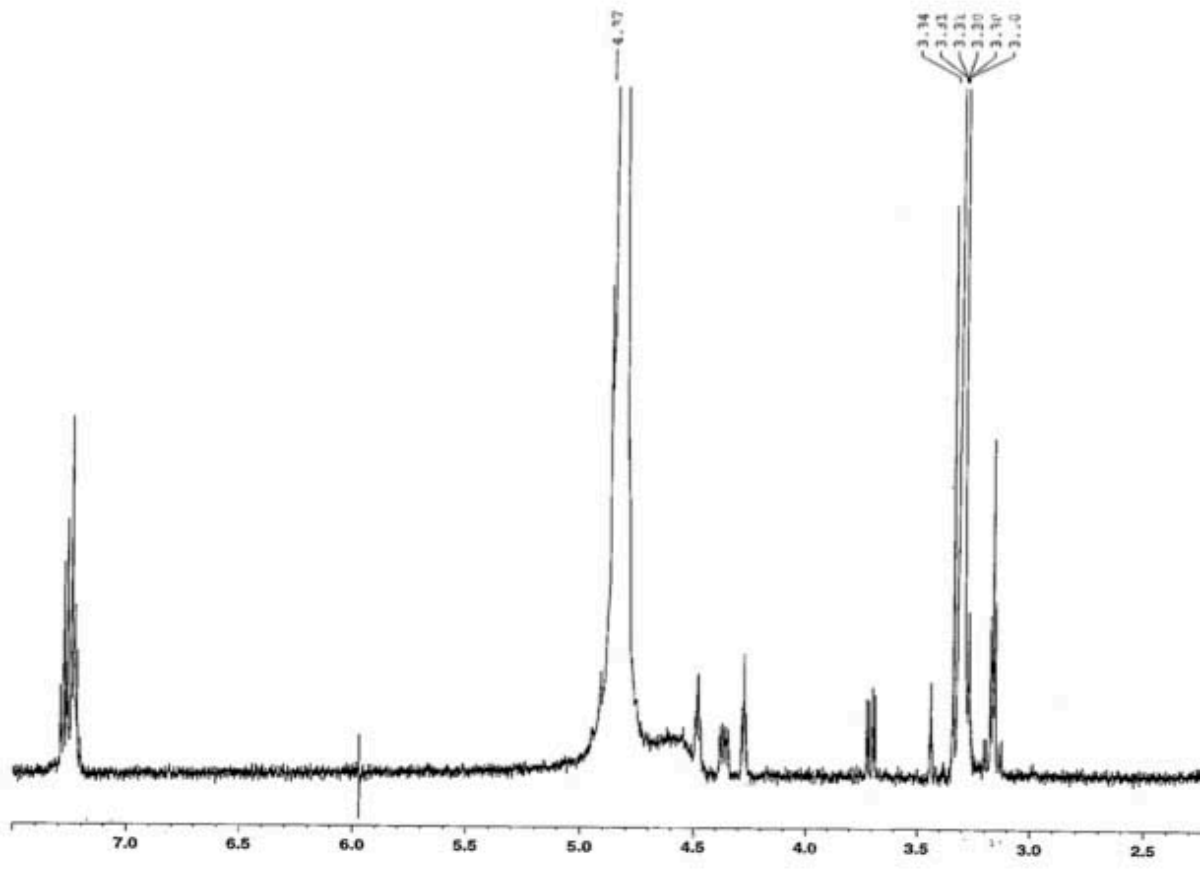
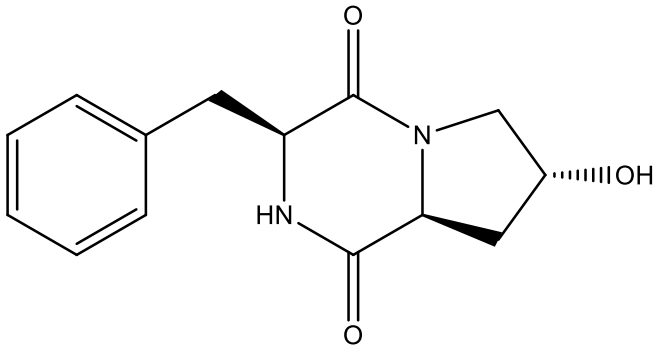


Figure 4.20: 500 MHz ^{1}H NMR spectrum of PA-3

Table 4.3: 500 MHz ^{1}H NMR Spectral data of PA-3

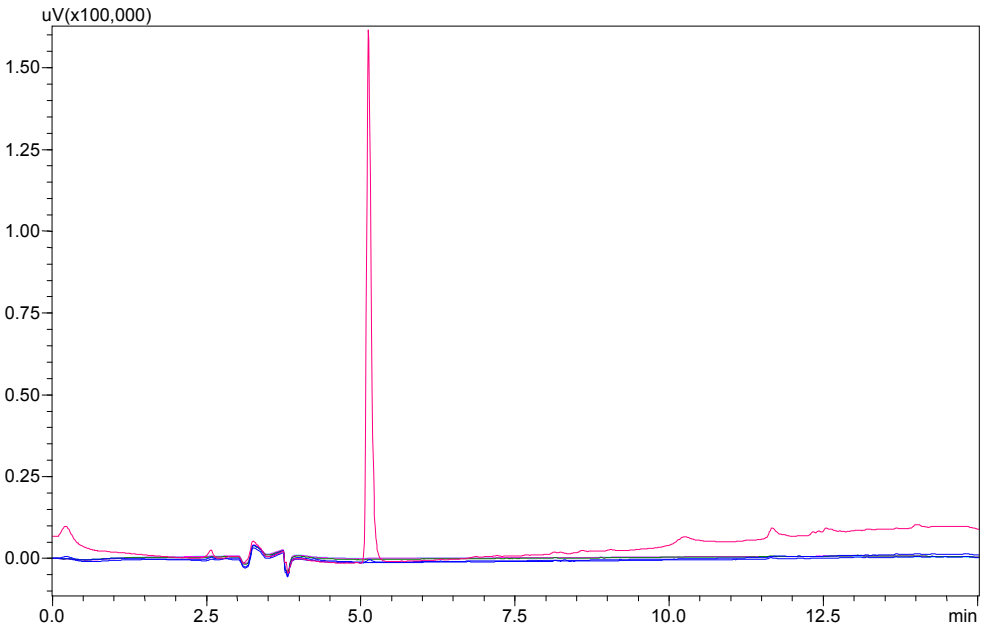
Position	δ_H recorded ($CDCl_3$)	cyclo(Phe-4-OH-Pro) ($CDCl_3$)
1	-	-
2	-	-
3	4.52-4.50 (1H, m)	4.48 (1H, m)
4	-	-
5	-	-
6	3.22-3.12 (1H, m), 3.75-3.68 (1H, dd)	3.29 (1H, m), 3.71 (1H, dd)
7	4.32-4.28 (1H, m)	4.28 (1H, m)
8	1.42-1.38 (1H, dd), 2.08-2.10 (1H, dd)	1.39 (1H, dd), 2.07 (1H, m)
9	4.40-4.35 (1H, dd)	4.37 (1H, dd)
10	3.21-3.12 (2H, m)	3.17 (2H, m)
11	-	-
12-16	7.32-7.27 (5H, m)	7.27-7.22 (5H, m)



(3)

4.4.4. Characterization of PA-4

PA-4 was obtained as yellowish amorphous powder, showing melting point of 178-179 °C. The purity of the compound was determined through TLC studies and verified by RP-HPLC analysis. A single peak chromatogram (Figure 4.21) showing retention time of 5.10 min was obtained when analysed using gradient method (Kinetex 5µ C_{18} 100A, 250 x 4.60 mm; Phenomenex and H_2O: ACN mobile phase). The IR spectrum (Figure 4.22) measured using DRA technique showed broad absorption bands for hydroxyl group at 3450 cm^{-1} (O-H stretch), N-H group at 3273 cm^{-1} and *cis*-amide functional group at 1680, 1617 cm^{-1} (CO stretch), 1460 cm^{-1} (N-H in-plane vibration), 1303 cm^{-1} (*cis*-CONH) and 1440 cm^{-1} (N-H bend). The molecular formula was assigned as $C_{11}H_{18}N_2O_3$ based on congregated spectral data and FAB mass spectrum (Figure 4.23) where the protonated molecular ion peak was found discerning at *m/z* 227 $[M+H]^+$. The fragmentation pattern witnessed **PA-4** to be made up of hexahydropyrrolo-pyrazine-dione nucleus (*m/z* 154, 136, 107). Although the mass spectrum of **PA-4** displayed similar pattern as that of **PA-1** to **PA-3**, the proton and carbon NMR spectra were found to be dissimilar.



(Detection at 214, 250, 280, 300, 320 and 350 nm)

Figure 4.21: The HPLC chromatogram of PA-4

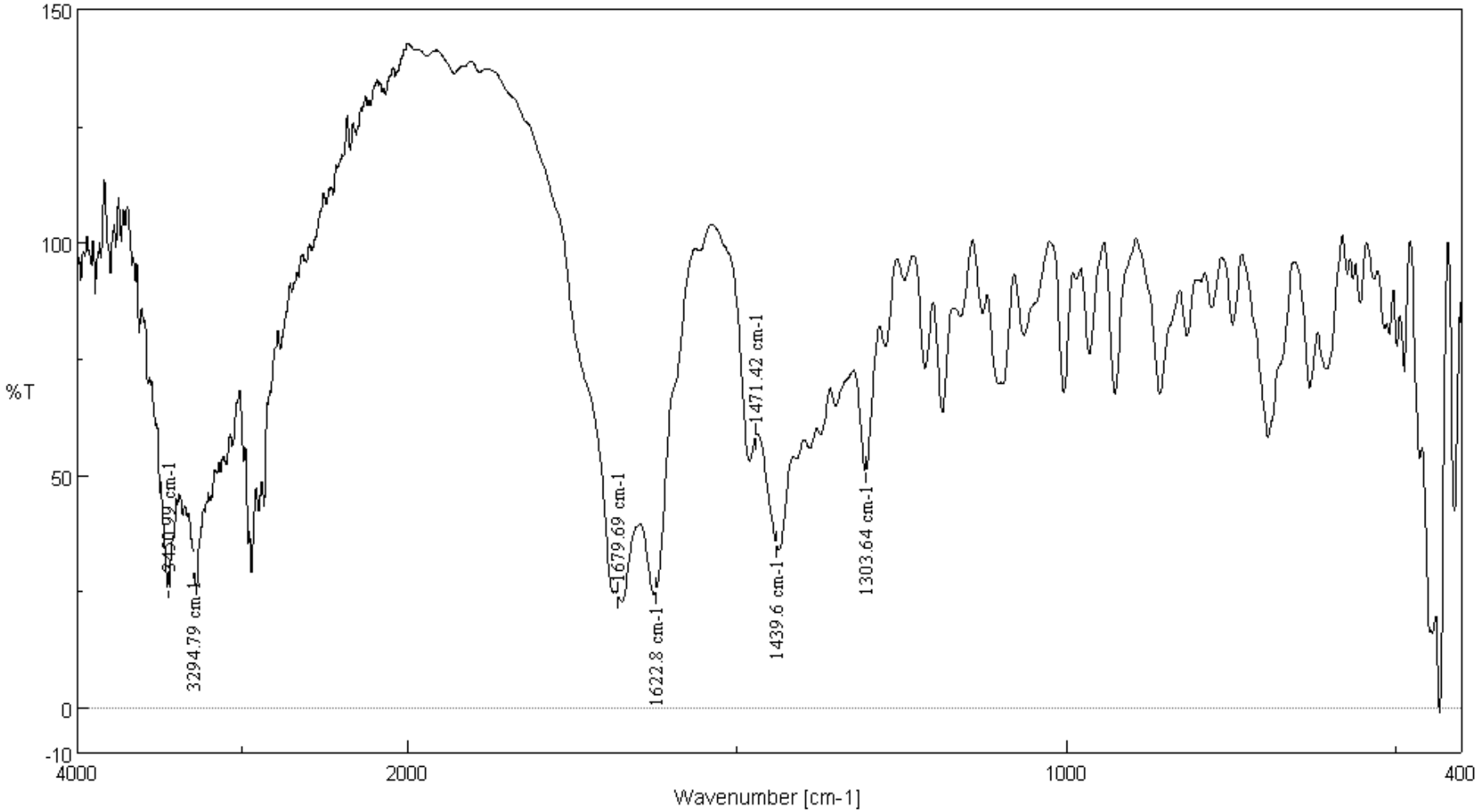


Figure 4.22: IR spectrum of PA-4

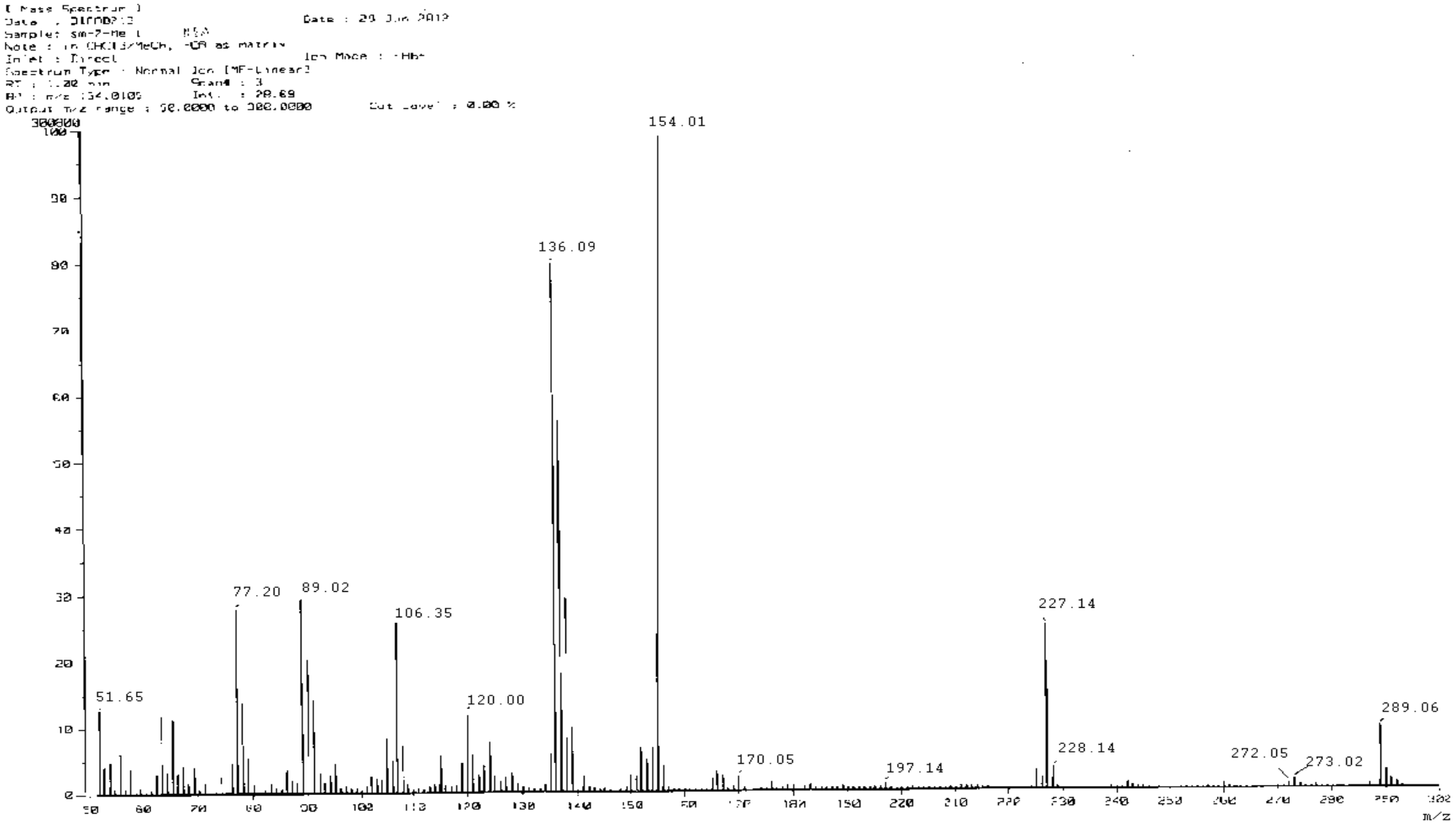
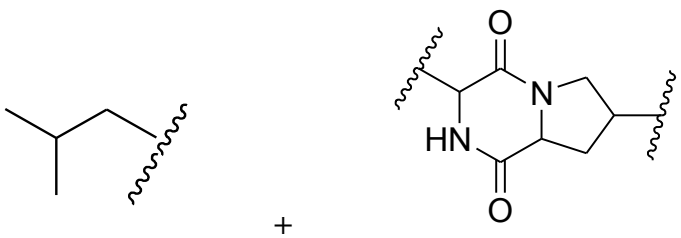


Figure 4.23: FAB mass spectrum of PA-4

The 125 MH_Z ^{13}C NMR (Figure 4.24) spectrum measured using CD_3OD displayed eleven carbon signals, which included two amide carbonyls (δ_C 171.6 and 167.6 ppm), three methylene carbons (δ_C 53.7, 67.0 and 36.7 ppm), four methine carbons (δ_C 57.3, 53.2, 37.9 and 20.8 ppm) along with two methyl carbons (δ_C 24.5 and 24.1 ppm). The ^1H NMR spectrum (Figure 4.25) showed two doublets of close chemical shift values along with a one proton septet (δ_H 4.51 ppm) and two protons multiplet (δ_H 2.27 and 2.08 ppm) revealing the presence of isobutyl group in **PA-4**. Further the signals due to methylene protons of proline part in the PMR spectrum was different than **PA-1** and **PA-2**, however it was similar to **PA-3**, suggesting the presence of a substitution at Pro-γ. The substituent as corroborated from the IR spectrum (*vide supra*) could be a hydroxyl group which was well decided from the difference in molecular weights (17 amu) of **PA-4** (MW 226) and derived part structure PA-4a (MW 209).



+

C_4H_9 (MW 57) $C_7H_8N_2O_2$ (MW 152)

PA-4a (MW 209)

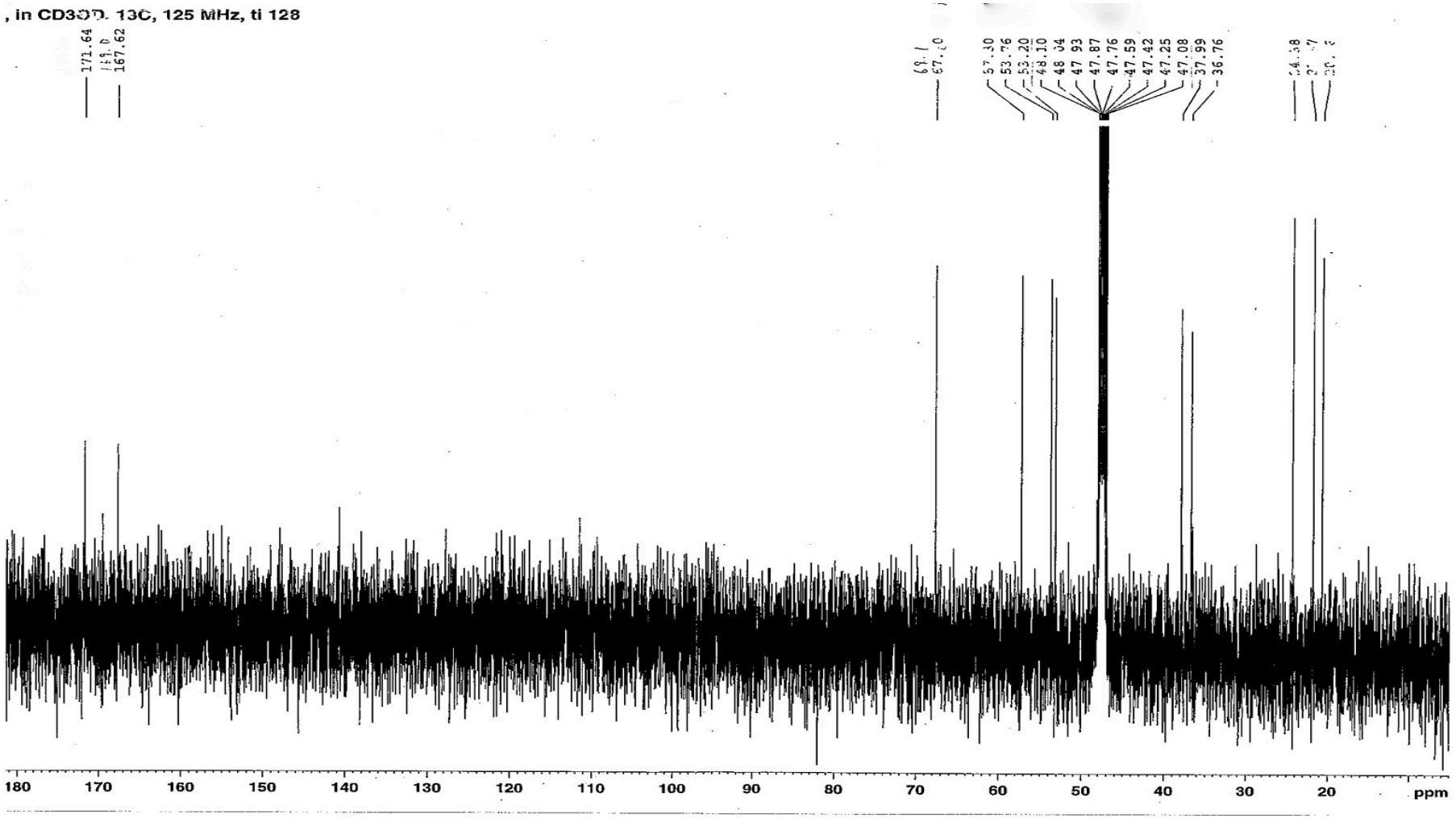


Figure 4.24: 125 MHz ^{13}C NMR spectrum of PA-4

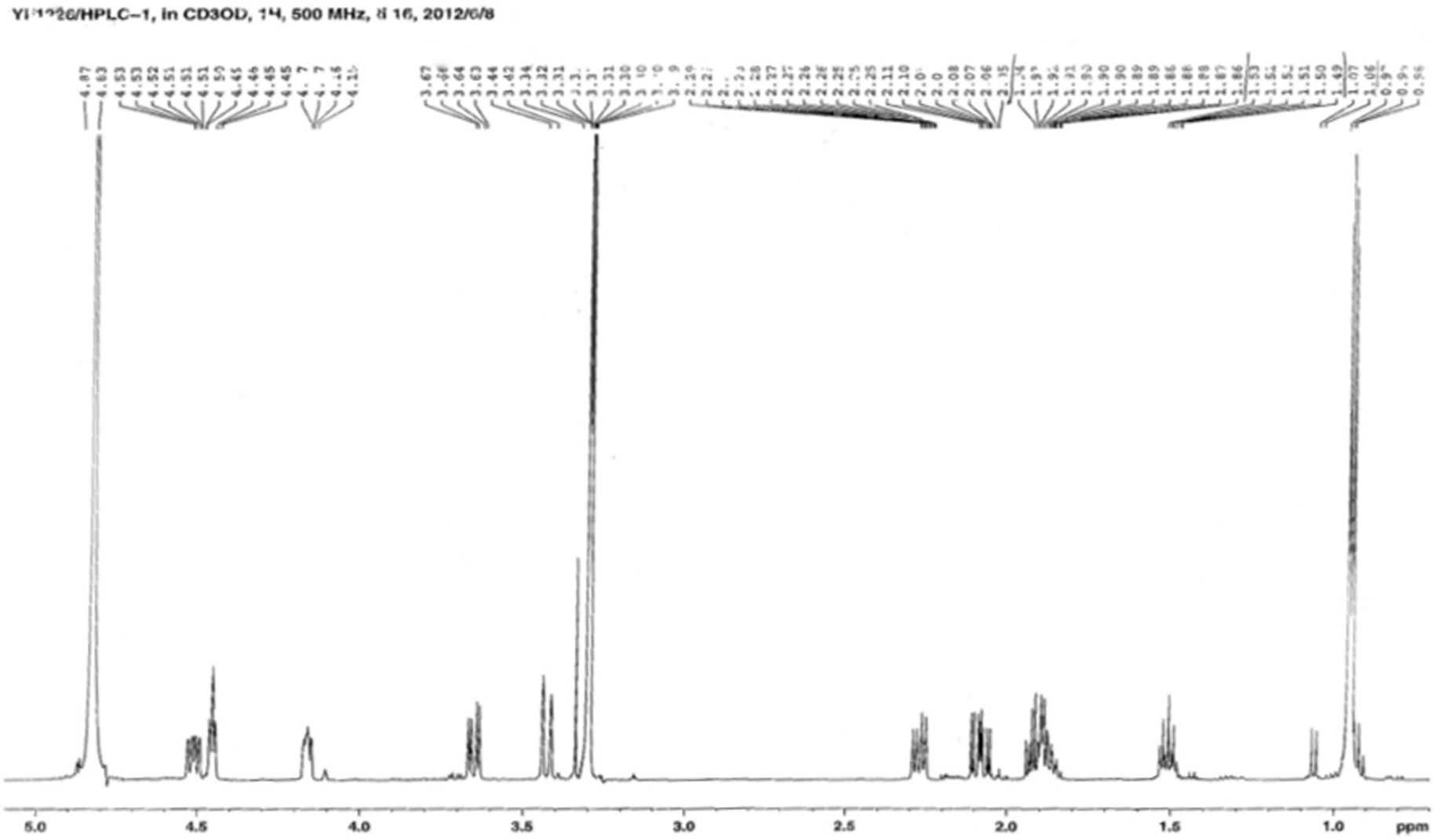


Figure 4.25: 500 MHz ^{1}H NMR spectrum of PA-4

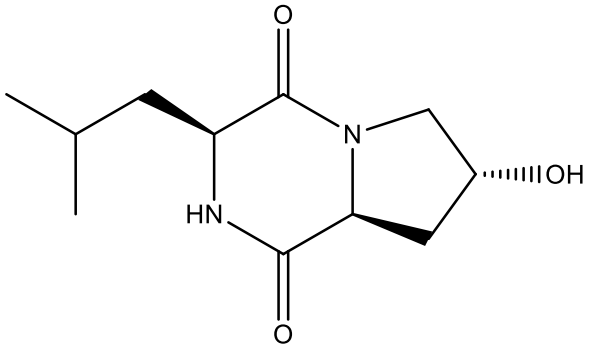
Finally the exact position of substituents, isobutyl group and hydroxyl group at C-3 and C-7, respectively was confirmed by comparing the observed ^{1}H NMR data of **PA-4** with that of **PA-3** and reported values. **PA-4** was finally identified as (3*S*,7*R*,9*S*)-hexahydro-7-hydroxy-3-(2-methylpropyl)pyrrolo[1,2-a] pyrazine-1,4-dione (**4**). The stereochemistry at 3rd, 7th and 9th positions were unambiguously assigned by the comparison of $[\alpha]^{25}_{D}$ [-135.2 (*c* 0.71, MeOH)] carbon and proton NMR data of **PA-4** with cyclo(*trans*-4-OH-(L)-Pro-(L)-Leu) and cyclo(*cis*-4-OH-(D)-Pro-(L)-Leu) (Furtado *et al*., 2005) as shown in Table 4.4 and 4.5 respectively.

Table 4.4: 125 MHz ^{13}C NMR spectral data of PA-4

Position	δ_C recorded (CD_3OD)	cyclo(*trans*-4-OH-L-Pro-L-leu) (CD_3OD)	cyclo(*cis*-4-OH-L-Pro-L-leu) (CD_3OD)
1	171.6	171.9	169.8
2	-	-	-
3	53.2	54.0	56.2/56.1
4	167.6	167.8	167.7
5	-	-	-
6	53.8	56.0	54.0
7	67.7	69.0	68.1
8	36.8	37.0	36.8
9	57.3	58.0	56.2/56.1
10	38.0	38.0	42.2
11	24.4	25.0	24.6
12	20.9	21.0	21.5
13	22.0	22.1	23.0

Table 4.5: 500 MHz ^{1}H NMR spectral data of PA-4

Position	δ_H recorded (CD_3OD)	cyclo(*trans*-4-OH-L-Pro-L-leu) (CD_3OD)	cyclo(*cis*-4-OH-L-Pro-L-leu) (CD_3OD)
1	-	-	-
2	-	4.56 (1H, br s)	-
3	4.17-4.14 (1H, m)	4.18-4.13 (1H, m)	4.00 (1H, m)
4	-	-	-
5	-	-	-
6	3.65 (1H, dd, *J* = 4.5, 12.8 Hz), 3.43 (1H, d, *J* = 12.7 Hz)	3.65 (1H, dd, *J* = 4.2, 12.6 Hz), 3.43 (1H, dd, *J* = 0.5, 12.6 Hz)	3.65 (1H, dd, *J* = 12.3, 3.5 Hz), 3.49 (1H, dd, *J* = 12.3, 5.6 Hz)
7	4.45 (1H, t, *J* = 4.4 Hz)	4.45 (2H, brt, *J* = 4.2 Hz)	4.56 (1H, m)
8	2.27 (1H, dd, *J* = 6.5, 13.3 Hz), 2.08 (1H, ddd, *J* = 4.3, 11.2, 13.2 Hz)	2.27 (1H, ddd, *J* = 1.0, 6.5, 13.1 Hz), 2.07 (1H, ddd, *J* = 4.2, 11.1, 13.1 Hz)	2.53 (1H, m), 2.17 (1H, m)
9	4.51 (1H, ddd, *J* = 1.3, 6.6, 11.2 Hz)	4.51 (1H, ddd, *J* = 1.2, 6.5, 11.1 Hz)	4.46 (1H, dd, *J* = 8.3, 8.1 Hz)
10	1.94-1.85 (1H, m), 1.53-1.48 (1H, m)	1.94-1.83 (1H, m), 1.53-1.46 (1H, m)	1.70 (1H, m)
11	1.94-1.85 (1H, m)	1.94-1.85 (1H, m)	1.54 (1H, m)
12	0.95 (3H, d, *J* = 6.3 Hz)	0.95 (3H, d, *J* = 6.5 Hz)	0.94 (3H, d, *J* = 6.1)
13	0.95 (3H, d, *J* = 6.3 Hz)	0.96 (3H, d, *J* = 6.5 Hz)	0.91 (3H, d, *J* = 6.1 Hz)



(4)

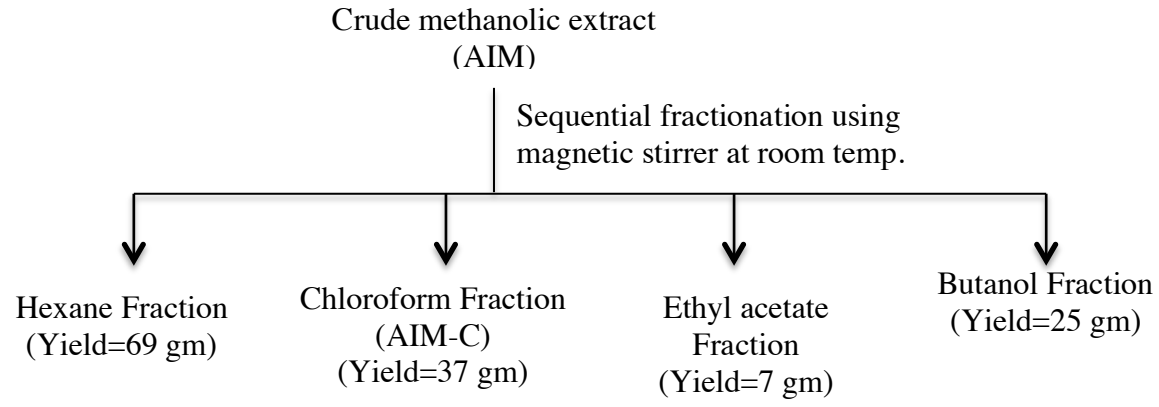
Existence of these cyclic dipeptides in the culture broth of *P. aeruginosa* is reported for the first time. Cyclo(D-Ala-L-Val) and cyclo(L-Pro-L-Tyr) are the compounds reported so far from *P. aeruginosa* (Holden *et al*., 1999). Dioxopiperazines like cyclo (D-Ala-L-Val) and cyclo (L-Pro-L-Tyr) had been reported as a new class of quorum sensing autoinducers in *Pseudomonas* strains (Ortiz-Castro *et al*., 2011; González *et al*., 2006; Degrassi *et al*., 2002). Besides, cyclo(L-Leu-L-Pro), cyclo(L-Phe-L-Pro), cyclo(L-Val-L-Pro), cyclo(L-Trp-L-Pro), and cyclo(L-Leu-L-Val) isolated from the deep-sea bacterium *Streptomyces fungicidicus* had been reported to exhibit antifouling effects (Li *et al*., 2006). Moreover, dioxopiperazines like cyclo(L-phenylalanyl-L-prolyl) had shown radio-protective effect on irradiated rat lung (Lee *et al*., 2008). These biologically effective 2,5-dioxopiperazines which are also known as diketopiperazines or cyclic dipeptides, are bio-synthesized in microorganisms by multifunctional assembly of nonribosomal peptide synthases and CDP synthases (Bonnefond *et al*., 2011; Lautru *et al*., 2002).

4.5. EXTRACTION AND LC-MS ANALYSIS OF *ABUTILON INDICUM*

Aerial parts of *A. indicum* (Family: Malvaceae) were extracted using petroleum ether and methanol sequentially in Soxhlet extractor. The methanolic extract (**AIM**) was concentrated under reduced pressure and fractionated using various organic solvents as shown in scheme 2. The residue obtained from $CHCl_3$ soluble fraction (**AIM-C**) was subjected for further chemical investigation as it was identified to possess interesting phenolic compounds on the basis of TLC studies.

AIM-C was subjected for LC-MS analysis, which documented the presence of 11 peaks, signaling small molecules having molecular weight in the range of 220 - 438 (Figure 4.26).

Scheme 2



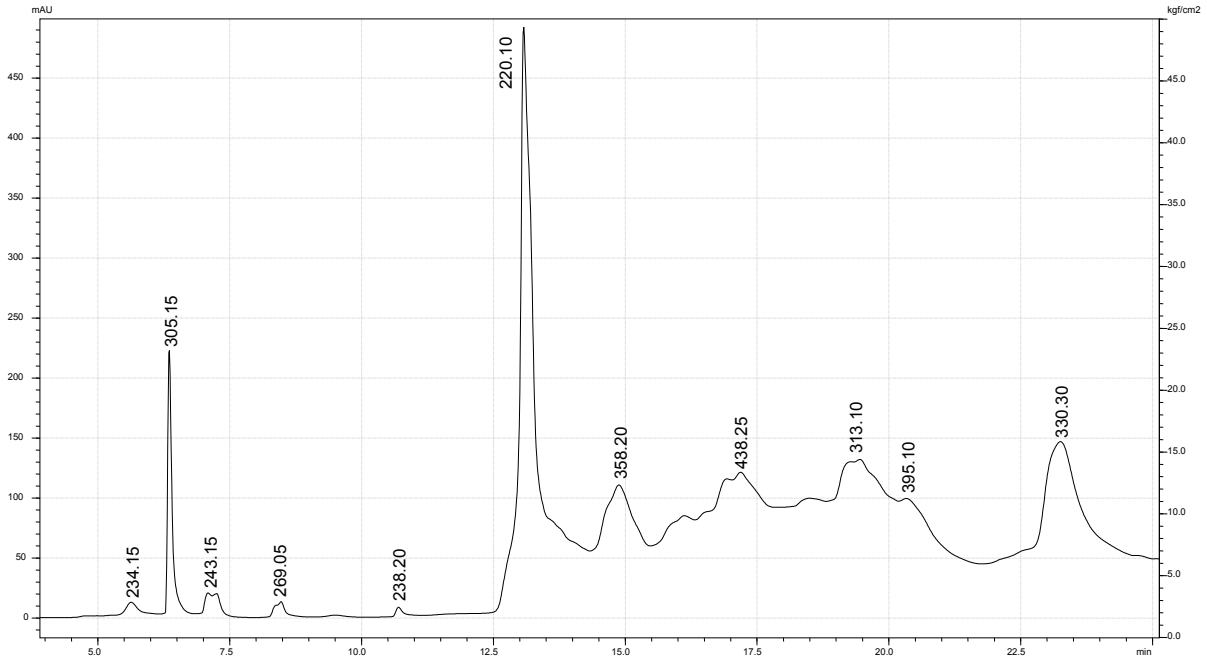
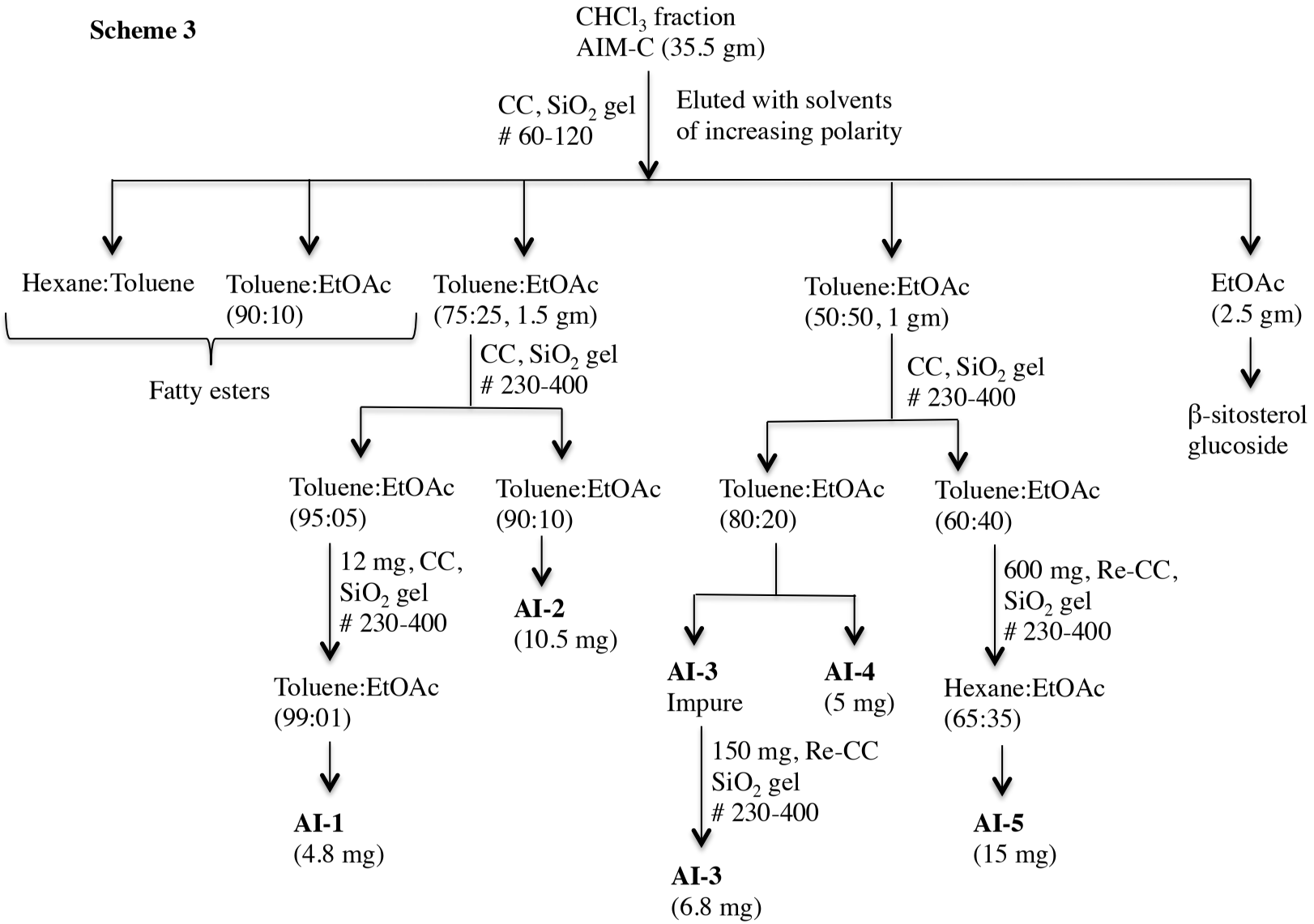


Figure 4.26: LC-MS fingerprint of AIM-C

4.6. ISOLATION OF CHEMICAL CONSTITUENTS OF *ABUTILON INDICUM*

The residue obtained from **AIM-C** was divided into two parts. One part (1.5 gm) was preserved for pharmacological screening and the second part (35.5 gm) was purified by column chromatography over silica gel and eluting with solvents of increasing polarity. Repeated column chromatography yielded five compounds designated as **AI-1**, **AI-2**, **AI-3**, **AI-4** and **AI-5**. The isolation procedure of these compounds is outlined in scheme 3.

Scheme 3


CHCl3 fraction
AIM-C (35.5 gm)
CC, SiO2 gel
60-120
Eluted with solvents
of increasing polarity
Hexane:Toluene
Toluene:EtOAc
(90:10)
Fatty esters
Toluene:EtOAc
(75:25, 1.5 gm)
CC, SiO2 gel
230-400
Toluene:EtOAc
(95:05)
12 mg, CC,
SiO2 gel
230-400
Toluene:EtOAc
(99:01)
AI-1
(4.8 mg)
Toluene:EtOAc
(90:10)
AI-2
(10.5 mg)
Toluene:EtOAc
(50:50, 1 gm)
CC, SiO2 gel
230-400
Toluene:EtOAc
(80:20)
AI-3
Impure
AI-4
(5 mg)
150 mg, Re-CC
SiO2 gel
230-400
AI-3
(6.8 mg)
Toluene:EtOAc
(60:40)
600 mg, Re-CC,
SiO2 gel
230-400
Hexane:EtOAc
(65:35)
AI-5
(15 mg)
EtOAc
(2.5 gm)
β-sitosterol
glucoside

4.7. CHARACTERIZATION OF ISOLATED CONSTITUENTS OF *ABUTILON INDICUM*

4.7.1. Characterization of AI-1

AI-1 was isolated as white amorphous solid, showing melting point of 138-140 °C. It was found to be freely soluble in MeOH, $CHCl_3$-MeOH mixture and benzene, but insoluble in n-hexane. The molecular formula of **AI-1** was settled as $C_{10}H_{10}O_3$ based on ESI mass spectrometrically derived molecular $[M-H]^-$ peak at *m/z* 177 as shown in Figure 4.27 and collected NMR data. The purity of **AI-1** was confirmed by TLC studies under different solvent systems [(R_f = 0.72 (Benzene:EtOAc, 1:1) and 0.54 (Hexane:EtOAc, 2:8)] and heating the developed plates sprayed with 10% methanolic H_2SO_4. The phenolic nature of **AI-1** was confirmed through purple colour change when the TLC spot was sprayed with $FeCl_3$ solution.

The IR spectrum of **AI-1** measured using DRA technique expounded absorption bands spread in the high frequency region at 3372 cm^{-1} for Ar-OH, 1685 cm^{-1} for conjugated ester carbonyl and 1590, 1517 and 1439 cm^{-1} for aromatic functionalities (Figure 4.28). The 500 MHz ^1H NMR spectrum of **AI-1** was measured by dissolving it in $CDCl_3$ (Figure 4.29). The structural information secured from the interpretation of the proton NMR spectrum is given in Table 4.6.

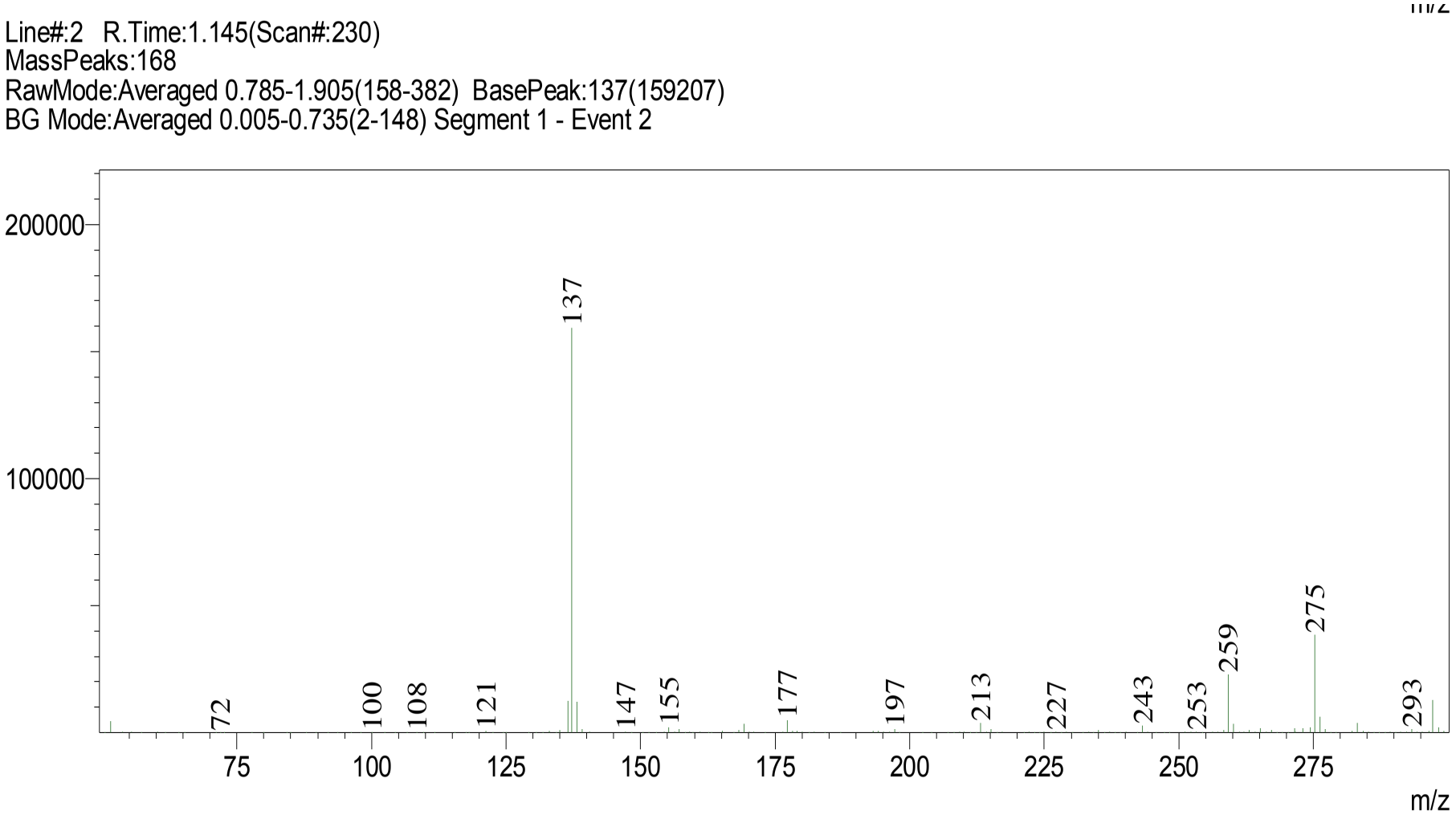


Figure 4.27: ESI mass spectrum of AI-1

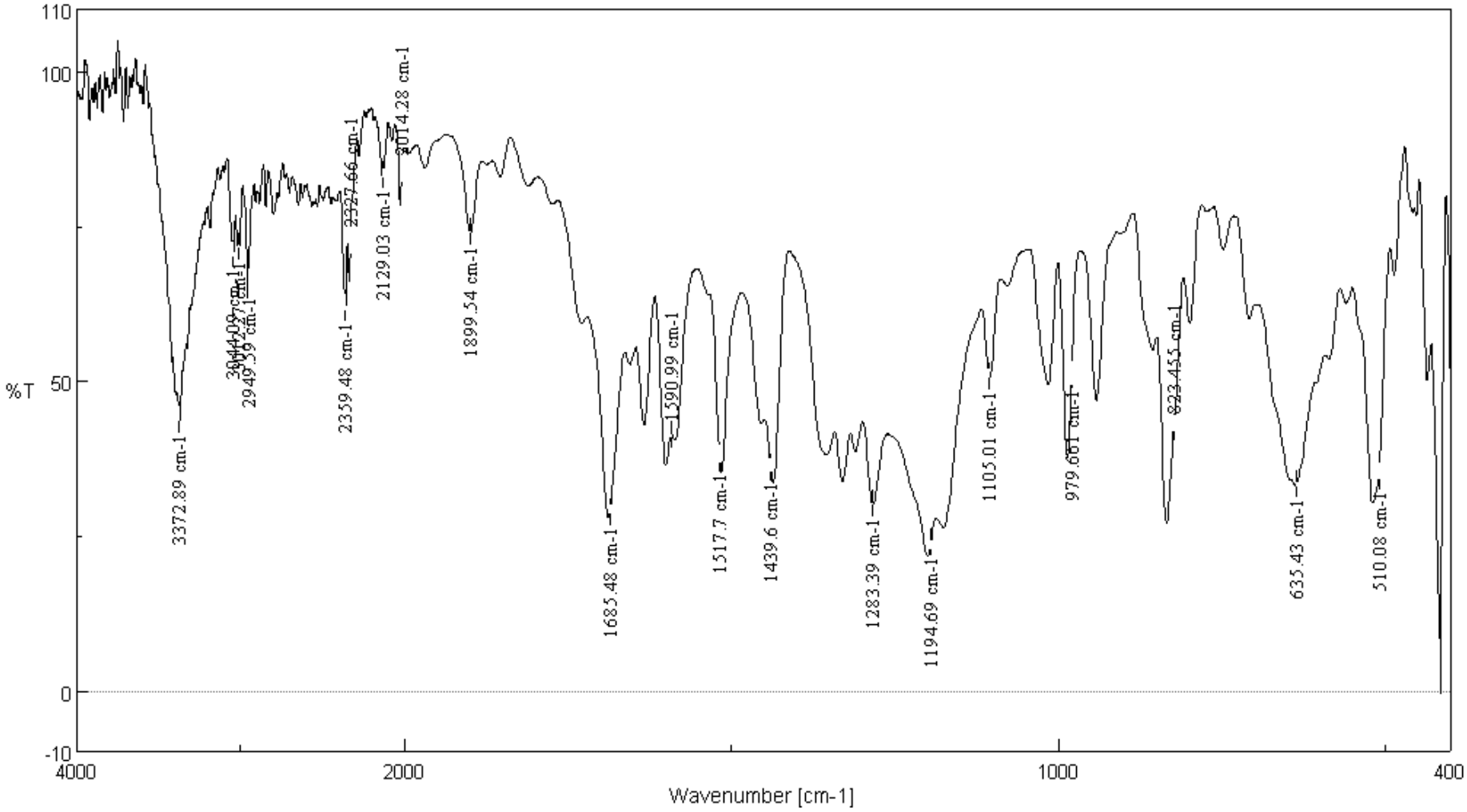


Figure 4.28: IR spectrum of AI-1

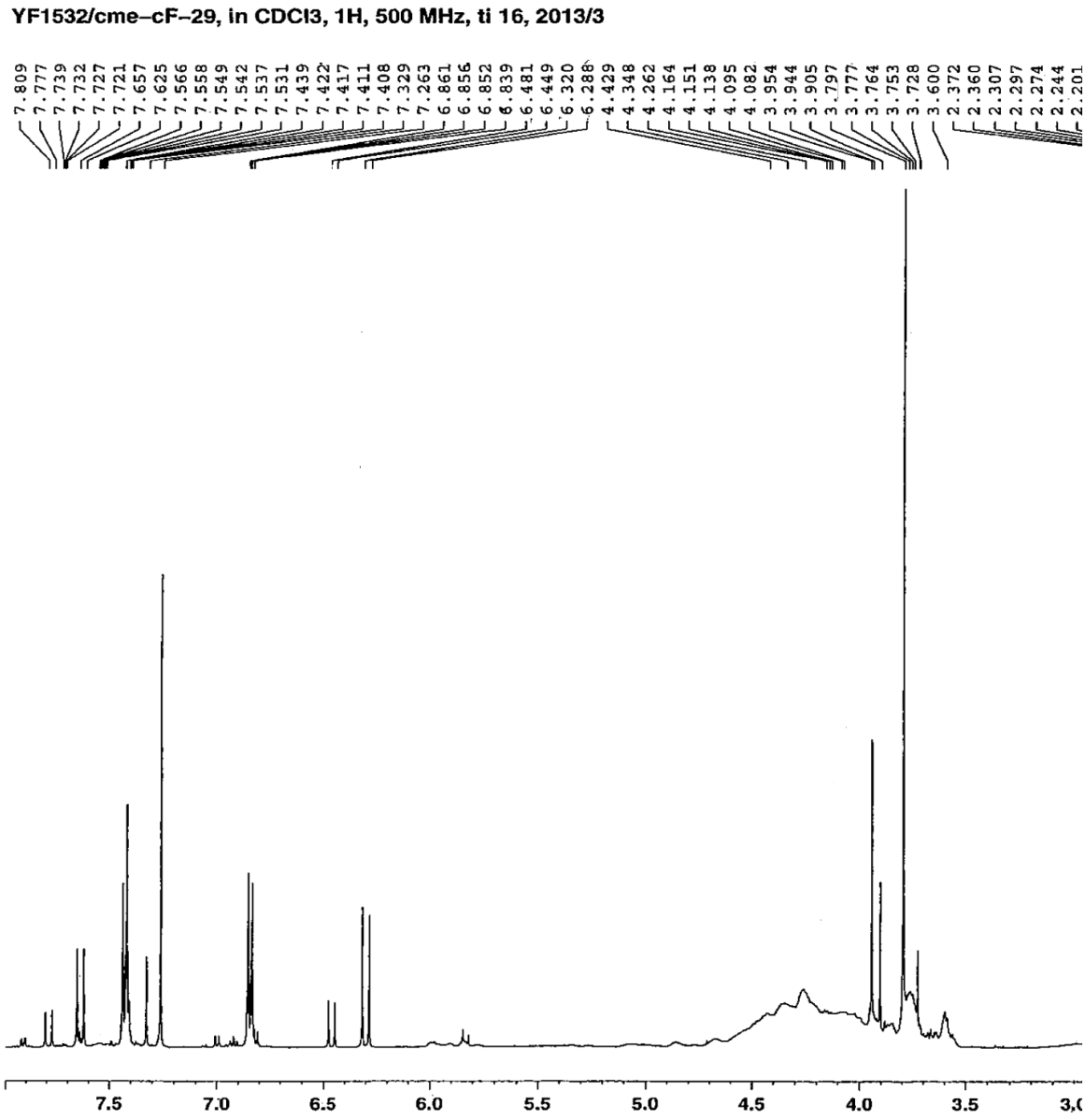


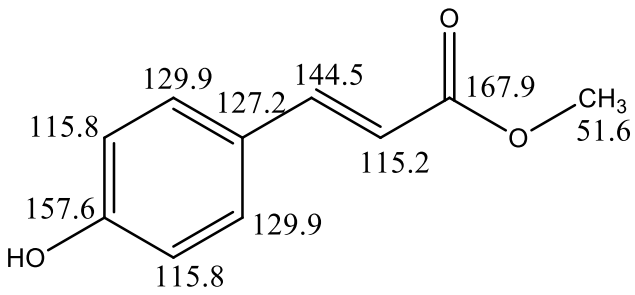
Figure 4.29: 500 MHz ^{1}H NMR spectrum of AI-1

Table 4.6: 500 MHz ^{1}H NMR spectral data of AI-1

Chemical Shift (δ ppm)	Integral Proton count	Splitting pattern (*J* Hz)	Probable assignment
7.64	1H	d (16.0)	-CH-
7.43	2H	d (8.5)	-CH-
6.86	2H	d (8.5)	-CH-
6.30	1H	d (16.0)	-CH-
3.78	3H	s	-O-CH_3

The ^{1}H NMR spectrum showed the presence of methoxyl proton at δ_H 3.78 ppm as singlet. The downfield region of the spectrum exhibited signals for aromatic protons of A_2B_2 type at δ_H 6.86 and 7.43 ppm as doublets for four protons. These two sets of protons showed mutual coupling demonstrating same coupling constant (J = 8.5 Hz). In view of these observations, presence of 1,4-disubstituted aromatic nucleus in **AI-1** having hydroxyl group was confirmed.

The remaining signals (δ_H 6.30 and 7.64 ppm) in the downfield region of the spectrum were identified to be due to olefinic protons adjacent to carbonyl i.e. of α, β-unsaturated protons. This information helped us to assume **AI-1** to be phenolic compound substituted with an α, β-unsaturated methyl ester carbonyl group at its *p*-position. This assumption gained support from ^{13}C NMR spectrum (Figure 4.30). The signal at δ_C 167.9 ppm confirmed the presence of ester carbonyl and its corresponding methoxyl signal was found to be discerned at δ_C 51.6 ppm. The signals for olefinic carbons were found at δ_C 144.5 and δ_C 115.2 ppm. The remaining aromatic carbons showed signals at δ_C 129.9, 127.2, 115.8 and 157.6 ppm.



AI-1

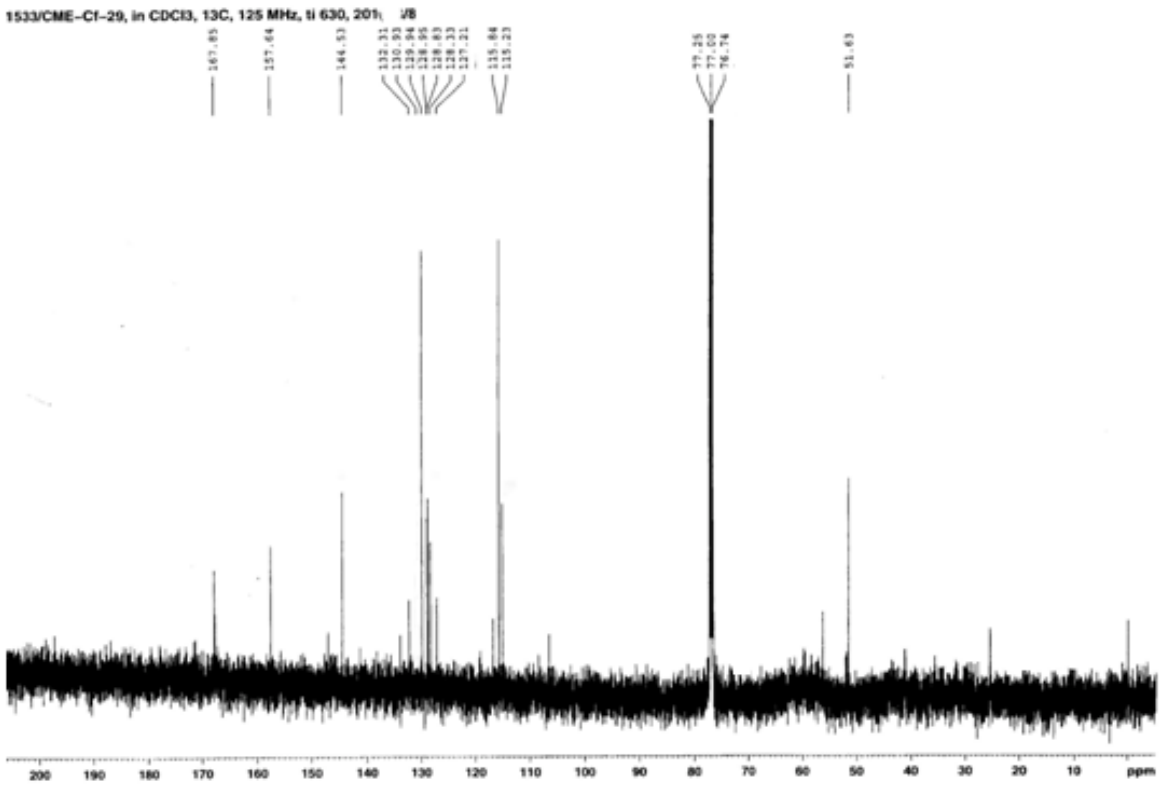
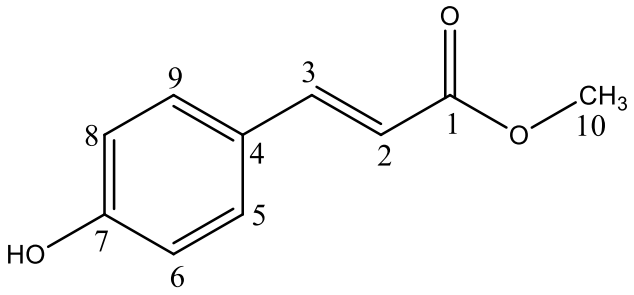


Figure 4.30: 125 MHz ^{13}C NMR spectrum of AI-1

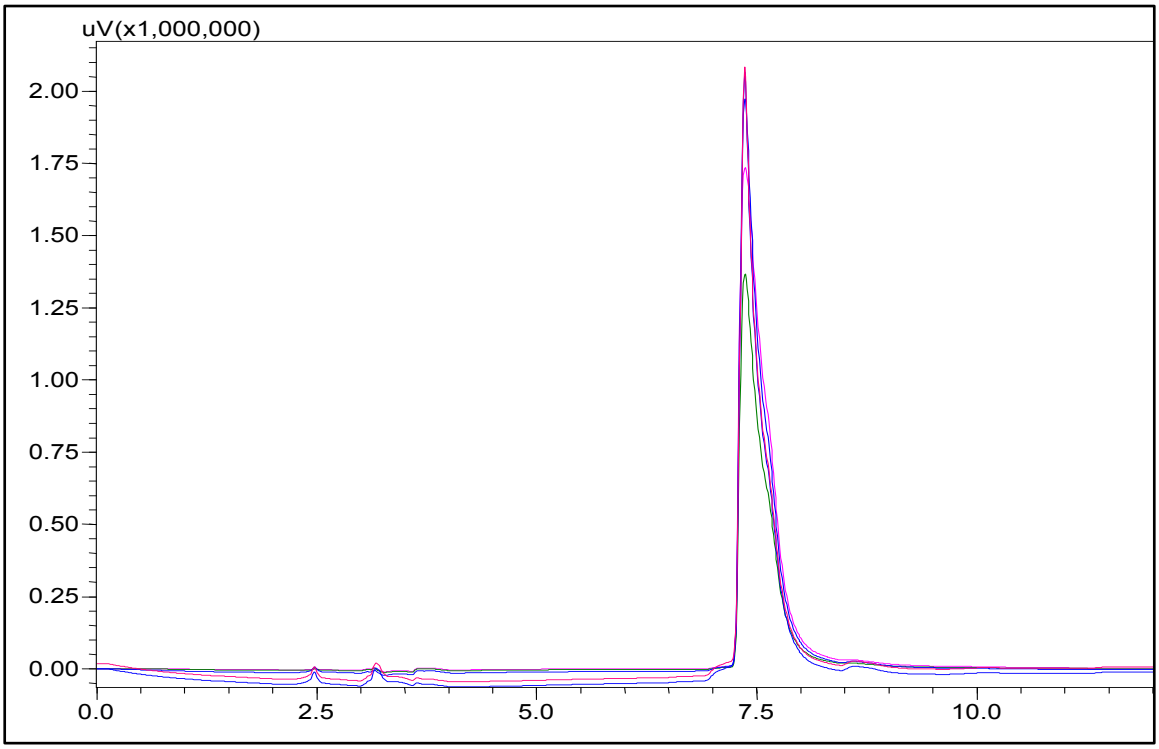
Based on the above gathered structural information, **AI-1** was determined as *p*-hydroxy cinnamic acid methyl ester (4-coumaric acid methyl ester). The relationship between the protons at C-2 and C-3 was finally identified as *trans* from the higher coupling constant value of 16 Hz. **AI-1** was finally identified as methyl *trans-p*-coumarate (**5**) (Chiang *et al*., 2003).



5

4.7.2. Characterization of AI-2

AI-2 was obtained as colourless crystals, showing melting point of 158-161 °C. It was found to be freely soluble in MeOH, $CHCl_3$-MeOH mixture and insoluble in n-hexane. The homogenous nature of **AI-2** was clarified by TLC using different solvent systems [Benzene:EtOAc (1:1), R_f 0.47 and hexane:EtOAc (2:8), R_f 0.54]. Also, the compound exhibited single peak chromatogram at R_t 7.39 min under HPLC analysis using reverse phase C_{18} column and detection at 200, 220, 254, 280 and 300 nm (Figure 4.31).



(Detection at 200, 220, 254, 280 and 300 nm)

Figure 4.31: The HPLC chromatogram of AI-2

The molecular formula of **AI-2** was settled as $C_{10}H_{10}O_4$ and it was further corroborated by the ESI mass spectrometrically derived molecular ion peak $[M-H]^-$ at *m/z* 193 (Figure 4.32). **AI-2** was identified to be aromatic (1617, 1529, 1446 cm^{-1}) having -OH group (3471 cm^{-1}) and conjugated ester carbonyl group (1684 cm^{-1}) through IR spectroscopy (Figure 4.33).

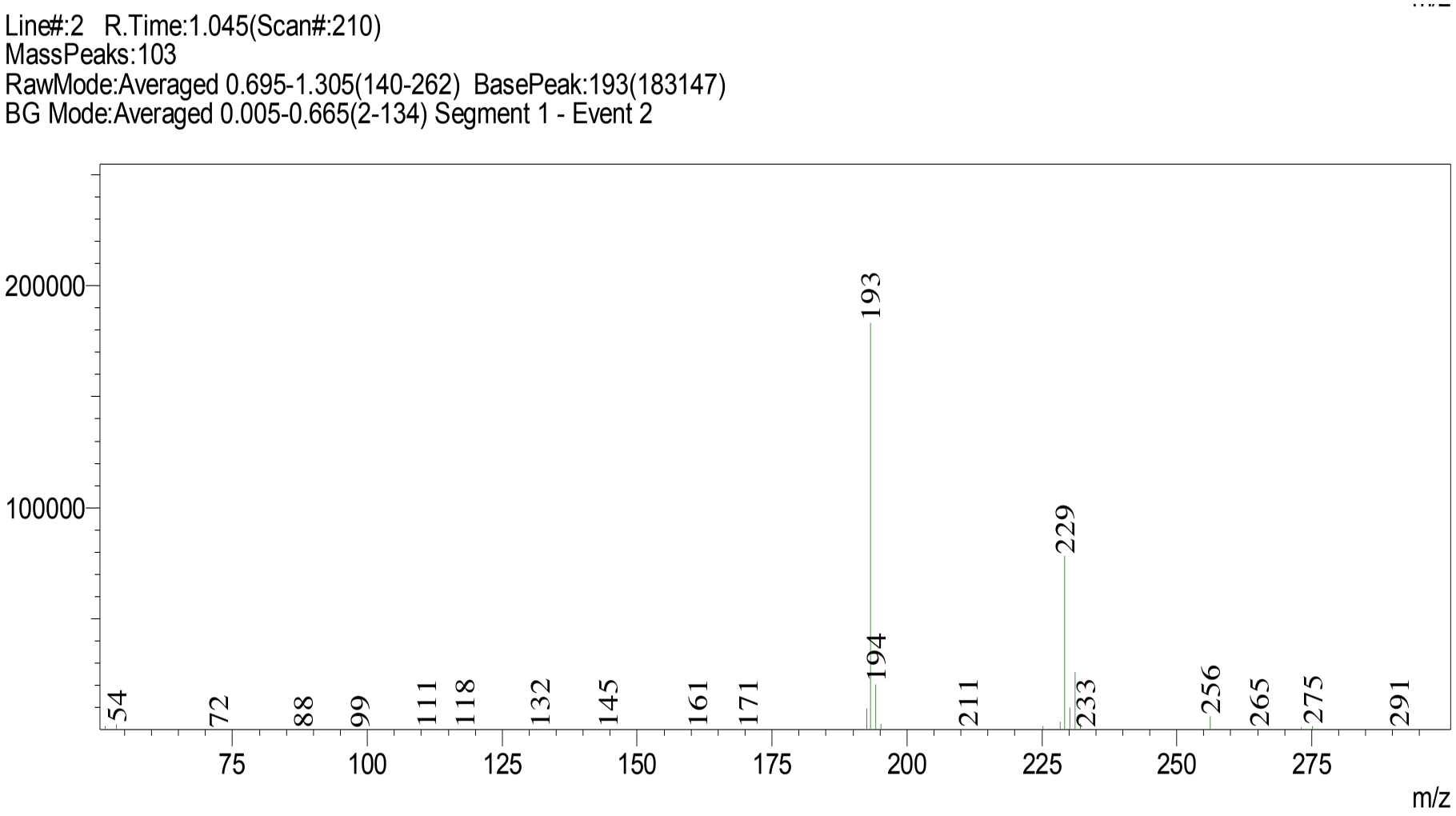


Figure 4.32: ESI mass spectrum of AI-2

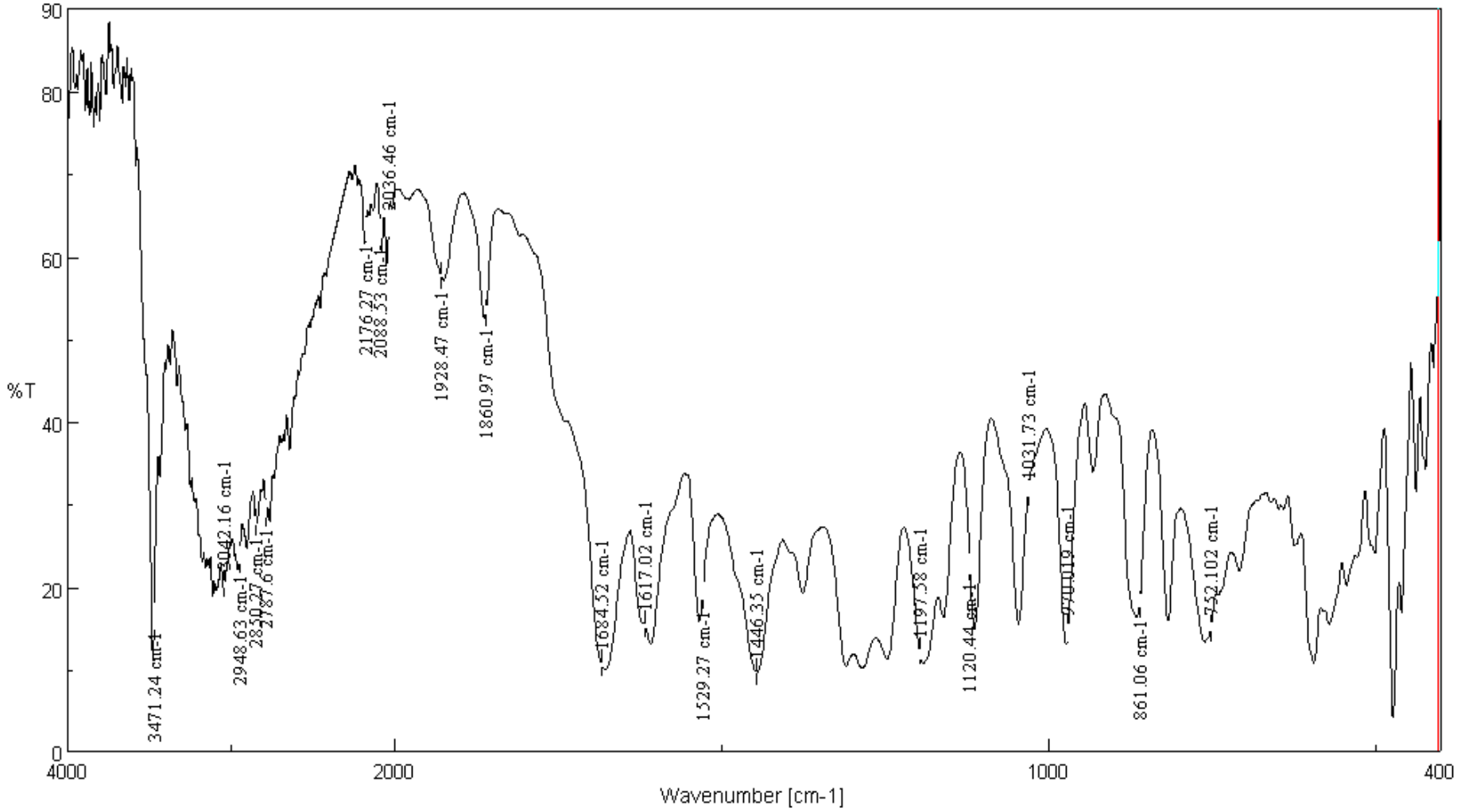


Figure 4.33: IR spectrum of AI-2

The ^{1}H NMR spectrum of **AI-2** (Figure 4.34) was measured at 500 MHz by dissolving in $CDCl_3$. The structural information obtained from the interpretation of the proton NMR spectrum is given in Table 4.7.

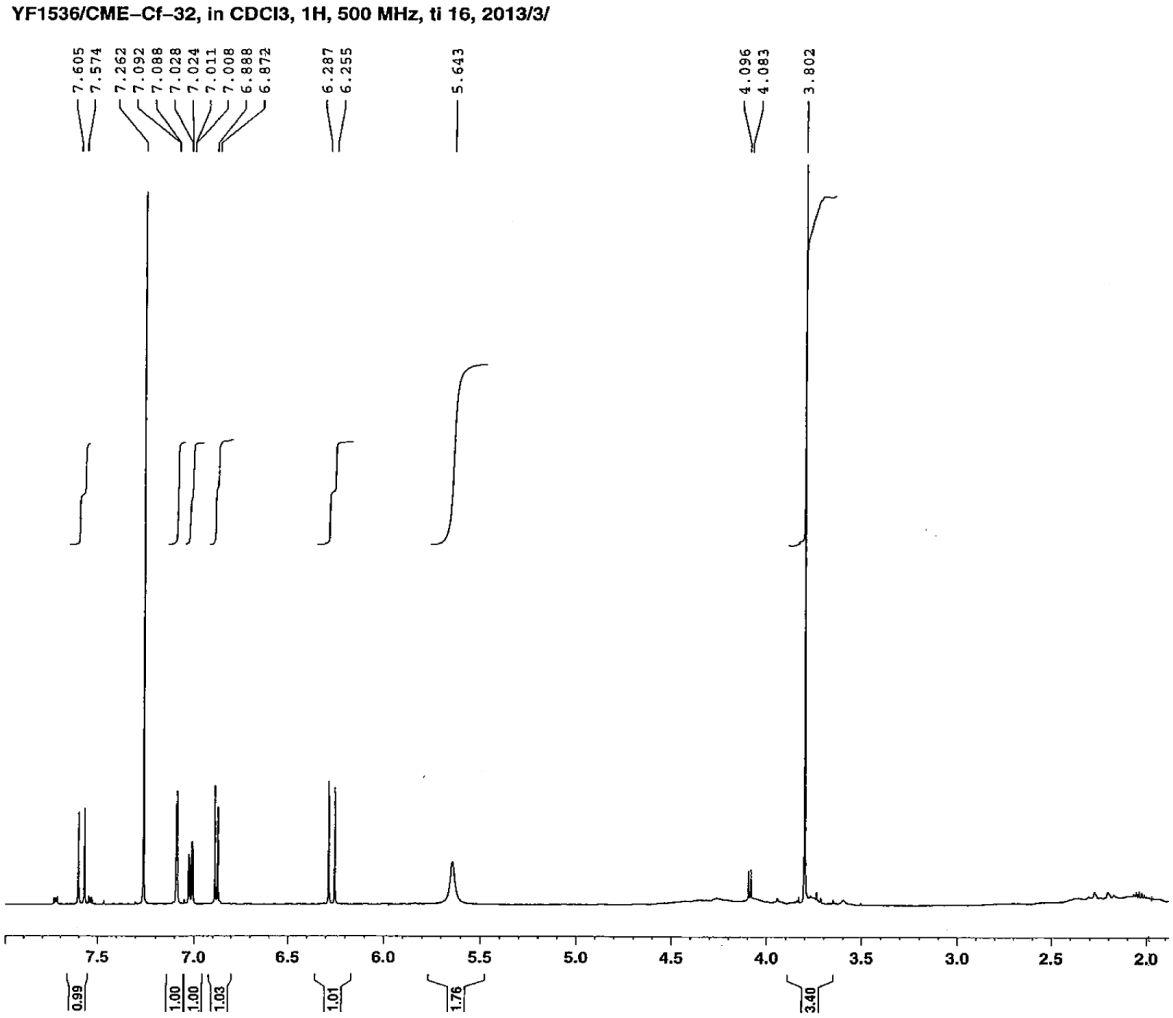


Figure 4.34: 500 MHz ^{1}H NMR spectrum of AI-2

Table 4.7: 500 MHz ^{1}H NMR spectral data of AI-2

Chemical Shift (δ ppm)	Integral Proton count	Splitting pattern (*J* Hz)	Probable assignment
7.59	1H	d (16.0)	-CH
7.09	1H	d (2.0)	-CH
7.02	1H	dd (2.0 & 8.5)	-CH
6.88	1H	d (8.0)	-CH
6.27	1H	d (16.0)	-CH
5.64	2H	br s	-OH
3.80	3H	s	-O-CH_3

The PMR spectrum showed a broad singlet for two protons, might be accountable for exchangeable protons of hydroxyl group. The appearance of three proton singlet at δ_H 3.80 due to methoxyl group and the mutually coupled signals resonating at δ_H 7.59 and 6.27 ppm due to olefenic protons in *trans* relationship (J = 16.0 Hz) signified the presence of methyl propenoate fragment as found in **AI-2**. The downfield region of the spectrum clearly indicated the presence of aromatic ring of ABX substitution pattern. A doublet at δ 6.88 ppm showed an ortho coupling (J = 8.0 Hz) and the doublet at δ 7.09 ppm showed meta coupling (J = 2.0 Hz) and the double doublet at δ 7.02 ppm showed ortho and meta coupling (J = 2.0, 8.0 Hz). The above gathered information well explained that **AI-2** has a 1,3,4-trisubstituted benzene nucleus attached with methyl prop-2-enoate and two hydroxyl groups.

The ^{13}C NMR spectrum of **AI-2** (Figure 4.35) was consistent with the above structure interpretation of trisubstituted benzene derivative. The resonance signals at δ 169.7 and 51.9 ppm confirmed the presence of methoxyl ester group and signals at 146.9 and 116.4 ppm confirmed olefenic carbons of methyl prop-2-enoate substituent.

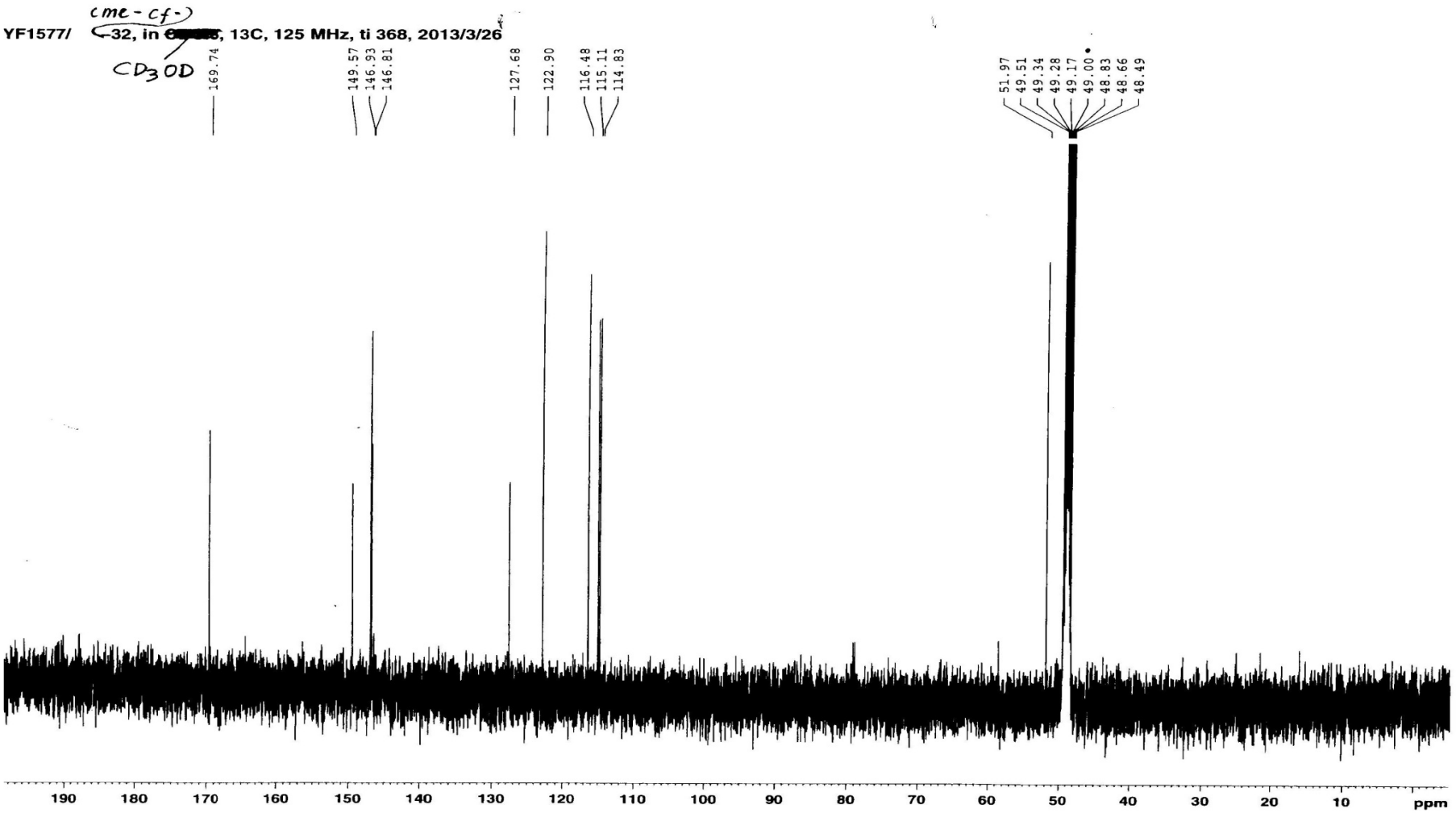
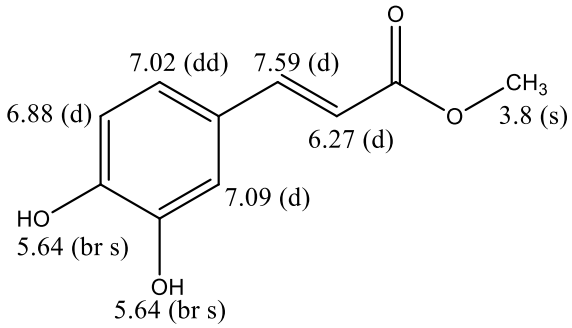
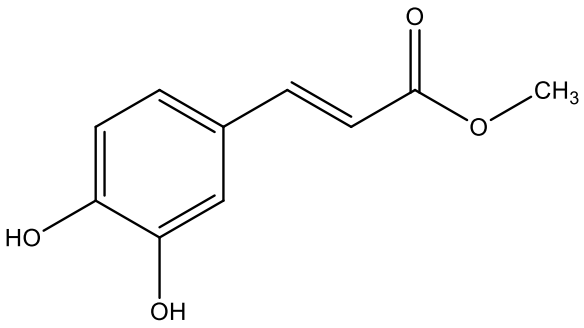


Figure 4.35: 125 MHz ^{13}C NMR spectrum of AI-2

Finally, **AI-2** was identified as (*E*)-methyl-3-(3,4-dihydroxyphenyl)acrylate or methyl caffeate (**6**). The observed spectral data were found to be in good agreement with reported data for caffeic acid methyl ester (Džubák *et al*., 2006).



AI-2



6

4.7.3. Characterization of AI-3

AI-3 was obtained as colourless crystals, showing m.p. of 205-209 °C. It was found to be freely soluble in MeOH, $CHCl_3$ and benzene. The purity of **AI-3** and its phenolic nature (positive result with $FeCl_3$ reagent) was identified through TLC studies using different solvent systems (Hexane:EtOAc, 7:3, R_f = 0.45 and $CHCl_3$:MeOH, 1:1, R_f = 0.55). The presence of broader absorption bands at 3042 – 2772 cm^{-1} (Ar-COOH), 3482 cm^{-1} (Ar-OH) and an intense band at 1679 cm^{-1} in the IR spectrum (Figure 4.36) of **AI-3** indicated the presence of aromatic nucleus having -OH and -COOH functional groups.

The molecular formula was determined as $C_9H_{10}O_5$ based on congregated spectral information, which was validated from $[M-H]^-$ peak observed at *m/z* 197 under ESI-MS analysis (Figure 4.37). The carbon NMR spectrum measured at 125 MHz (Figure 4.38) revealed three pairs of chemically and magnetically equivalent carbons [δ 56.8 (Ar-O**C**H_3), 108.4 (Ar-**C**H-) and 148.9 (Ar-**C**H-) ppm)] and three non-equivalent carbons [(δ 170.0 (Ar-COOH), 144.8 (Ar-quarternary carbon) and 122.0 (deshielded Ar-quarternary carbon) ppm] accounting for nine carbons of **AI-3**.

The proton NMR spectrum measured at 500 MHz (Figure 4.39) described **AI-3** to be a 1,3,4,5-tetrasubstituted compound having two methoxyls (δ 3.87 ppm), a carboxylic acid and a hydroxyl groups. The methoxyl groups were found to be positioned at C-3 and C-5 based on the equivalent chemical shift value of C-2 and C-6 protons. The possibility of attachment at C-2 and C-6 was ruled out based on the appearance of highly deshielded δ value of aromatic protons (7.32 ppm). Finally, the compound was identified as 4-hydroxy-3,5-dimethoxy benzoic acid (syringic acid) (**7**), which was unambiguously confirmed by comparison of the observed NMR data with that of reported data (Sidana *et al*., 2013).

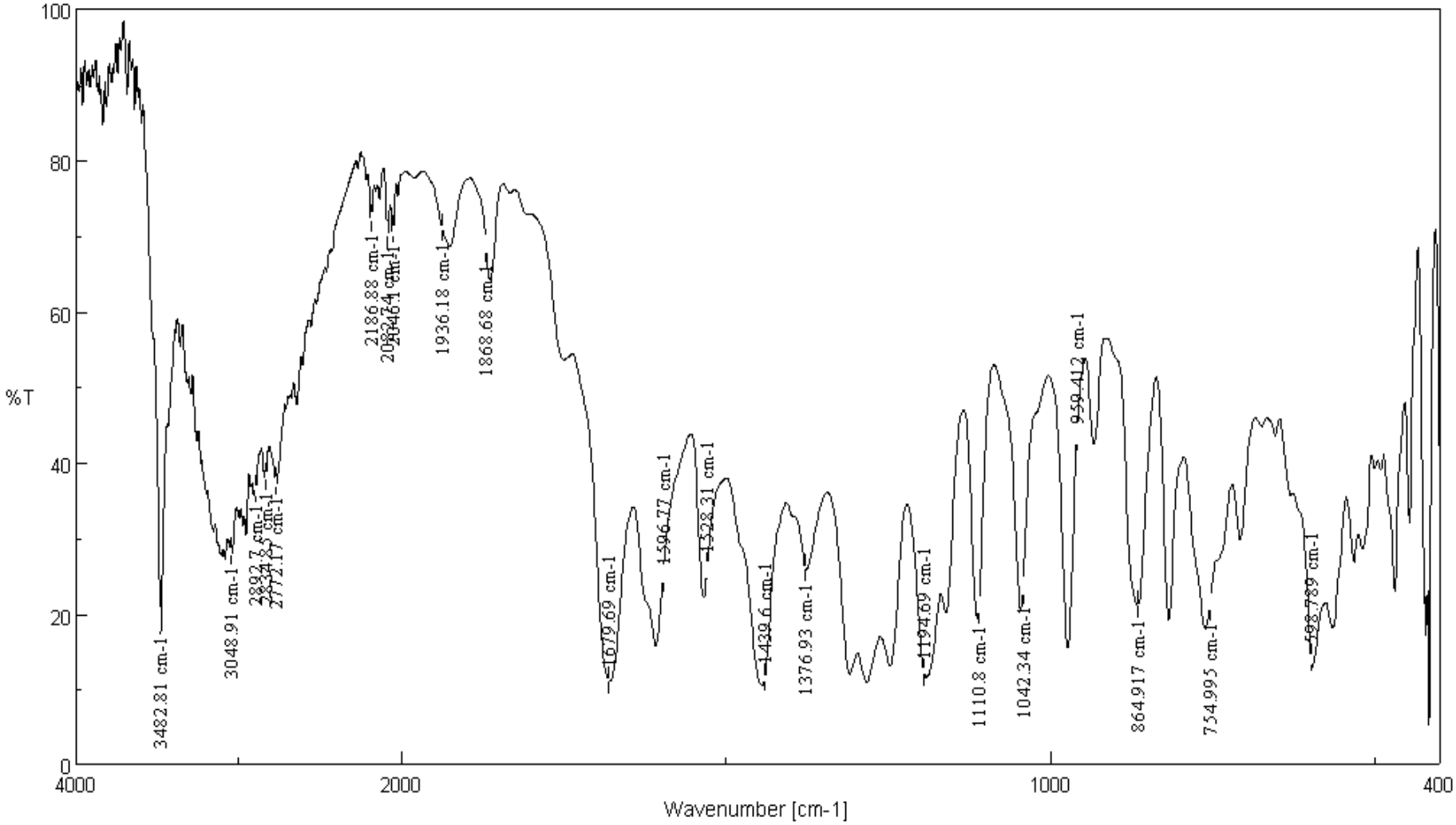


Figure 4.36: IR spectrum of AI-3

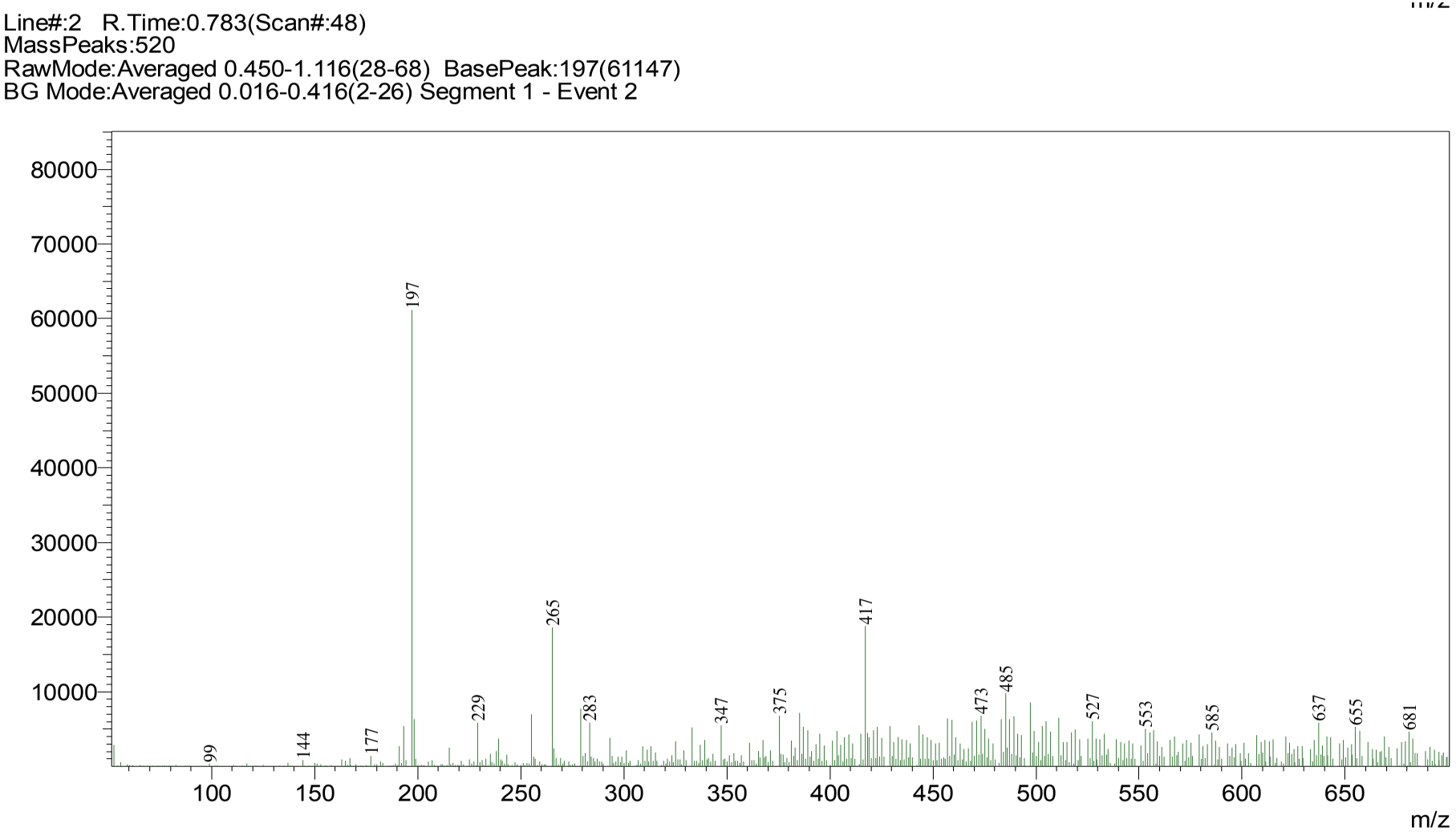


Figure 4.37: ESI mass spectrum of AI-3

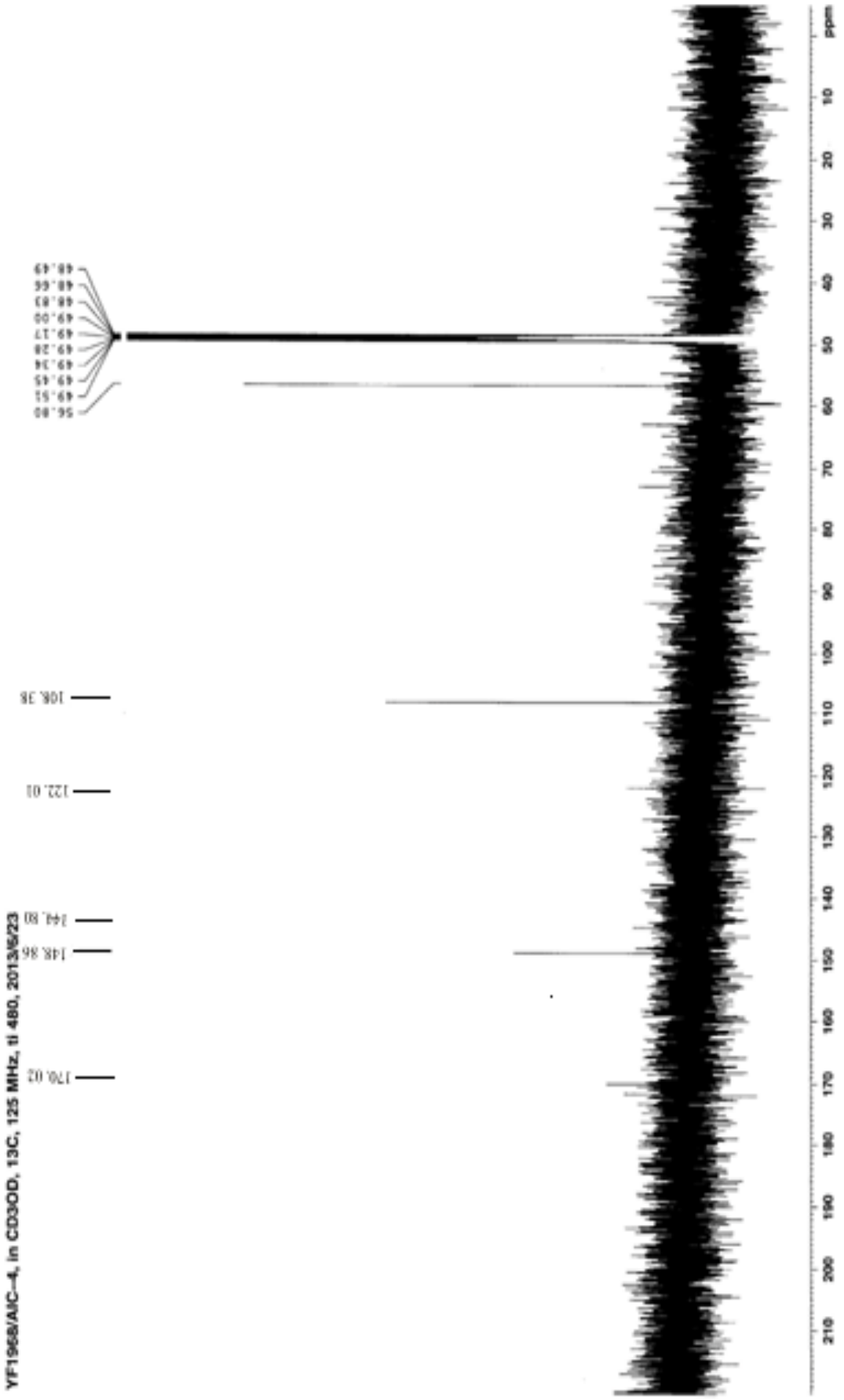


Figure 4.38: 125 MHz ^{13}C NMR Spectrum of AI-3

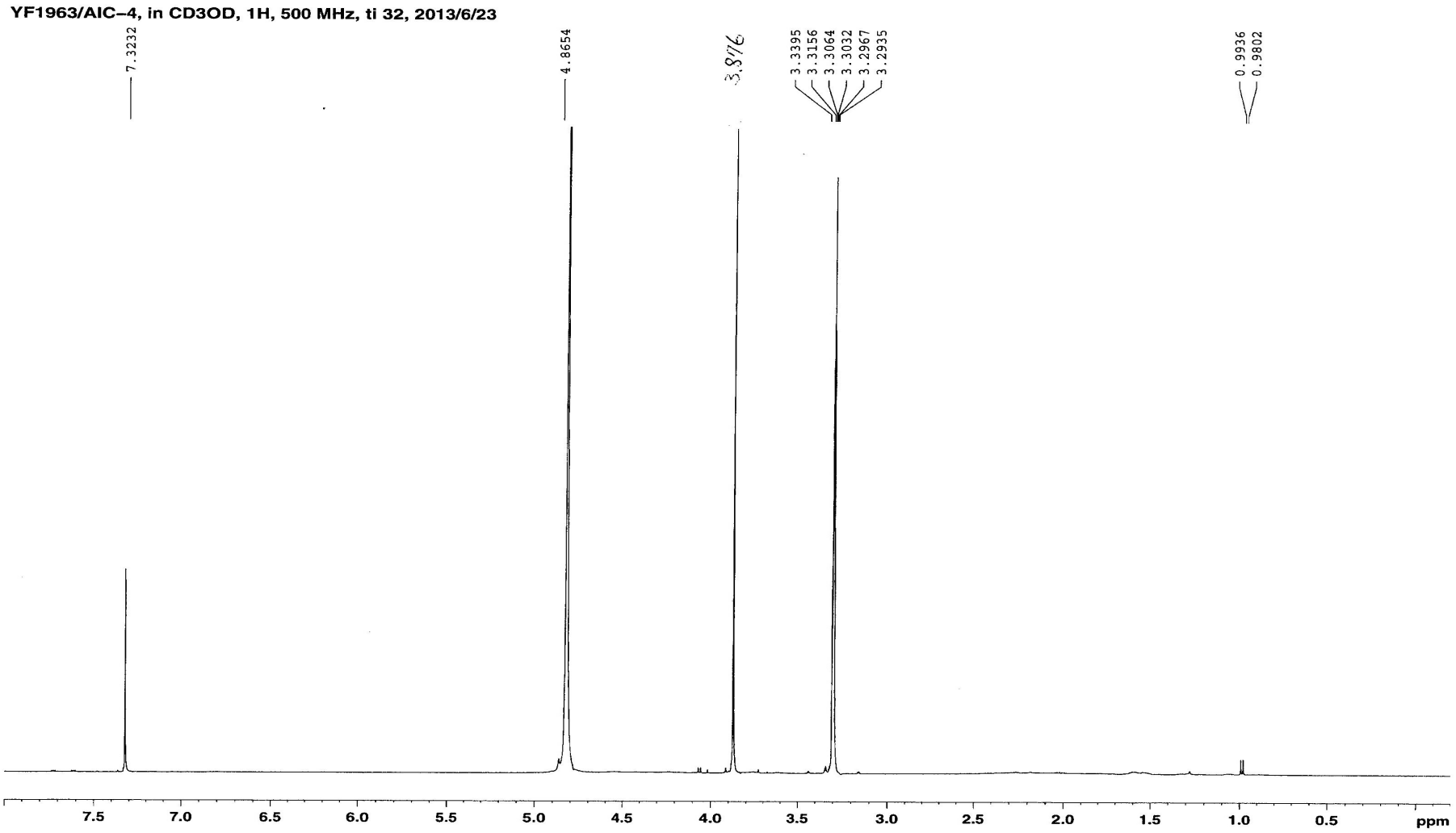
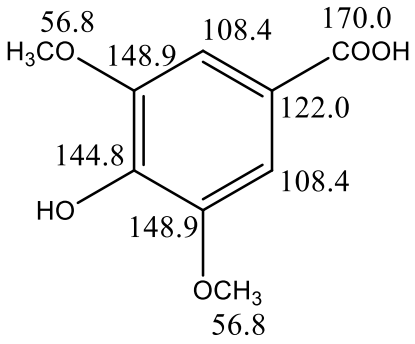
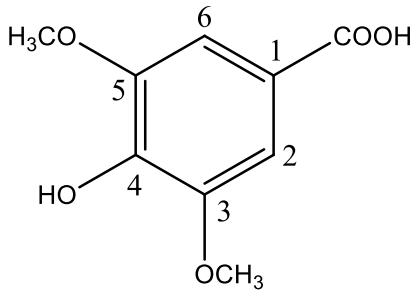


Figure 4.39: 500 MHz ^{1}H NMR spectrum of AI-3


56.8
H3CO
148.9
108.4
170.0
COOH
122.0
144.8
108.4
HO
148.9
OCH3
56.8

AI-3


6
H3CO
1
COOH
5
2
HO
4
3
OCH3

7

4.7.4. Characterization of AI-4

AI-4 was isolated as white amorphous powder showing melting point of 210-213 °C. It was found to be freely soluble in MeOH, $CHCl_3$- MeOH mixture and insoluble in hexane. The homogenous nature of **AI-4** was demonstrated by TLC studies using $CHCl_3$:MeOH (4:6), R_f 0.54 and Toluene:EtOAc (1:1), R_f 0.34.

The molecular formula of **AI-4** was settled as $C_8H_8O_4$ on the basis of results of NMR spectra and it was further corroborated by the ESI-mass spectrophotometrically derived molecular ion peak at m/z 167 $[M-H]^-$ (Figure 4.40). The IR spectrum (Figure 4.41) showed absorption bands for aromatic -OH (3487 cm^{-1}) and -COOH (3050-2900 cm^{-1}) functional groups. The presence of -COOH group was explicitly confirmed from the existence of resonance signal at δ 168.5 ppm in the ^{13}C NMR spectrum (Figure 4.42).

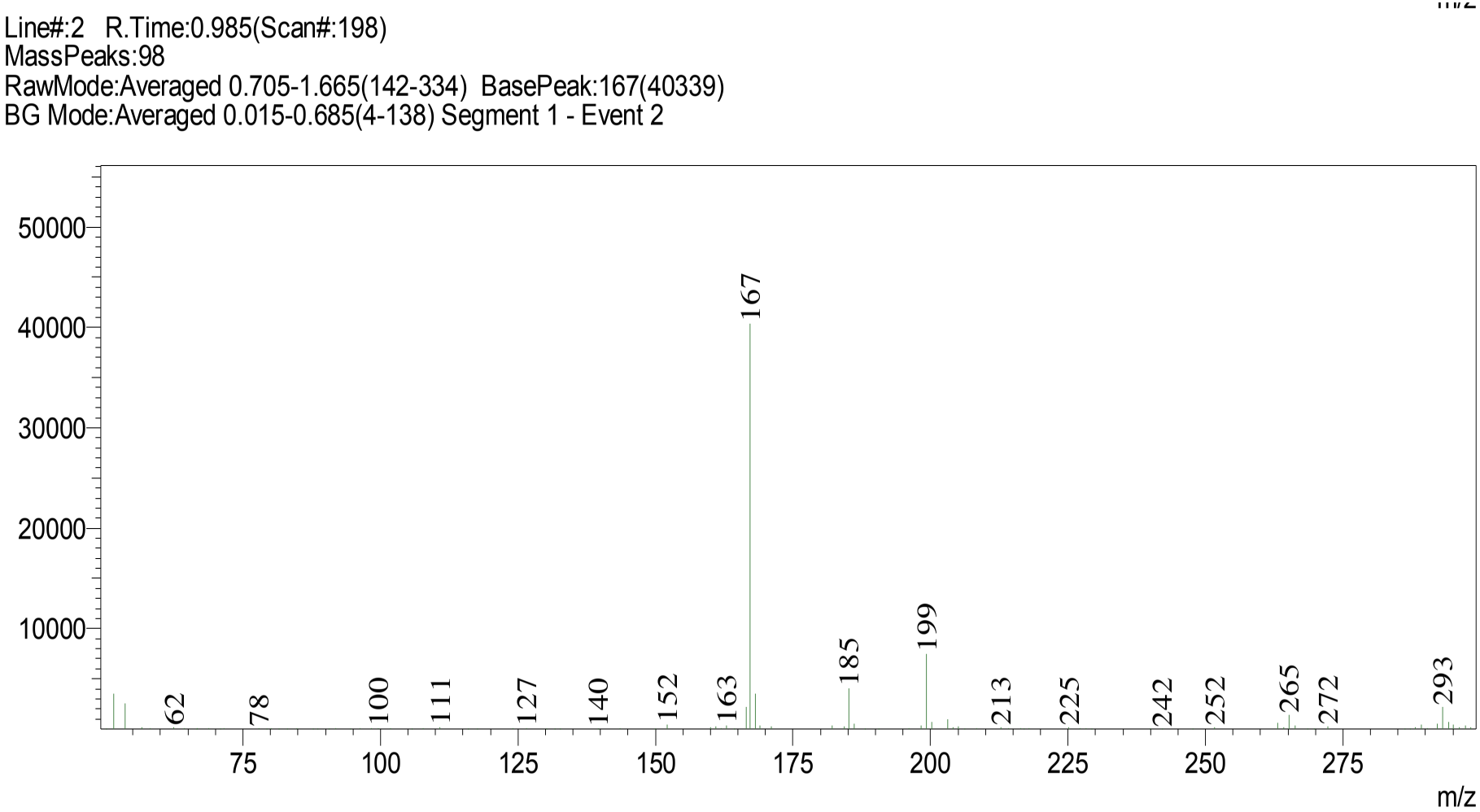


Figure 4.40: ESI mass spectrum of AI-4

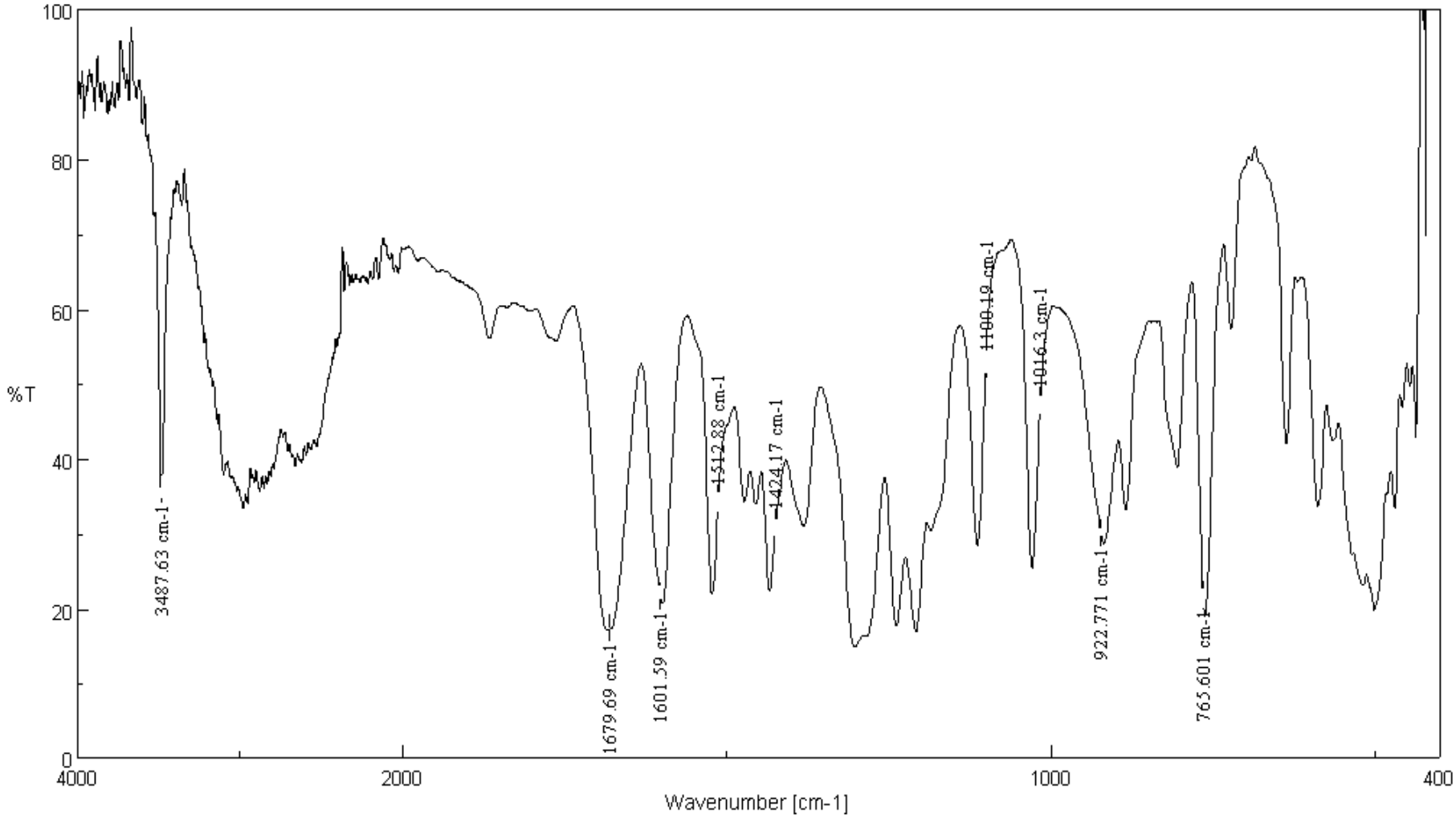


Figure 4.41: IR spectrum of AI-4

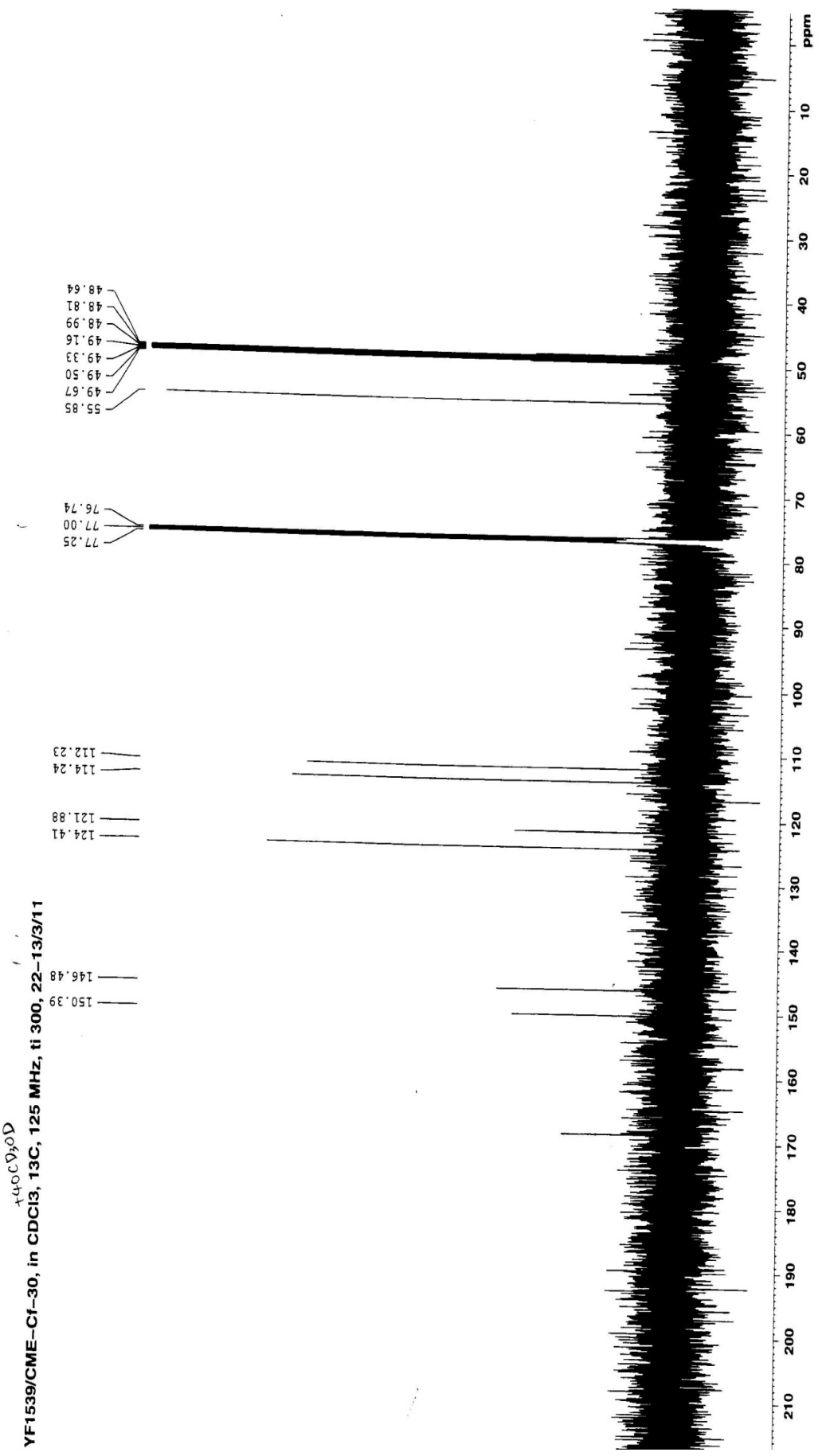
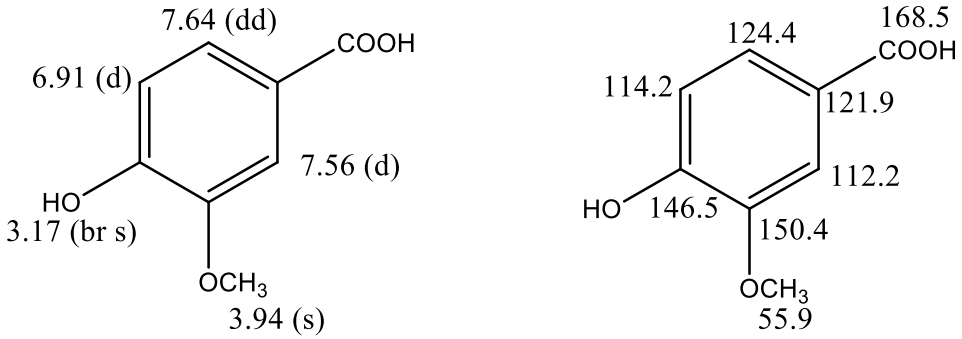


Figure 4.42: 125 MHz ^{13}C NMR spectrum of AI-4

The ^{1}H NMR spectrum of **AI-4** was done by dissolving in $CDCl_3$ under 500 MHz frequency (Figure 4.43). The upfield region of the spectrum showed a singlet at δ 3.94 ppm integrating for three protons due to aromatic methoxyl and a broad singlet integrating for one proton, which may be due to an exchangeable hydroxyl proton. Further, the downfield region of the spectrum clearly indicated the presence of aromatic protons of ABX pattern [δ 6.91 (d, J = 8.5 Hz), 7.56 (d, J = 2.0 Hz), 7.64 (dd, J =8.5, 2.0 Hz)].

The PMR and the CMR suggested **AI-4** to possess 1, 3, 4-trisubstituted benzene derivative attached with a carboxylic, hydroxyl and methoxyl group. Comparison of the measured spectral data with those of reported information in the literature showed consistency with vanillic acid i.e., 4-hydroxy-3-methoxy benzoic acid (**8**) (Yu *et al*., 2006). The structure of **AI-4** depicted with ^{13}C NMR data is presented below.



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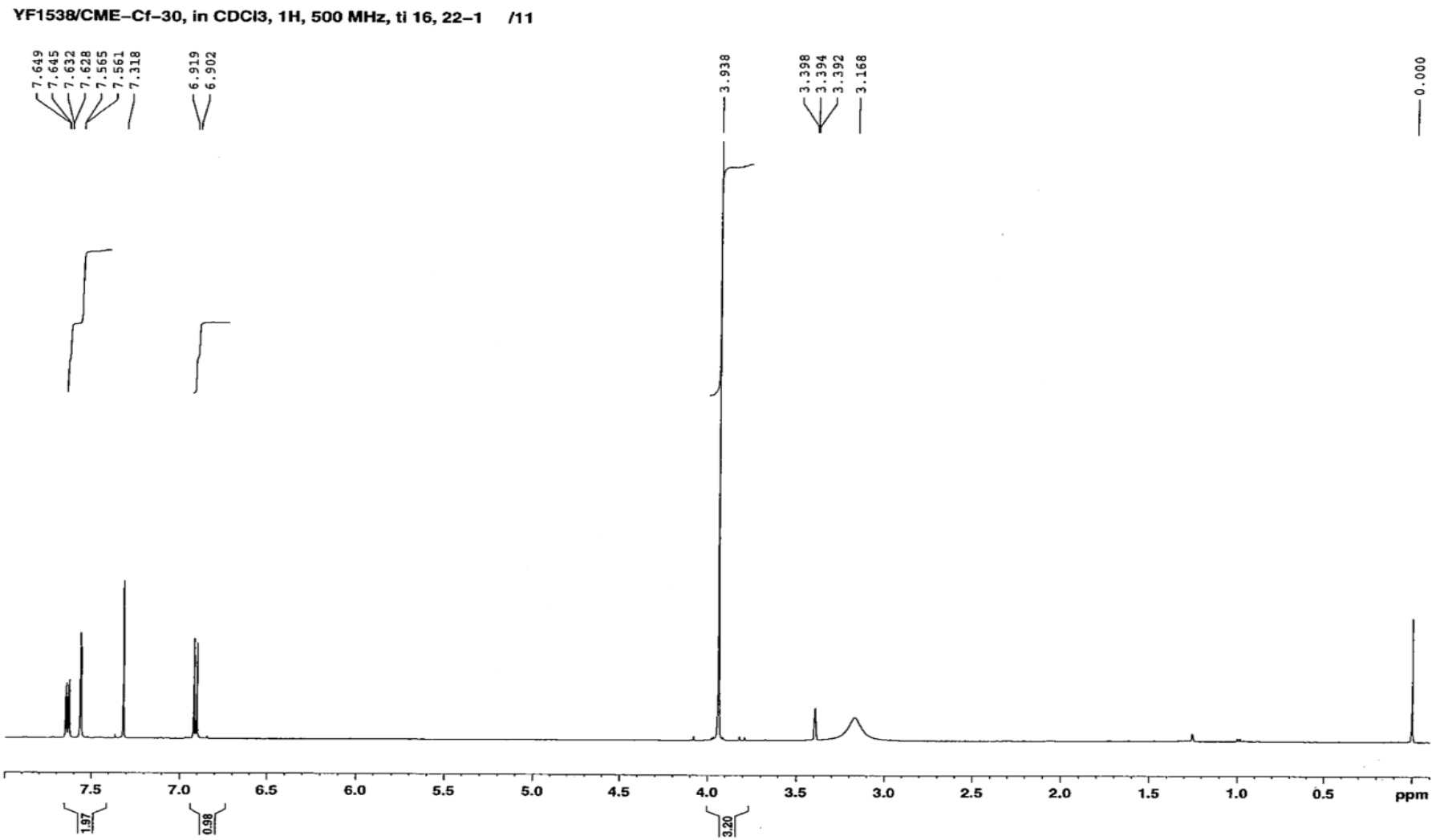


Figure 4.43: 500 MHz ^{1}H NMR spectrum of AI-4

4.7.5. Characterization of AI-5

AI-5 was isolated as white amorphous powder, showing m.p. of 100-103 °C and $[\alpha]^{25}_{D}$ +10.2 (*c* 0.1, MeOH). The purity of the compound was assessed through TLC studies by heating the developed plates sprayed with 10% methanolic Sulphuric acid. Under TLC studies, the compound exhibited positive reaction with phosphomolybdic acid indicating its reducible nature. **AI-5** was found to be freely soluble in $CHCl_3$, MeOH and benzene. It was sparingly soluble in hexane but insoluble in water. The IR spectrum (Figure 4.44) of this less-polar compound was found to exhibit absorption bands for acid carbonyl (1760 cm^{-1}), alcoholic -OH group (3550 cm^{-1}) and characteristic stretching and bending vibration bands for methylene and methine C-H bonds, suggesting the presence of long chain fatty ester.

The molecular formula was settled as $C_{18}H_{34}O_5$ based on elemental analysis and $[M+Na]^+$ derived base peak at *m/z* 353 and $[M-H]^-$ derived molecular ion peak at *m/z* 329 [Figure 4.45]. The occurrence of eighteen carbons in **AI-5** was further corroborated from the 125 MHz ^{13}C NMR spectrum, which clarified the existence of one carboxylic carbon, two olefenic carbons, three oxymethine carbons, eleven methylene carbons and one methyl carbon (Figure 4.46, Table 4.8). Affirmatively, the 500 MHz proton NMR spectrum displayed closely resonating double doublets [δ 5.71 ppm (*J* = 5, 15 Hz) and 5.66 ppm (*J* = 5, 15 Hz)] of olefenic protons and three deshielded single proton signals (δ 4.05, 3.89 and 3.40 ppm) of oxymethine protons (Figure 4.47). The up-field region of the spectrum showed signals for methyl (δ 0.93 ppm, t) and methylene protons [δ 1.28-1.33 ppm, and 1.44-1.60 ppm (m, 22H)]. Based on the collective spectral data and degree of unsaturation (DBE = 2), **AI-5** was confirmed as an 18-carbon fatty acid having one double bond. Of the total five oxygen atoms, two were accounted for acid group and the remaining three could be assumed as exchangeable functional group -OH.

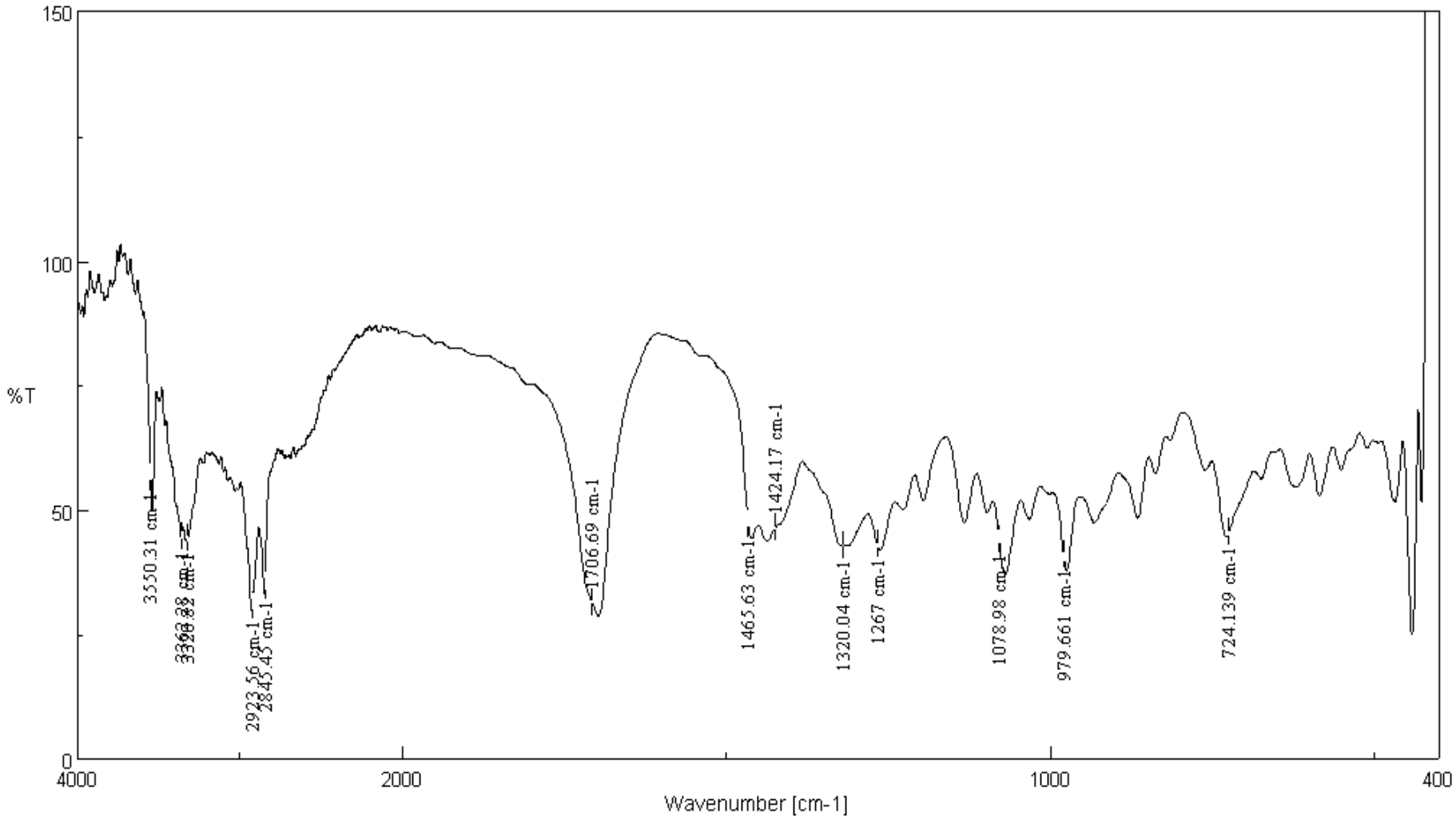


Figure 4.44: IR spectrum of AI-5

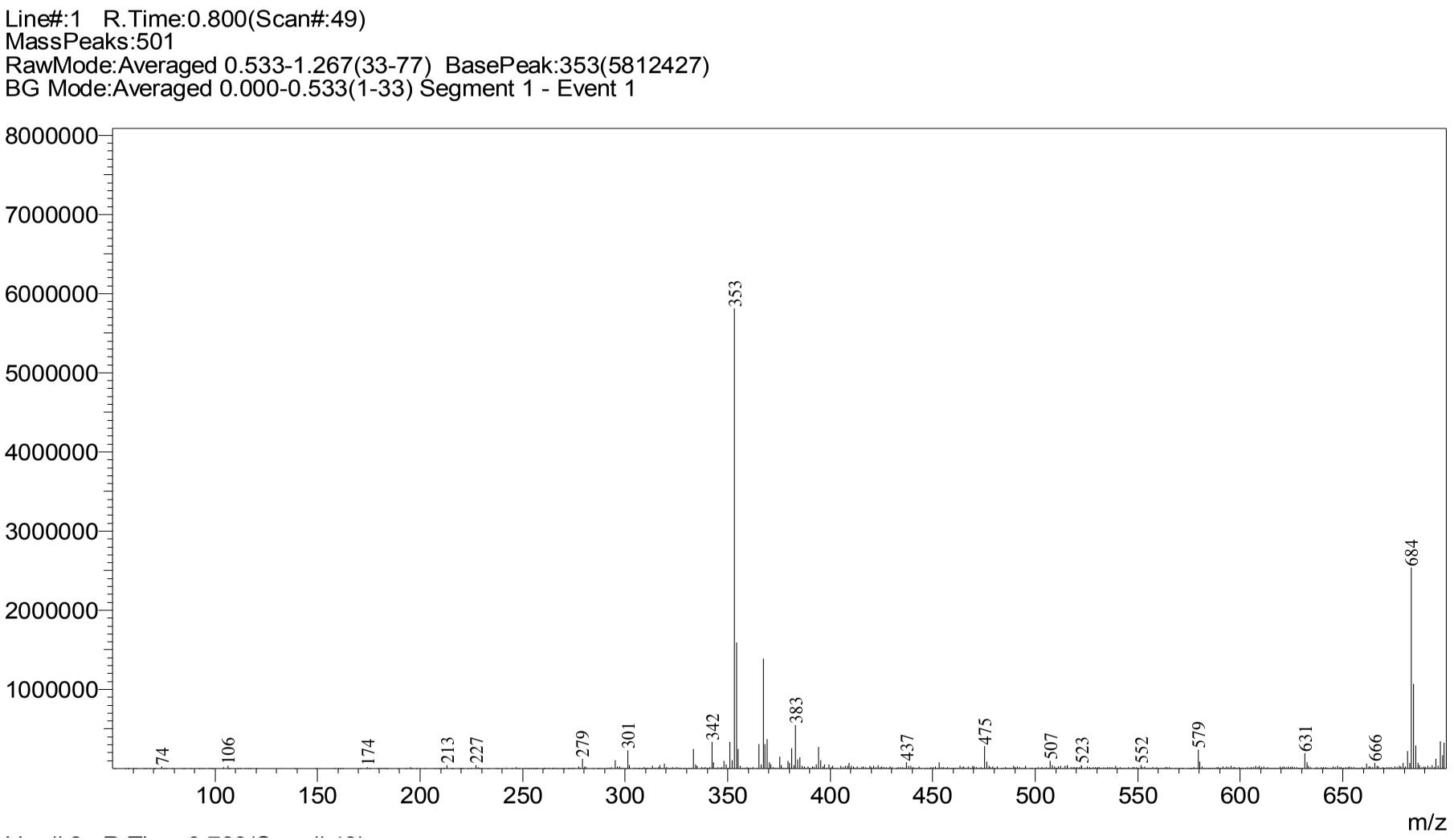


Figure 4.45: ESI mass spectrum of AI-5

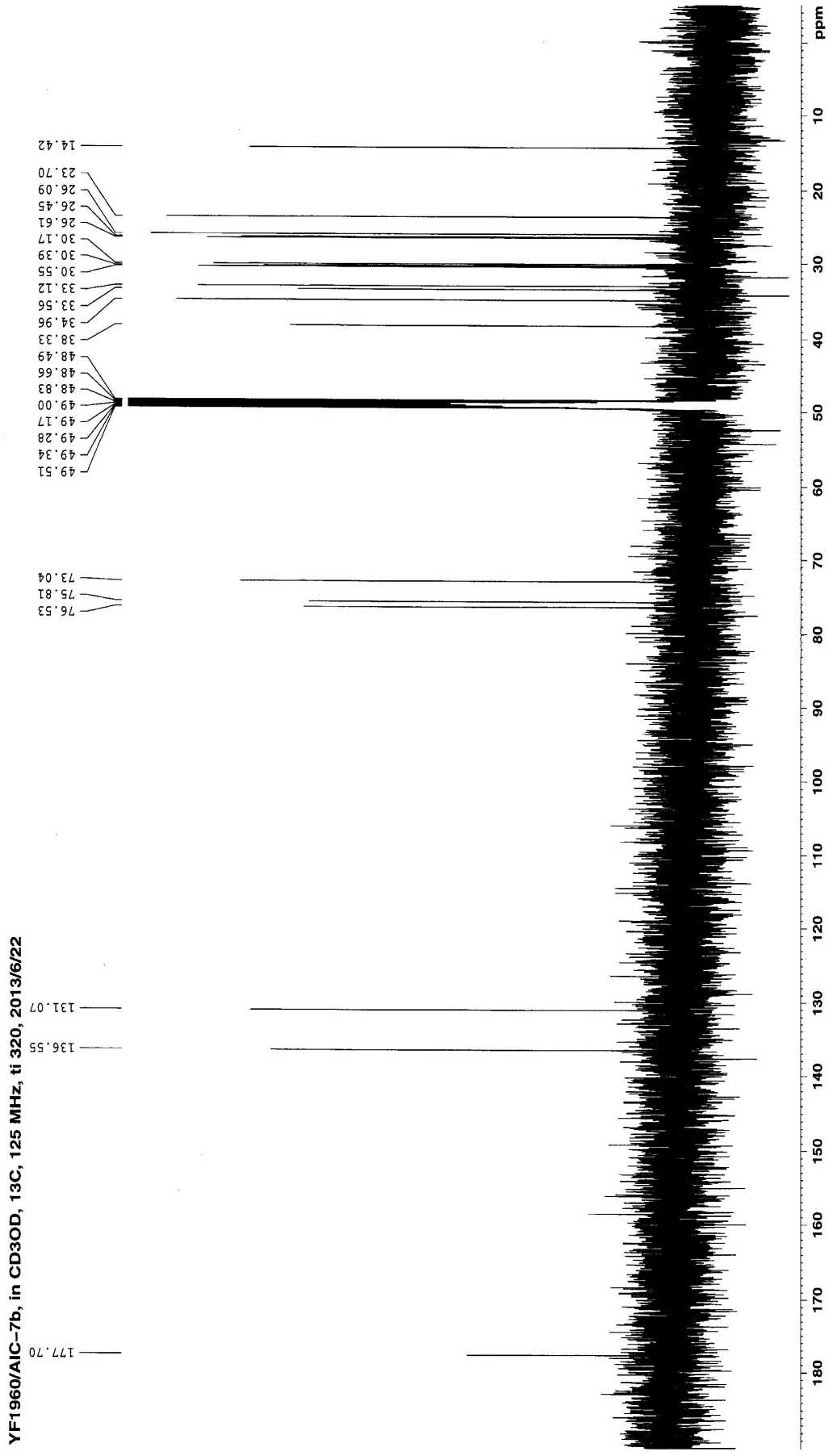


Figure 4.46: 125 MHz ^{13}C NMR spectrum of AI-5

Table 4.8: 125 MHz ^{13}C NMR spectrum of AI-5

Position	δ_C recorded (CD_3OD)	δ_C reported (CD_3OD)
1	177.7	177.6
2	136.5	136.5
3	131.0	131.1
4	76.5	76.5
5	75.8	75.8
6	73.0	73.0
7	38.3	38.3
8	34.9	34.9
9	33.5	33.5
10	33.1	33.1
11	30.5	30.5
12	30.3	30.4
13	30.1	30.2
14	26.6	26.6
15	26.4	26.4
16	26.0	26.1
17	23.7	23.7
18	14.4	14.4

The exact positions of attachment of -OH groups were confirmed through correlation spectroscopy. The 500 MHz ^{1}H-^{1}H COSY spectrum (Figure 4.48) explained the correlation between olefenic protons and oxymethine protons and hence the double bond should be flanked by methine protons attached to hydroxyl groups (secondary alcohols). Two of the three oxymethine protons were found to be positioned next-to-next as evidenced from the correlation between them (δ 3.89 and 3.40 ppm) (**AI-5**). Search of literature revealed identity of observed ESI-MS, ^{1}H- and ^{13}C NMR data with 9*S*,12*S*,13*S*-trihydroxy-10*E*-octadecenoic acid i.e. pinellic acid (Sunnam *et al*., 2013). Thus the structure of **AI-5** was determined and is shown as **9**.

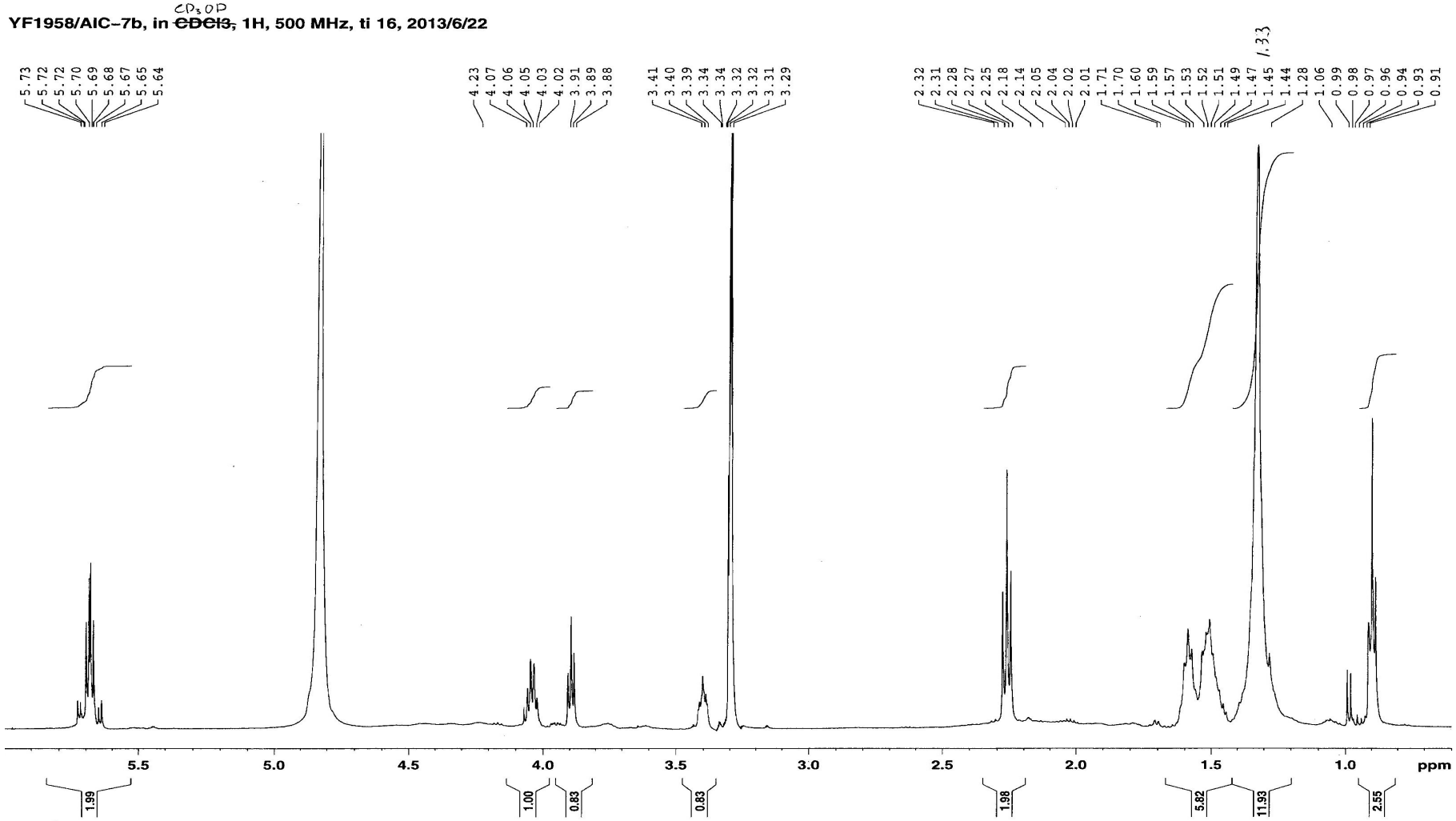
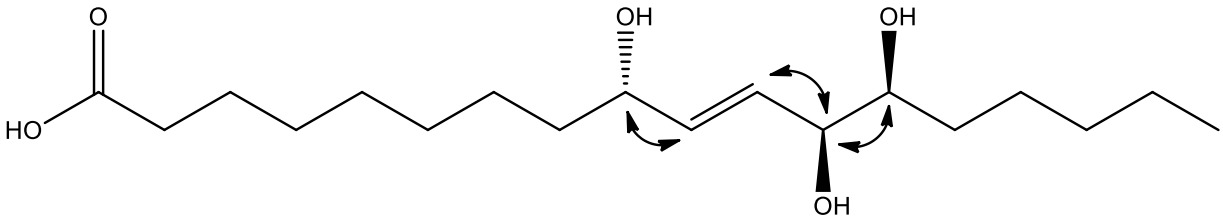


Figure 4.47: 500 MHz ^{1}H NMR spectrum of AI-5



AI-5

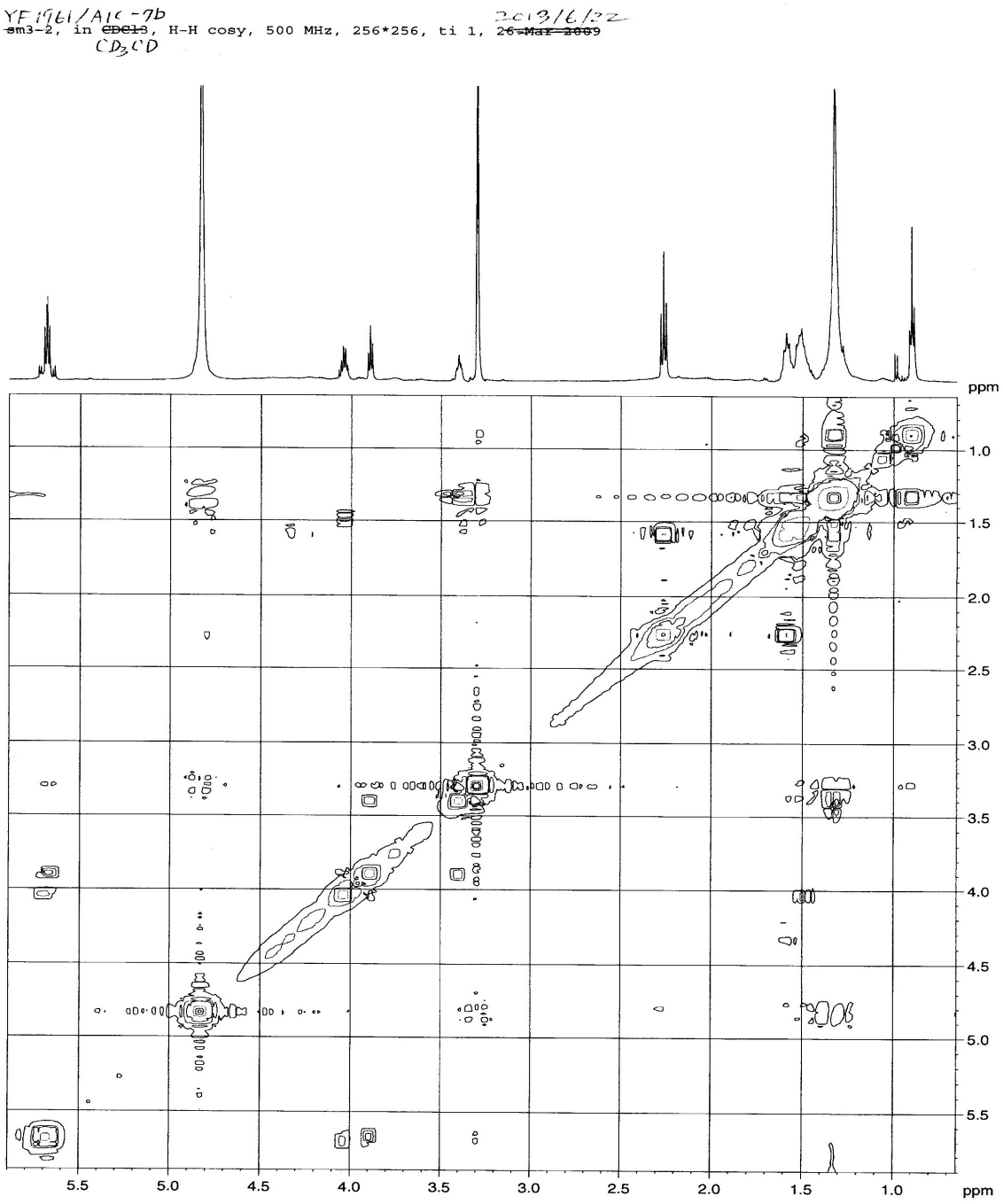
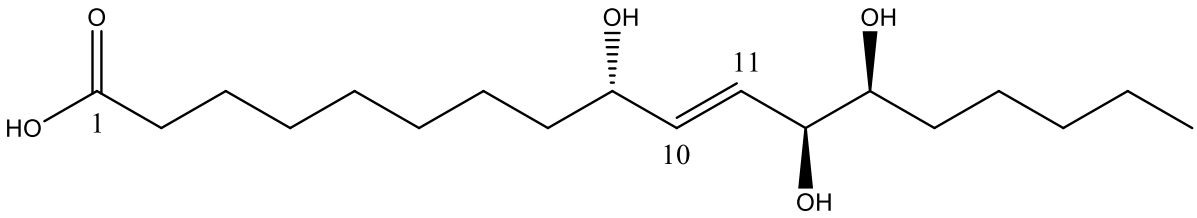


Figure 4.48: ^{1}H-^{1}H COSY spectrum of AI-5



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4.8. IN-VITRO SCREENING OF MOTHER EXTRACTS AND ISOLATED COMPOUNDS USING LPS-INDUCED PRODUCTION OF TNF-α MODEL

The effect of **EEPA**, **AIM**, **AIM-C** and nine isolated pure compounds on the production of TNF-α was tested using LPS-induced TNF-α production in mouse macrophage cell lines RAW 264.7. Prednisolone, a corticosteroid used in the treatment of a wide range of inflammatory and autoimmune conditions (Czock *et al*., 2005) was used as reference standard.

LPS is an outer membrane component of gram-negative bacteria and is a potent activator of monocytes and macrophages. It triggers the abundant secretion of many cytokines from macrophages including IL-1β, IL-6 and TNF-α and the major cell surface receptor for LPS on macrophages is CD14.

4.8.1. Measurement of TNF-α production by ELISA

During acute or chronic inflammation, excessive TNF-α is produced as pro-inflammatory cytokine, which results in the systemic inflammatory response syndrome, severe tissue damage, and septic shock. Effect of **EEPA**, **AIM**, **AIM-C** and nine single compounds isolated from **EEPA** and **AIM-C** on modulating the production levels of TNF-α was tested by stimulating macrophage cell line RAW 264.7 using LPS and the results were compared with standard drug, prednisolone.

The RAW 264.7 cells were treated with different concentrations of extract and compounds for 1 h followed by 6 h of LPS (1 μg/ml) incubation. After incubation, the supernatant was collected and estimated for the concentration of TNF-α by ELISA as per the manufacturers instruction. **EEPA**, **PA-1**, **PA-2**, **PA-3**, **PA-4** and **AI-2** exhibited promising results by down regulating levels of TNF- α whereas **AIM**, **AIM-C**, **AI-1**, **AI-3**, **AI-4** and **AI-5** did not show any inhibition at tested concentration levels. The detailed results of active extract and compounds are presented in Table 4.9, Figure 4.49, Figure 4.50 and Figure 4.51.

Table 4.9: Effect of EEPA on LPS-induced TNF-α production in RAW 264.7 macrophages

Treatment	Concentration	Concentration of TNF-α (pg/ml) ± SEM
Blank	NA	295.87 ± 26.84
LPS	1 μg/ml	2403.47 ± 43.18
EEPA + LPS	100 μg/ml	840.87 ± 195.38***
EEPA + LPS	50 μg/ml	1442.17 ± 33.68***
EEPA + LPS	25 μg/ml	1586.57 ± 48.34***
EEPA + LPS	12.5 μg/ml	1691.07 ± 53.70***
EEPA + LPS	6.25 μg/ml	1840.97 ± 71.83**
Prednisolone	10 μM	1545.67 ± 39.29***

(Cells were treated with the indicated concentrations of EEPA and standard prednisolone for 1 h and then incubated with LPS (1 μg/ml) treatment for 6 h. The TNF-α concentration was determined by ELISA kit. The values were presented as mean ± SEM from triplicate. $^{***}p < 0.001$, $^{**}p < 0.01$ vs LPS control).

The results were compared with standard drug, prednisolone (10 μM), which exhibited 42.8% inhibition of TNF-α concentration. Around 74.2% protein inhibition was observed with 100 μg/ml treatment of **EEPA** (Figure 4.49) and the inhibition effect was found to be increasing with the increase in concentration giving IC_{50} of 38.79 μg/ml (Figure 4.49).

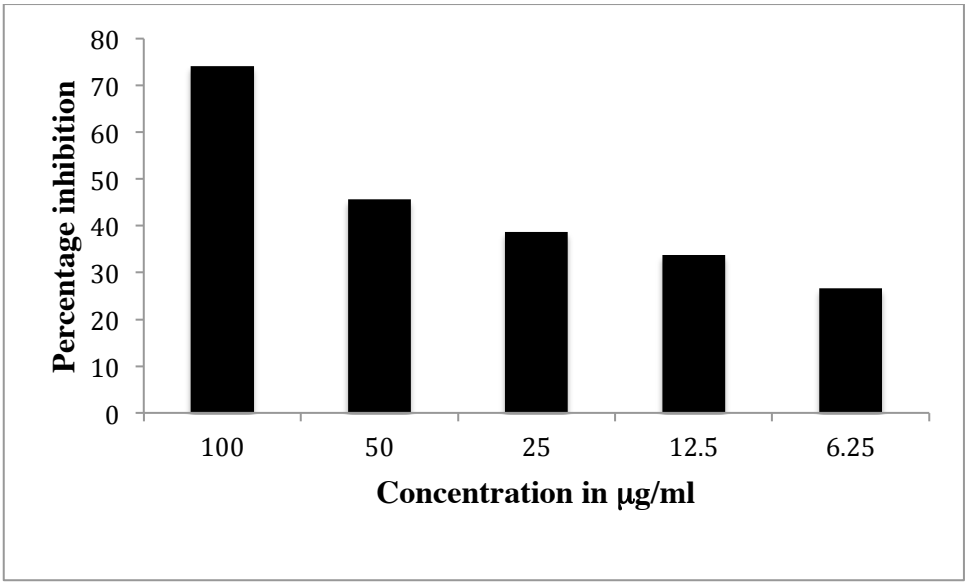


Figure 4.49: Percentage inhibition of EEPA on LPS-induced TNF-α production in RAW 264.7 macrophages (cells were treated with indicated concentrations of EEPA for 1 h and then incubated with LPS (1 µg/ml) for 6 h. The TNF-α concentration was determined by ELISA kit).

Among the four isolated diketopiperazines, cyclo(Gly-L-Pro) (**PA-1**) showed 60.2% inhibition followed by compounds **PA-3** (53.8%), **PA-4** (49.3%) and **PA-2** (45.0%) at 100 µM concentration (Figure 4.50). Compounds **PA-1** and **PA-3** were found to be equally active to the clinically used drug prednisolone at 10 µM concentration. All cyclic dipeptides showed significant effect even at 1 µM concentration.

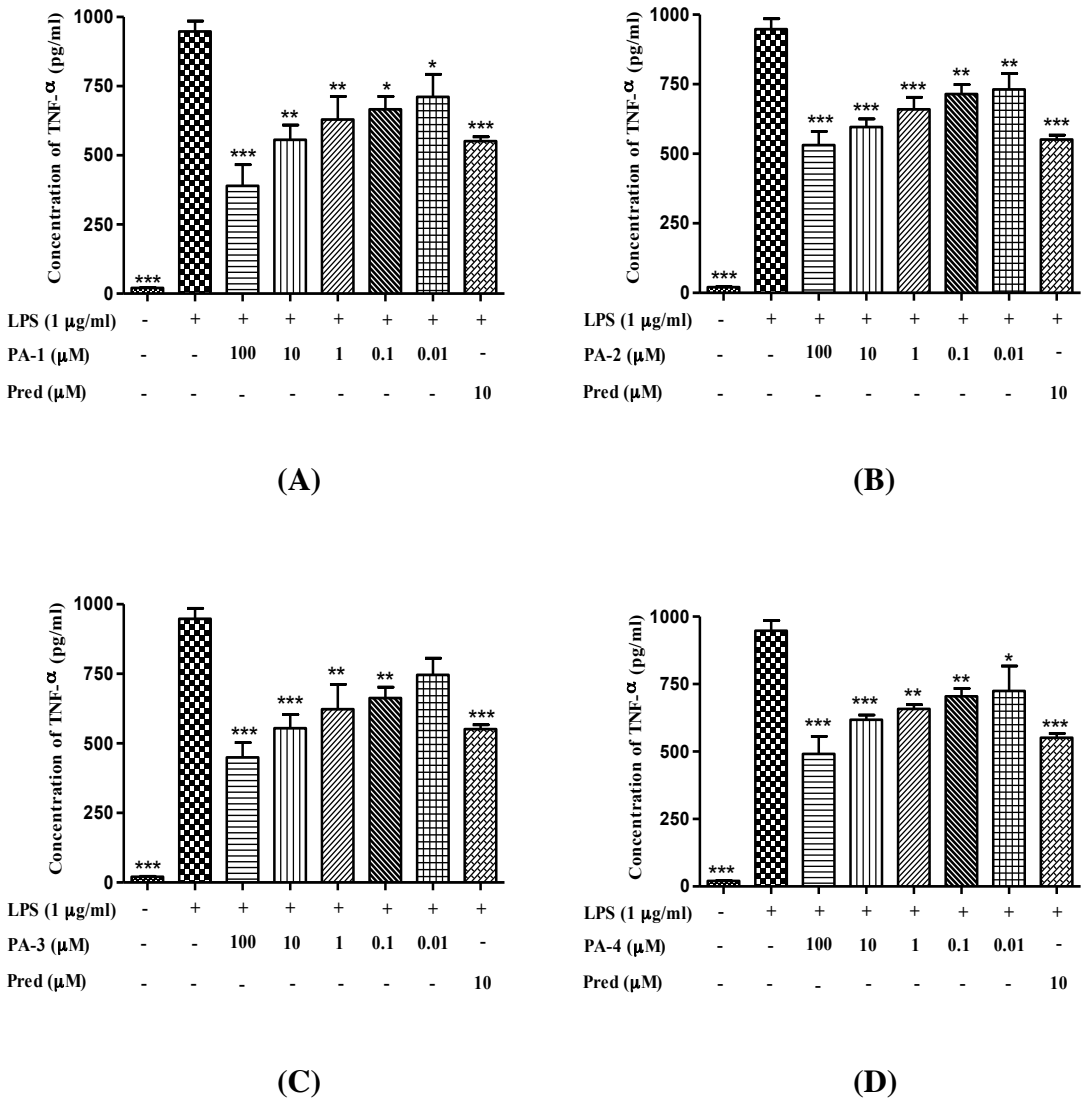


Figure 4.50: Effect of PA-1 (A), PA-2 (B), PA-3 (C) and PA-4 (D) on LPS-induced TNF-α production in RAW 264.7 macrophages (cells were treated with the indicated concentrations of PA-1, PA-2, PA-3, PA-4 and standard prednisolone for 1 h and then incubated with LPS (1 μg/ml) for 6 h. The TNF-α concentration was determined by ELISA kit. The values were presented as mean ± SEM from triplicate. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs LPS control).

The plant derived samples **AIM**, **AIM-C**, **AI-1**, **AI-3**, **AI-4** and **AI-5** did not show any inhibition on TNF-α levels under in-vitro LPS-induced TNF-α production model. However **AI-2** at 100 μM concentration showed significant inhibition ($p < 0.001$) on TNF-α production levels (Figure 4.51). While all *P. aeruginosa* derived samples were effective even

at 0.01 μM concentration, **AI-2** was not so. The percentage inhibition exhibited by **AI-2** at 100 μM concentration was found to be 55% where as, at 10 μM it was found to be 24%.

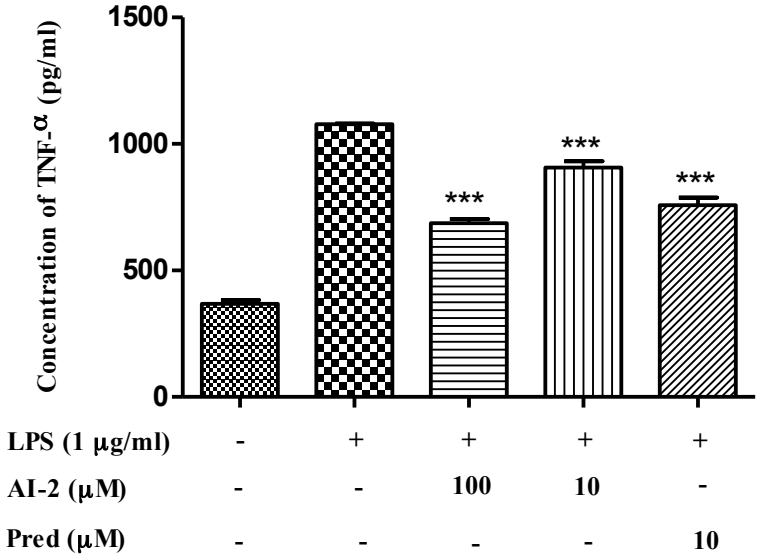


Figure 4.51: Effect of AI-2 on LPS-induced TNF-α production in RAW 264.7 macrophages (cells were treated with the indicated concentrations of AI-2 and standard prednisolone for 1 h and then incubated with LPS (1 μg/ml) for 6 h. The TNF-α concentration was determined by ELISA kit. The values were presented as mean ± SEM from triplicate. $^{***}p < 0.001$ vs LPS control).

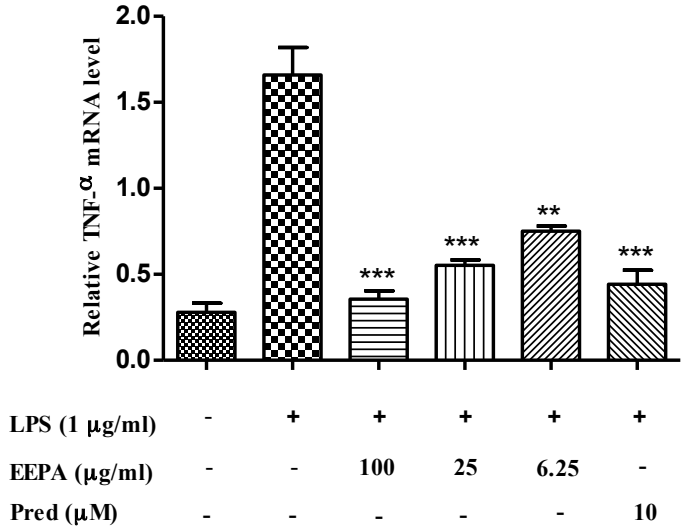
The significantly active **EEPA** and **PA** compounds were further screened for their effect on relative mRNA levels of TNF-α.

4.8.2. Measurement of relative mRNA levels of TNF-α by quantitative RT-PCR

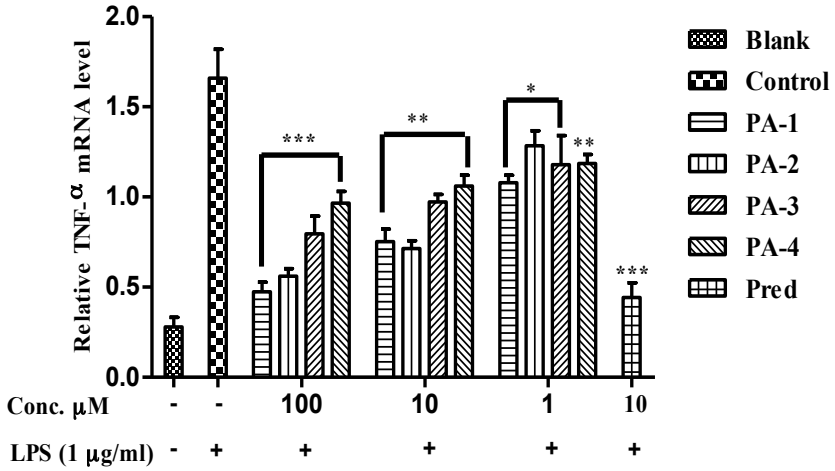
To study the inhibition effect of active **EEPA** and **PA** compounds in detail, the cellular TNF-α expression was also assessed using quantitative RT-PCR. Quantitative RT-PCR is used to quantify mRNA in both relative and absolute terms. It can be applied for the quantification of mRNA expressed from endogenous genes, and transfected genes of either stable or transient transfection. It is the most sensitive method as yet in quantitative analysis of mRNA.

Pretreatment with **EEPA** and **PA-1** to **PA-4** diminished LPS-induced inflammatory cytokine transcription significantly ($p < 0.001$). There was no basal change in the cytokines

expression following incubation with test samples without LPS. At higher concentration (100 μg/ml), **EEPA** diminished LPS-stimulated TNF-α mRNA levels of 1.65 ± 0.16 to 0.35 ± 0.04. More than 50% suppression was found even at lower concentrations of 6.25 μg/ml (Figure 4.52).



(A)



(B)

Figure 4.52: Effect of EEPA (A) and PA-1 to PA-4 (B) on mRNA expression levels of TNF-α (measured by quantitative RT-PCR in RAW 264.7 cells treated with the indicated concentration of EEPA with or without 1 μg/ml LPS for 6 h. Data represent the mean ± SEM from triplicate. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs LPS control).

Among the single compounds, **PA-1** and **PA-2** were found to reduce the LPS-elevated mRNA expression levels of TNF-α by three folds. Reduction of the same by **PA-3** was found to be two folds. Compared to LPS control, nevertheless all compounds were found to be significantly diminishing mRNA expression levels of TNF-α (Figure 4.52).

To understand the in-vivo performance of **EEPA**, proline based cyclic dipeptides and **AI-2**, an acute in-vivo study on rat was carried out. As the natural **PA-1** to **PA-4** and **AI-2** were obtained in small amounts, synthetic attempts were made. Synthesis of **PA-4** and **AI-2** was successfully achieved.

4.9. CHEMICAL SYNTHESIS OF CYCLO(*TRANS*-4-HYDROXY-L-PROLINE-L-LEUCINE) (PA-4)

Literature reported the synthesis of **PA-4** by dipeptide formation from *N*-carbobenzoxy-hydroxyproline and leucine *n*-butyl ester followed by deprotection of the carbobenzoxy group of the hydroxy-proline amino moiety (Ienaga *et al*., 1987). We instead started from commercially available *trans*-4-hydroxy-L-proline methyl ester hydrochloride and *N*-(*tert*-butoxycarbonyl)-L-leucine. Coupling of the protected amino acids using EDC · HCl afforded the dipeptide, which upon treatment with trifluoroacetic acid at 70 °C in toluene smoothly yielded cyclo(*trans*-4-hydroxy-L-Proline-L-Leucine) (**PA-4**). The synthetic **PA-4** was identical with that obtained from *P. aeruginosa* and showed the same physico-chemical properties as reported in literature (Furtado *et al*., 2005).

4.10. CHEMICAL SYNTHESIS OF METHYL CAFFEATE (AI-2)

AI-2 was directly synthesized by condensation reaction using caffeic acid as starting material in presence of methanol and concentrated H_2SO_4 as reported in literature (Dzubak *et al*., 2006). The reaction mixture was purified by column chromatography with increasing polarity i.e. hexane (100%) till EtOAc:Hexane (85:15). The purified product was confirmed by co-TLC and mixed m.p. studies with authentic natural **AI-2**.

4.11. IN-VIVO SCREENING FOR THE ESTIMATION OF TNF-α PRODUCTION

The acute toxicity study of **EEPA** on female wistar rats was done by taking 2000 mg/kg dose and was found to be non-toxic. Based on acute toxicity data, dose of **EEPA** was decided for the in-vivo study.

The consequence of oral administration of **EEPA** (750, 500, 250 and 125 mg/kg), **PA-4** (50 mg/kg) and **AI-2** (10 and 50 mg/kg) followed by LPS (0.3 mg/kg; i.p.) injection in rats was determined by measuring plasma TNF-α concentrations using ELISA kit. In control rats, injection of LPS caused approximately 200 times increase in TNF-α concentration. The LPS mediated increase in plasma TNF-α was found to be reduced in all test groups. Results demonstrated a significant reduction in plasma TNF-α by **EEPA** at various dose levels ($p<0.001$) compared to LPS control group. A drastic reduction of around 80% was observed in rats treated with 500 mg/kg dose levels of **EEPA**. Lower dose of **EEPA** (125 mg/kg) also exhibited 44.7% decrease in TNF-α release. Cyclo(*trans*-4-hydroxy-L-Pro-L-leu) (**PA-4**), one of the isolates of **EEPA** displayed, 51% drop of TNF-α concentration (Figure 4.53) where as **AI-2** did no show any inhibition at 10 and 50 mg/kg dose. Further, results proved that *A. indicum* reported to posses anti-inflammatory activity was not effective in inhibiting TNF-α production.

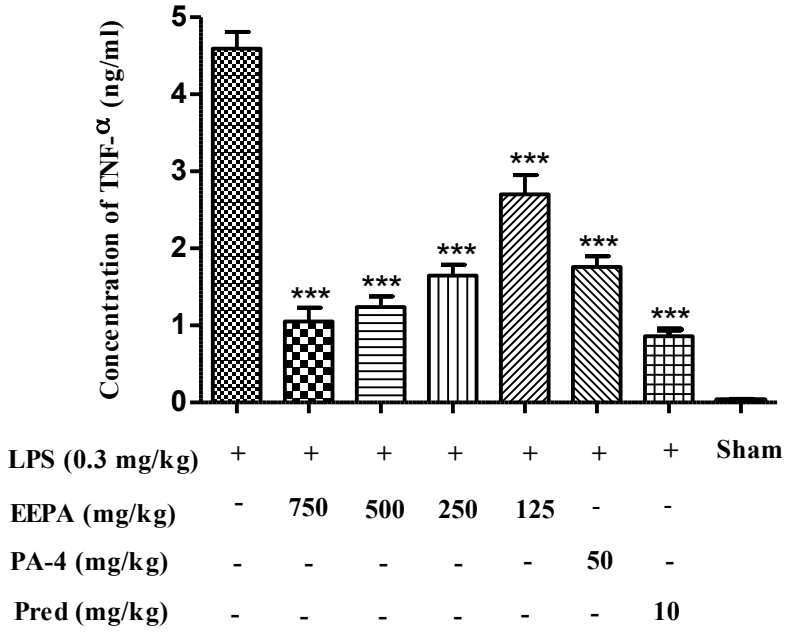


Figure 4.53: In-vivo screening of EEPA and PA-4 on TNF-α production (extract was orally administered. LPS 0.3 mg/kg was injected i.p. after 1 h of test compound incubation. 90 min of post LPS injection blood was collected via retro-orbital puncture; plasma was used for the estimation of TNF-α by ELISA. Data represent the mean ± SEM from triplicate. $^{***}p < 0.001$ Vs LPS control).

Autoimmune diseases involve both humoral and cellular immune mechanisms comprising cytokine-mediated activation of T and B lymphocytes and the recruitment and activation of macrophages. The critical role of TNF-α in the pathophysiology of inflammation is by mediating the induction of other cytokines such as cyclooxygenase, prostaglandins and metalloproteinases (Feldmann *et al*., 1996; Cheon *et al*., 2002). Thus the outcome of in-vitro and in-vivo experiments disclosed the potent effect of culture broth extract of *P. aeruginosa* and its secondary metabolites in down-regulating a key cytokine, TNF-α, which also contributes to systemic features of fever, myalgia and weight loss (Van Vollenhoven, 2009). The study also explored the possibility of these cyclic dipeptide molecules to inhibit or control the expression of other pro-inflammatory cytokines.

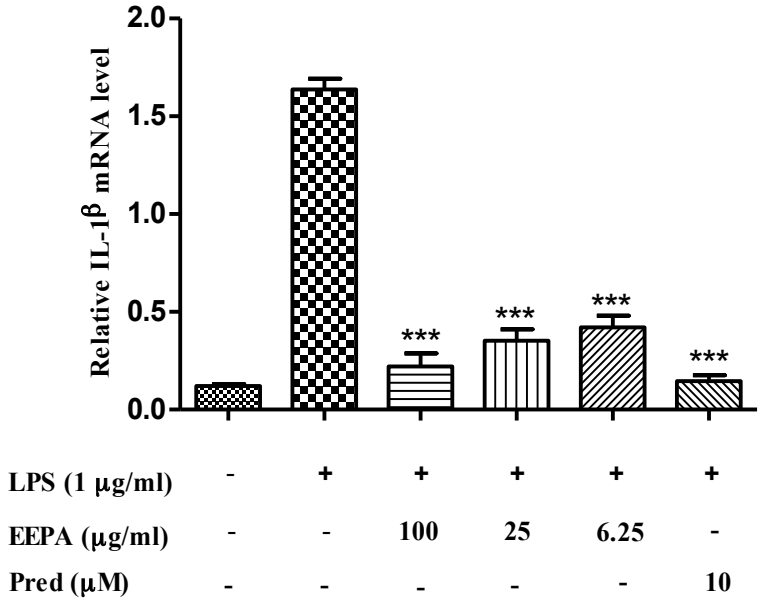
A recent study has revealed the anti-inflammatory effect of analogous diketopiperazines, (3*S*,8a*S*)-3-isobutyl-hexahydro-pyrrolo[1,2-a]pyrazine-1,4-dione and (3*S*,8a*S*)-3-(4-hydroxy-benzyl)-hexahydro-pyrrolo[1,2-a]pyrazine-1,4-dione isolated from *P. aeruginosa* and *P. fluroescens*, respectively by inhibiting TNF-α in peripheral blood nuclear cells (Rupesh *et al*., 2012). Also identification of immunomodulating dipeptides like cyclo(Arg-Lys) and cyclo(Asp-Lys) (Blazickova *et al*., 1994) and pro-inflamamtory NF-κB signal suppressing cyclo(His-Pro) (Minelli *et al*., 2012) further encouraged us to carry out extensive evaluation. Hence the TNF-α inhibitory **EEPA** and **PA-1** to **PA-4** compounds were further evaluated for their effect on other cytokines, IL-1β and IL-6.

4.12. MEASUREMENT OF RELATIVE mRNA LEVELS OF IL-1β AND IL-6 BY QUANTITATIVE RT-PCR

The activated macrophages released a number of different pro-inflammatory mediators, including IL-1β, IL-6, TNF-α and NO. Hence the study was extended to understand the mechanism of action of **EEPA** and active **PA** compounds in greater detail. The effect on cellular expression of IL-1β and IL-6 was also determined using quantitative RT-PCR.

EEPA and its isolates showed significant effect in down regulating both IL-1β and IL-6. The effect of **EEPA** was more pronounced on IL-1β compared to IL-6 (Figure 4.54). Compound **PA-1** was found to be potentially active in decreasing the expression levels of IL-1β from 1.63 ± 0.05 to 0.31 ± 0.10 at 100 μM and IL-6 levels from 1.19 ± 0.06 to 0.35 ± 0.04. Nevertheless compound **PA-2** over all displayed diminution of IL-1β and IL-6 under tested conditions. **PA-2** was found to be more effective than **PA-1** in controlling the expressions of IL-6. Results further demonstrated a concentration dependent decrease in

cytokine levels by dioxopiperazine isolates. A comparative chart with standard drug prednisolone is presented in Figure 4.55.



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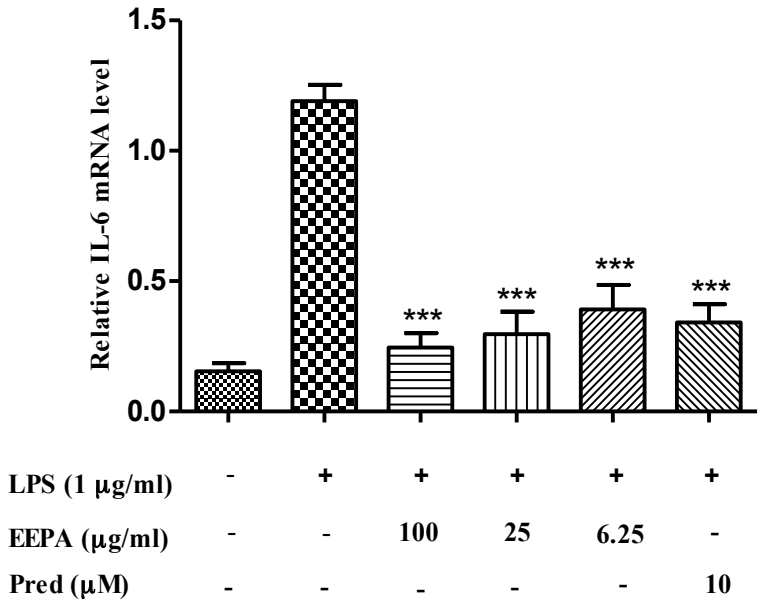
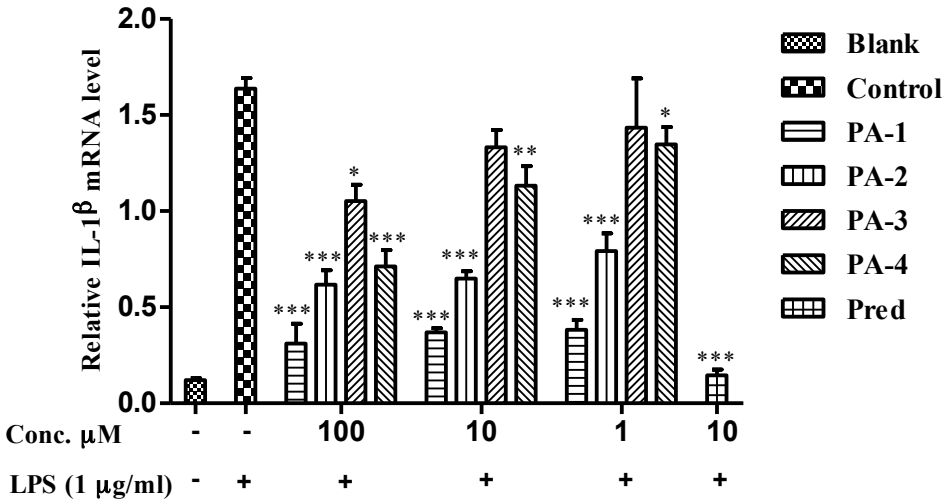
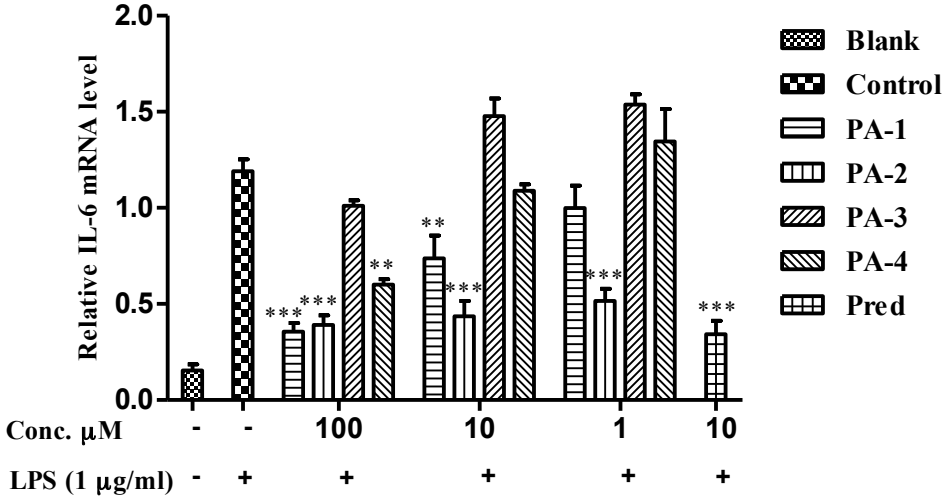


(B)

Figure 4.54: Effect of EEPA on mRNA expression levels of IL-1β (A) and IL-6 (B) (measured by quantitative RT-PCR in RAW 264.7 cells treated with the indicated concentrations with or without 1 µg/ml LPS for 6 h. Data represent the mean ± SEM from triplicate. $^{***}p < 0.001$ vs LPS control).



(A)

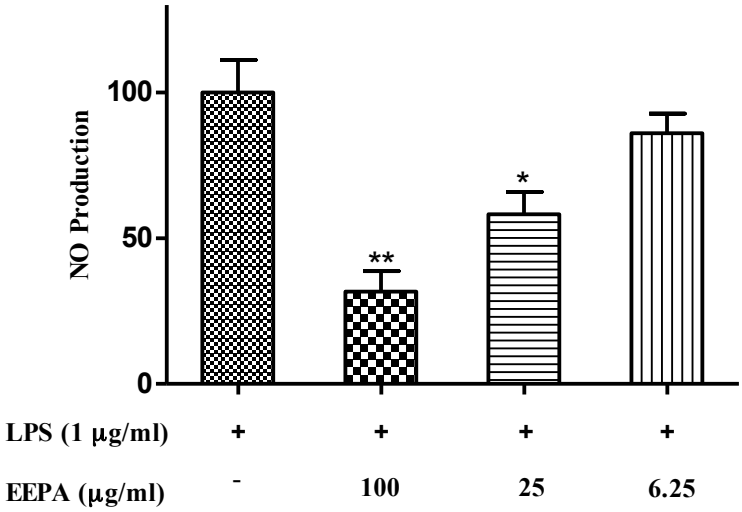


(B)

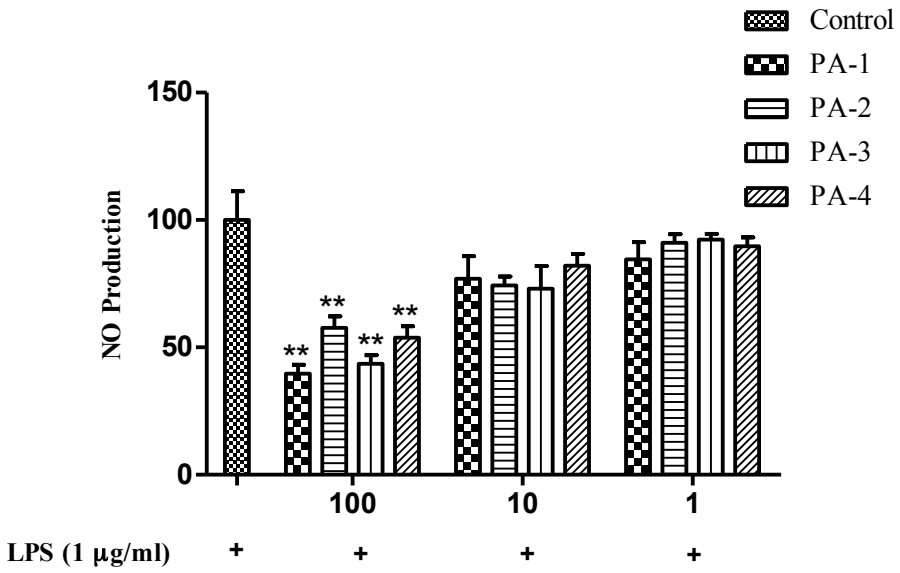
Figure 4.55: Effect of PA-1 to PA-4 on mRNA expression levels of IL-1β (A) and IL-6 (B) (measured by quantitative RT-PCR in RAW 264.7 cells treated with the indicated concentrations with or without 1 µg/ml LPS for 6 h. Data represent the mean ± SEM from triplicate. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs LPS control).

4.13. MEASUREMENT OF NITRIC OXIDE PRODUCTION

Nitric oxide (NO) has been proved to possess antibacterial and virustatic properties due to which it exerts a role in host defense. However, excessive NO production will lead to damage of host cells due its cytotoxic potential (Wong *et al*., 1995). Hence NO is a pivotal regulator in states of inflammatory conditions. This is produced by iNOS from L-arginine upon stimulation of macrophages by exposure to bacterial LPS. Hence the study was extended to measure the NO production subsequent to the inhibitory effect exhibited by **EEPA** and cyclic dipeptides on mRNA level of TNF-α, IL-1β and IL-6 in LPS stimulated RAW 264.7 cells. The percentage NO production by **EEPA** and compounds **PA-1** to **PA-4** as determined by the Griess method is presented in Figure 4.55. **EEPA** showed more than 50% reduction at 100 μg/ml concentration. Among the cyclic dipeptides, cyclo(Gly-L-Pro) (**PA-1**) exhibited significant control over NO production compared to the other compounds **PA-2** to **PA-4**, which added support to its pro-inflammatory cytokine inhibitory effect. No basal NO production was found when the cells were incubated with only the test samples without LPS (Figure 4.56).



(A)



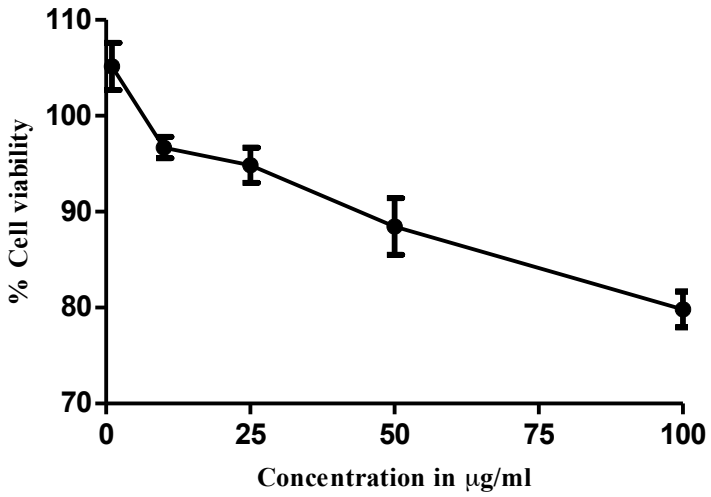
(B)

Figure 4.56: Effect of EEPA and PA-1 to PA-4 on the nitric oxide (NO) production in LPS-stimulated RAW264.7 cells (cells were stimulated with 1 μg/ml of LPS only or LPS plus various concentrations of sample for 16 h. NO production was determined by the Griess reagent method. The data represent the mean ± SEM from triplicate. ** $p < 0.01$, $^{*}p < 0.05$ vs LPS control).

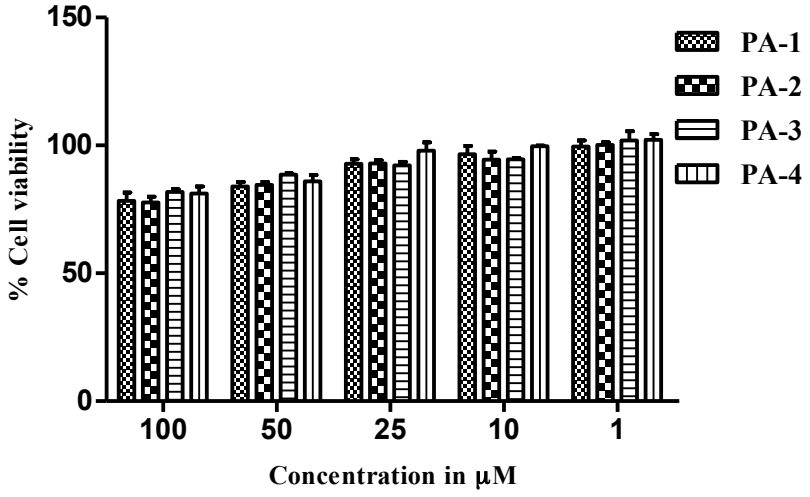
4.14. MTT ASSAY FOR TESTING CELL VIABILITY

Measurement of cell viability and proliferation forms the basis for numerous in-vitro assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. Hence to know the effect of **EEPA** and **PA-1** to **PA-4** on RAW 264.7 cell viability, MTT assay was performed.

Viability of cells treated with various concentrations of **EEPA** and **PA-1** to **PA-4** for 1 h followed by LPS incubation for 24 h was measured by MTT assay. Results proved less toxic behavior of **EEPA** (8% inhibition at 50 μg/ml) and isolated cyclic dipeptides (3-13% inhibition at 50 μM) as shown in figure 4.57.



(A)



(B)

Figure 4.57: Effect of EEPA (A) and PA-1 to PA-4 (B) on the viability of RAW 264.7 cells (cells were treated with various concentrations of EEPA for 1 h followed by LPS incubation for 24 h and cell viability was measured by MTT assay).

4.15. ANALYSIS OF LC-ESI-MS CHROMATOGRAM OF EEPA

LC-ESI-MS analysis of **EEPA** under PDA detection displayed around 8 (Figure 4.58) prominent peaks of having *m/z* values in the range of 154 – 311 other than the isolated cyclic dipeptides (**PA-1**, 154; **PA-2**, 245; **PA-3**, 260 and **PA-4**, 226) (Figure 4.59).

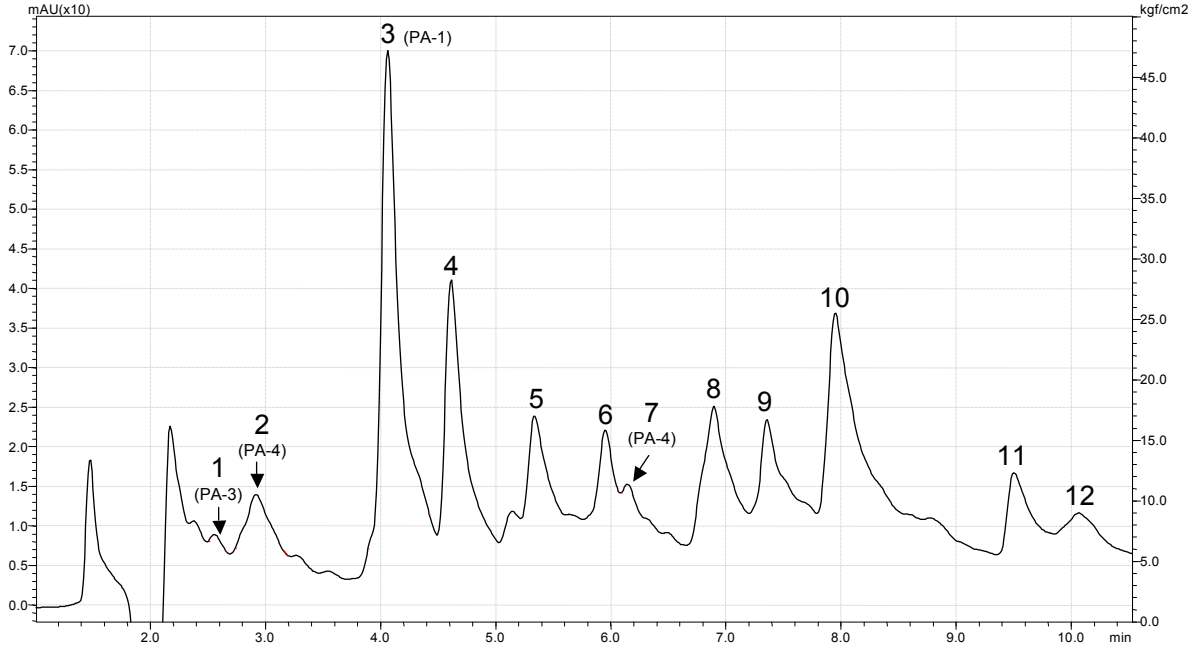
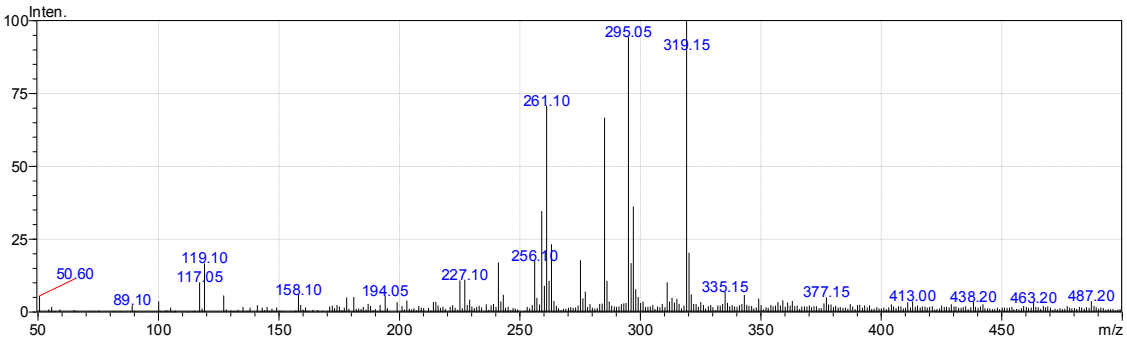
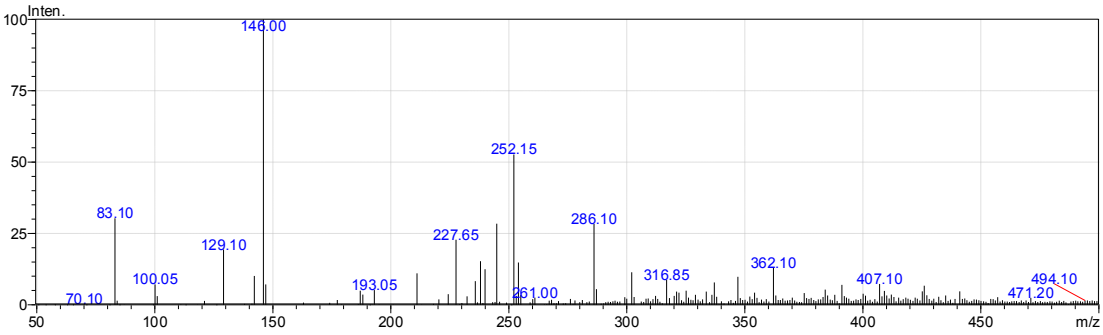


Figure 4.58: LC-ESI-MS fingerprint of EEPA

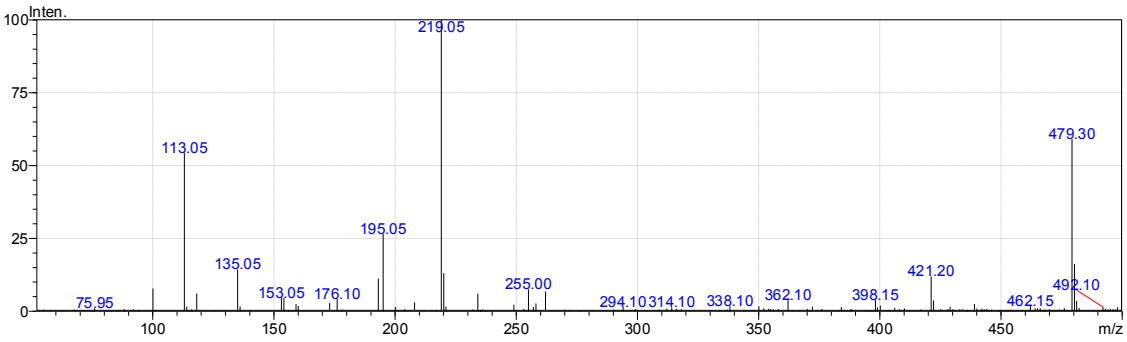
Chemical and biological correlation of **EEPA** based on LC-MS chromatogram revealed the involvement of eight other constituents in suppressing the major macrophage-derived inflammatory mediators TNF-α, IL-1β and IL-6 under LPS-induced shock. Also the chromatogram explored the more potentially active **PA-1** [cyclo(L-Proline-Glycine)] as the major constituent. Hence the highly significant effect of **EEPA** was clearly identified to be because of **PA-1**.



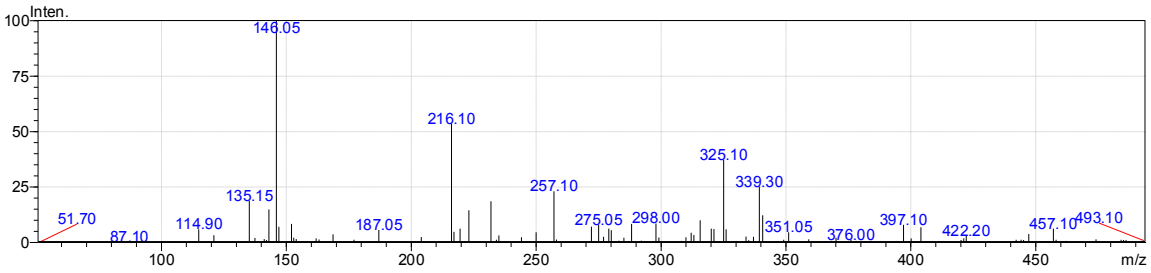
1st peak



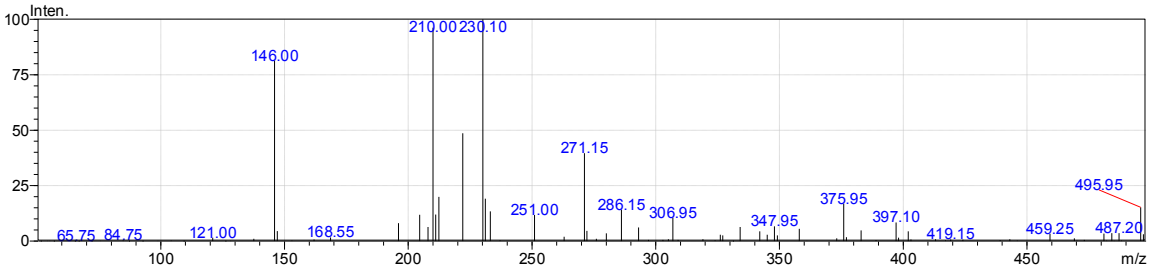
2nd peak



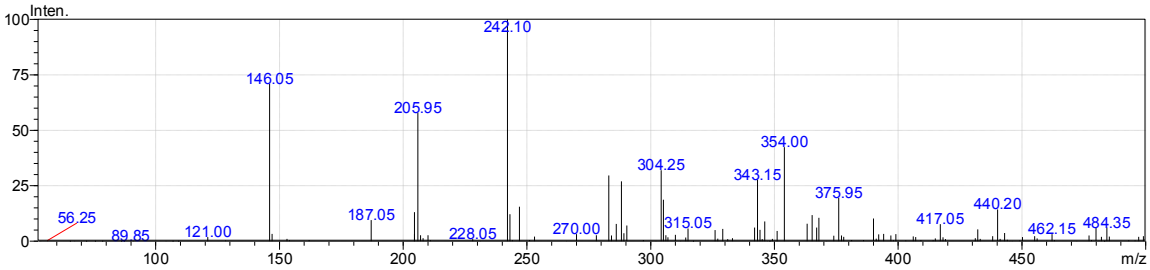
3rd peak



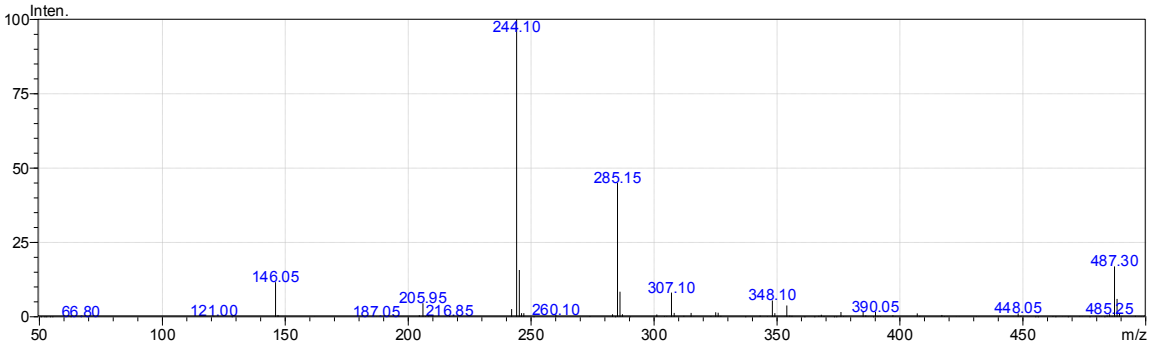
4th peak **Contd…**



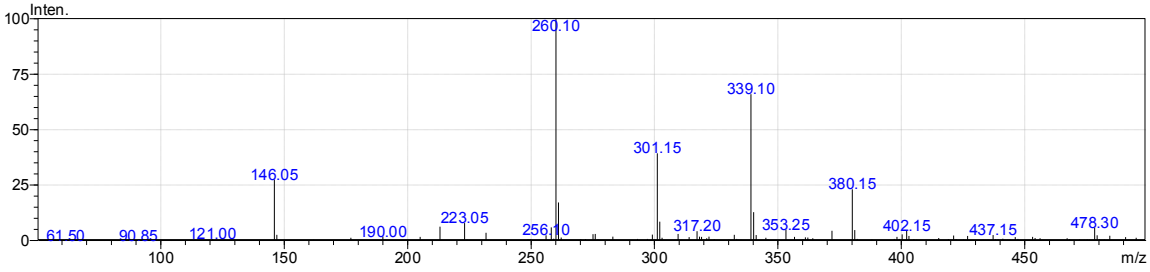
5th peak



6th peak

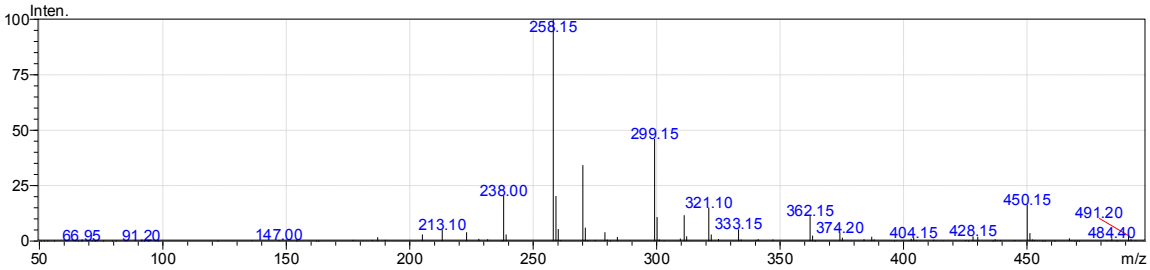


7th peak

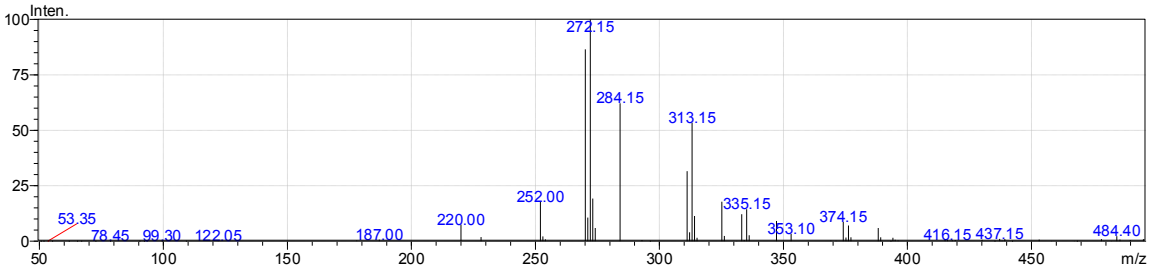


8th peak

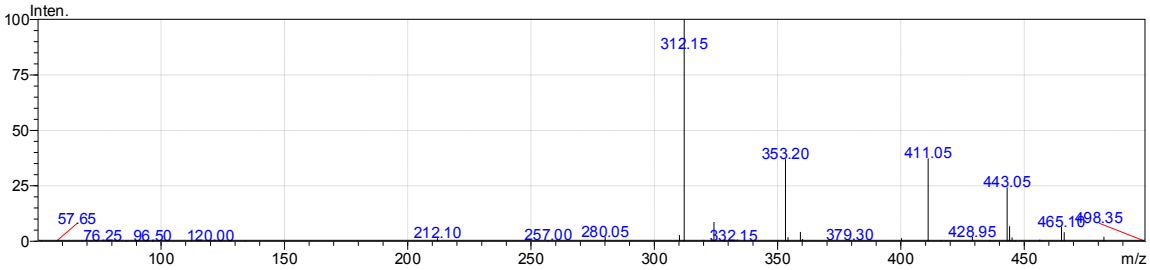
Contd…



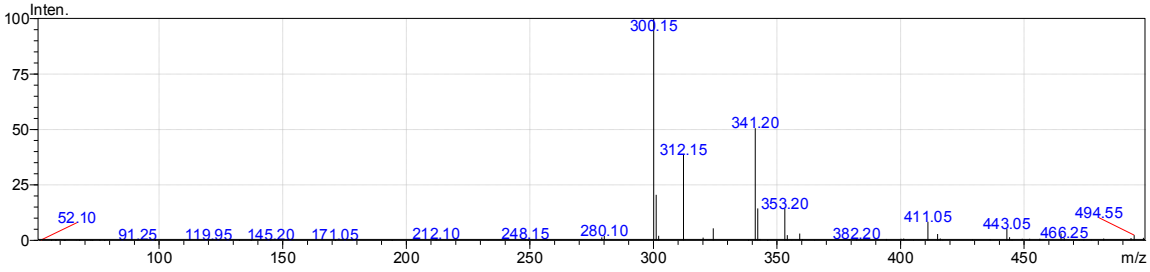
9th peak



10th peak



11th peak



12th peak

Figure 4.59: ESI mass spectra of EEPA constituents

CHAPTER 5

MATERIALS AND METHODS

5.1. REAGENTS

Chemicals	Make
HiPurA™ Bacterial Genomic DNA Purification Kit, HiPurA™ PCR Product Purification Kit, Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Antibiotic solution, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), Ethidium bromide, Molecular biology grade water	HiMedia Laboratories Pvt. Ltd.
Agarose gel	Lonza, ME, USA
1 Kb DNA Ladder	New England Bio labs.
TRI Reagent®, LPS (E. coli serotype 0111:B4), Prednisolone, Tween 20	SIGMA-ALDRICH
Verso cDNA Synthesis kit	Thermo Fisher Scientific, Inc.
KAPA SYBR® FAST qPCR Kit Master Mix (2x) Universal	KAPA BIOSYSTEMS
Silica gel (# 60-120, 230-400 and 100-200) and silica gel G and GF_{254}, Dimethyl Sulfoxide (DMSO)	Merck Specialties Private Limited
Anhydrous sodium sulphate (Na_2SO_4), Anhydrous calcium chloride ($CaCl_2$), Methylcellulose 4000 CPS	S. D. Fine-chem Ltd. Mumbai, India
Enzyme linked immunosorbent assay (ELISA) kits for TNF-α	eBiosciences Inc.

All reagents used were of analytical grade. All the consumables used for the cell culture work were sterilized. The extract and compounds were dissolved in 100% DMSO and added directly to the culture media before the addition of LPS. The final concentration of DMSO never exceeded 0.1%.

5.2. CHEMICAL AND PHYSICAL ANALYSIS

Apparatus / Instrument	Make / Model
Orbital shaking incubator	REMI Laboratory instruments, Mumbai, India
Rotary evaporator	Buchi R-210; Switzerland
Lyophilizer	SCANVAC, COOL SAFE™, Labogene, Denmark
UV cabinet	Bio technics, India
Polymerase Chain Reaction (PCR)	MJ Mini Personal thermal cycler; BIO-RAD, CA, USA
Melting point	VMP-DS; VEEGO, New Delhi, India
UV/Vis Spectrophotometer	Jasco V-650, Japan
High Performance Liquid Chromatography (HPLC)	Prominence; Shimadzu, Japan
Reverse phase column	Kinetex 5µ C18 100A, 250 x 4.60 mm; Phenomenex
Nuclear Magnetic Resonance (NMR) (tetramethylsilane (TMS) as an internal reference).	DRX 500; Bruker, ME, USA
Mass Spectroscopy (MS) and LC-ESI-MS	LCMS-2020; Shimadzu, Japan
Polarimeter (cell length = 20 cm)	PA-1R DELUXE Advanced polarimeter, New Delhi, India.
Multi detection reader	Spectramax M4, California, USA
Real Time-PCR (RT-PCR)	CFX Connect real-time PCR; BioRad, CA, USA

The chemical shift values in NMR spectra are reported in delta (δ ppm) units. MS were recorded in Electron Spray Ionization (ESI) mode. Analytical HPLC was performed on reverse phase column using water and acetonitrile (CH_3CN) in a stepwise gradient and Photo Diode Array (PDA) (SPD-M20A) system. Thin layer chromatography (TLC) was prepared using silica gel G/GF_{254} and spots were visualized by exposing to Iodine vapour and by

spraying 10% sulphuric acid (H_2SO_4) in methanol (MeOH) followed by heating the plates at 110 °C for 15 min. All the compounds were routinely dried over anhydrous $CaCl_2$ for 24 h in vacuo and were tested for purity by TLC or HPLC. Anhydrous Na_2SO_4 was routinely used for drying the organic solvents.

5.3. LEAD IDENTIFICATION FROM *PSEUDOMONAS AERUGINOSA*

5.3.1. Isolation of bacteria

Soil sample from the rhizosphere of groundnut crop was collected from Thandur village, Mahabubnagar District, Telangana State, India. Thirty-day-old plants were uprooted, and the roots with enclosed soil were collected in plastic bags. For the isolation of bacteria from the rhizospheric soil, 10 g of soil was mixed with 90 ml of sterile distilled water. Then, a serial dilution of the suspension (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) was prepared. About 0.1 ml from each dilution was spreaded on King's B medium and incubated at 28 °C until the bacterial colonies were formed. The bacterial colonies were incubated for 24 h and then the plates were viewed under UV transilluminator by observing fluorescence effect in King's B medium. The detected fluorescence spots were picked and streaked in fresh plates containing King's B media. The plates were then incubated and maintained for further use (Battu *et al*., 2009).

5.3.2. Identification of bacteria

5.3.2.1. Identification by using King's A media

The isolated bacteria were streaked into the freshly prepared and sterilized King's A media taken in a petri plate. Incubation of the petri plate was done at 28 °C for 48 h. A blue-green zone was formed around the colonies, which was extracted using 5 ml of chloroform and five drops of HCl was added. A colour change from blue to red was observed indicating the presence of pyocyanin, which is characteristic of *P. aeruginosa*.

5.3.2.2. Isolation of genomic DNA and PCR amplification of 16S rDNA

Isolated microorganism was grown in 5 ml of King's B broth at 37 °C for overnight by constant agitation. Using Genomic DNA Purification Kit according to manufacturer's protocol the genomic DNA was isolated. Genomic DNA concentration and purity was determined by measuring absorbance at 260 nm and 280 nm using Multi detection reader. Amplification of 16S rDNA was carried out using universal primers PS16F 5'TGGCTCAGATTGAACGCTGGCGG-3' and PS16R 5'GATCCAGCCGCAGGTTCCCCTAC-3' and genomic DNA as a template. 200 ng/µl of the extracted DNA was used as a template. The amplification was carried out in 50 µl reaction mixture containing 200 ng each of forward and reverse primer, 200 µM each dNTP, 1 unit of Taq DNA polymerase, 5 µl of 10X PCR buffer and 1.5 mM of magnesium chloride ($MgCl_2$). Amplifications were performed with a thermal cycler, after an initial 3 min denaturation at 94 °C, the samples were cycled for 40 sec at 94 °C, 30 sec annealing at 60 °C, and 90 sec extension at 72 °C for 35 cycles. PCR products were analyzed on 1% agarose gel and visualized with UV light after staining with ethidium bromide. The appropriate size amplification products were gel purified by using PCR Product Purification Kit according to the instructions of the manufacturer.

5.3.2.3. DNA Sequencing and sequence analysis of amplified products

Gel purified amplicons were sent for commercial sequencing at Merck Millipore, Bangalore, India. The amplicons were sequenced with previously described forward and reverse primers. Chromatograms containing mixed or overlapping peaks were excluded for further sequence analysis and the samples were resequenced to obtain clear chromatograms with reduced base noise.

BLAST search was performed to find out similar sequences in the Gene Bank database (Nucleotide Blast). Phylogenetic tree was constructed using Neighbour-joining (NJ)

method in MEGA version 5.0 to analyze the evolutionary relationship of isolated strain with the strains available in the database.

5.4. CULTIVATION OF *PSEUDOMONAS AERUGINOSA*

5.4.1. Preparation of *Pseudomonas aeruginosa* preculture

Twenty-five ml of King's B broth was poured in 100 ml capacity flask and was inoculated with 1 ml of freshly grown *P. aeruginosa* strain under laminar airflow system. This was allowed to incubate for a week at 30 °C and maintained for further use adhering to safety guidelines.

5.4.2. Cultivation of *Pseudomonas aeruginosa*

Twenty-five liters of King's B broth was inoculated with the preculture and allowed to grow at 30 °C for seven days. A small quantity of EtOAc was added to the culture flask to stop the growth of the bacteria and then the culture broth was centrifuged for 10 min at 10,000 rpm cautiously. The supernatant and the precipitate were collected separately. The supernatant was extracted using EtOAc in a separatory funnel with vigorous shaking. The EtOAc layer of the culture filtrate was collected. The extraction was repeated thrice and the EtOAc layers were pooled and evaporated under reduced pressure to yield a dry residue (13 g), which was designated as **EEPA**. The process was repeated twice to give more quantity of **EEPA**.

5.5. LC-MS ANALYSIS OF EEPA

LC-ESI-MS analysis was performed on LCMS-2020 Shimadzu system. The LC part was performed on LD-20AD pump with SIL-20ACHT autosampler. The conditions used for the LC separation were optimized as gradient mode water (A) and acetonitrile (B) given in Table 5.1. Experiment was carried out using Shimadzu HPLC packed column (Shim-Pack XR-ODS, 100 x 3.0 mm) at a flow rate of 0.3 ml/min. An SPD-M20A (diode array) detector was used to monitor the chromatographic process. The analysis was done in positive as well as negative ion mode. The conditions for the mass spectrometry were as follows: drying gas (nitrogen) flow 15 L/min; nebulizer flow 1.5 L/min; desolvation line temperature 250 °C; heat block temperature 250 °C; flow at split end before entering mass spectrometer i.e. 0.075 ml (to mass spectrometer): 0.225 ml (to PDA detector). The program Lab solutions (Release 5.42 SP6) was operated for system control of the MS and data acquisition. **EEPA** of 1000 µg/ml was injected to record the chromatogram.

Table 5.1: Gradient program proposed for the analysis of EEPA

Time (min)	**Mobile Phase (A%)**	**Mobile Phase (B%)**	**Profile**
0.00-0.01	60	40	Isocratic
0.01-7.00	60 → 10	40 → 90	Linear ramp to 90% B
7.00-9.00	10	90	Isocratic
9.00-12.00	10 → 95	90 → 05	Linear ramp to 95% A
12.00-15.00	96 → 60	05 → 40	Linear ramp to 40% B
15.01	Stop		

5.6. ISOLATION OF SECONDARY METABOLITES OF *PSEUDOMONAS AERUGINOSA*

EEPA (600 mg) was chromatographed over silica gel (25 g) starting with hexane–EtOAc (10:1) as mobile phase, which eluted out fatty components (glycerides and fatty acids). Continued elution with EtOAc–MeOH (1:0 to 8:1) yielded a mixture of three in fractions 15-16 (164 mg, eluted with EtOAc–MeOH, 10:1) and **PA-1** in fractions 18-19 (40 mg, eluted with EtOAc–MeOH, 9:1). The residue obtained from fractions 15–16 was rechromatographed over silica gel with $CHCl_3$–MeOH to give **PA-2** (18 mg, eluted with $CHCl_3$–MeOH, 20:1) and a 3:1 mixture of two (84 mg, eluted with $CHCl_3$–MeOH, 12:1). This mixture (15 mg) was further separated by Prep-HPLC (conditions: solvent, MeOH–H_2O (3:4); flow rate 0.8 ml/min; detection by UV at 243 nm, **PA-4** and **PA-3** eluted at 6.2 and 7.0 min, respectively) to give **PA-3** (2.5 mg) and **PA-4** (8 mg). The residue obtained from fractions 18-19 was rechromatographed over silica gel to give **PA-1** (16 mg, eluted with $CHCl_3$–MeOH, 9:1).

PA-1

Colourless crystal; m.p. 177-180 °C; $[\alpha]^{25}_D$ of -117.7 (c 1.55, MeOH); IR (ν_{max}): 1680, 1648, 1460, 1298, 1414, 1340 and 3180-3100 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): δ 3.57-3.49 (2H, m, 3-H), 2.03-1.91 (2H, m, 4-H), 2.03-1.91 (1H, m, 5a-H), 2.33-2.29 (1H, m, 5b-H), 4.24-4.20 (1H, dd, *J* = 8.9, 2.2 Hz, 6-H), 4.12 -4.08 (1H, d, *J* = 16.8 Hz, 9a-H), 3.73 (1H, d, *J* = 16.8 Hz, 9b-H); ^{13}C NMR (CD_3OD, 125 MHz): δ 170.6 (C-1), 165.0 (C-4), , 58.5 (C-9), 45.6 (C-3), 44.9 (C-6), 28.0 (C-7), 21.9 (C-6). FAB-MS: *m/z* found for $C_7H_{10}N_2O_2$: 155 $[M+H]^+$, 149, 135, 117, 93, 75 (100%), 57.5.

PA-2

White amorphous solid; m.p. 130-132 °C; $[\alpha]^{25}_{D}$ – 182.2 (*c* 0.09, MeOH); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.32-7.27 (5H, m, 12-16-H), 5.81 (1H, br s, 2-H), 4.28 (1H, dd, *J* = 3.2, 10.4 Hz, 3-H), 4.07 (1H, t, *J* = 8.2 Hz, 9-H), 3.67-3.54 (1H, m, 6-H), 3.54-3.67 (1H, m, 6-H), 2.80 (1H, dd, *J* = 10.4, 14.5 Hz, 10-H), 2.37-2.31 (1H, m, 8b-H), 2.06-1.83 (1H, m, 8a-H), 2.03-1.83 (2H, m, 7-H),; ^{13}C NMR ($CDCl_3$, 125 MHz): δ 169.4 (C-1), 165.1 (C-4), 136.0, 129.16 (C-12 and C-16), 129.24 (C-15 and C-13), 127.5 (C-14), 59.1 (C-3), 56.2 (C-9), 45.4 (C-6), 36.8 (C-10), 28.4 (C-8) and 22.5 (C-7); FAB-MS: *m/z* found for $C_{14}H_{16}N_2O_2$: 245.12 $[M+H]^+$, 154 (100%), 136, 107, 89, 77, 51.

PA-3

Amorphous solid; $[\alpha]^{25}_{D}$ – 7.0 (*c* 0.09, MeOH); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.32-7.27 (5H, m, 12-16-H), 4.52-4.50 (1H, m, 3-H), 4.40-4.35 (1H, dd, 9-H), 4.32-4.28 (1H, m, 7-H), 3.75-3.68 (1H, dd, 6b-H), 3.22-3.12 (1H, m, 6a-H), 3.21-3.12 (2H, m, 10-H), 2.10-2.08 (1H, dd, 8b-H) and 1.42-1.38 (1H, dd, 8a-H); FAB-MS: *m/z* found for $C_{14}H_{16}N_2O_3$: 261.12 $[M+H]^+$, 227, 154 (100%), 136, 107, 89, 77, 51.

PA-4

Yellowish amorphous powder; m.p. 178-179 °C; $[\alpha]^{25}_{D}$ [-135.2 (*c* 0.71, MeOH); IR (ν_{max}): 3450, 3273, 1680, 1617, 1460, 1303, 1440 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz): δ 4.51 (1H, ddd, *J* = 1.3, 6.6, 11.2 Hz, 9-H), 4.45 (1H, t, *J* = 4.4 Hz, 7-H), 4.17-4.14 (1H, m, 3-H), 3.65 (1H, dd, *J* = 4.5, 12.8 Hz, 6a-H), 3.43 (1H, d, *J* = 12.7 Hz, 6b-H), , 2.27 (1H, dd, *J* = 6.5, 13.3 Hz, 8a-H), 2.08 (1H, ddd, *J* = 4.3, 11.2, 13.2 Hz, 8b-H), 1.94-1.85 (1H, m, 10a-H), 1.94-1.85 (1H, m, 11-H), 1.53-1.48 (1H, m, 10b-H), 0.96 (3H, d, *J* = 6.3 Hz, 13-H) and 0.95 (3H, d, *J* = 6.3 Hz, 12-H); ^{13}C NMR (CD_3OD, 125 MHz): δ 171.6 (C-1), 167.6 (C-4), 67.7 (C-7), 57.3 (C-9), 53.8 (C-6), 53.2 (C-3), 38.0 (C-10), 36.8 (C-8), 24.4 (C-11), 22.0 (C-13) and 20.9 (C-12); FAB-MS: *m/z* found for $C_{11}H_{18}N_2O_3$, 227.14 $[M+H]^+$, 154 (100%), 136, 106, 89, 77, 51.

5.7. CHEMICAL INVESTIGATION OF *ABUTILON INDICUM*

5.7.1. Extraction of plant material

Around 5 kg of dried and powdered aerial parts of *A. indicum* was collected from Jawahar Nagar village, Shameerpet, Hyderabad and authenticated by Dr. V. Chelladurai Research Officer, Botanical Survey of India, Government of India. A herbarium of *A. indicum* is stored at Department of Pharmacy, BITS-Pilani Hyderabad Campus.

The powdered aerial part of *A. indicum* (5 kg) was subjected to Soxhlet extraction for 48 h, first with petroleum ether to remove fatty constituents and subsequently by methanol. The obtained organic extract was concentrated to one-eighth of original volume under reduced pressure using rotavapor and lyophilized to get a yield of 547.38 g (**AIM**).

5.7.2. Isolation of chemical constituents of *Abutilon indicum*

The crude methanolic extract was fractionated (Scheme 2) using different organic solvents starting from non-polar to polar solvents using mechanical stirrer. Solvents used were in the order of hexane, chloroform, ethyl acetate and butanol. The respective fractions were concentrated under reduced pressure using rotavapor. The chloroform soluble fraction (**AIM-C**) yielded 37 g of dry residue, which was divided into two parts of 1.5 g and 35.5 g for pharmacological screening and chromatographic purification, respectively.

5.7.3. Purification of chloroform fraction

The concentrated and dried **AIM-C** (35.5 g) was subjected to column chromatography using silica gel # 60-120 mesh size. The elution was started with hexane and continued with toluene followed by EtOAc. The column was monitored using TLC. The eluates, which were found to show interesting spots in TLC, were further taken for purification by CC.

Column elution with hexane followed by toluene and toluene:EtOAc (90:10) yielded fatty esters. The toluene:EtOAc (75:25) showed positive color change with ferric chloride

($FeCl_3$) reagent and hence were taken for re-column chromatography. The concentrated 1.5 g of eluate was adsorbed on silica gel (#230-400) and was loaded into column. The elution was started with 100% of toluene. Based on TLC observation, 12 mg toluene:EtOAc (95:05) eluate was further purified by re-CC using silica gel (#230-400). Toluene:EtOAc (99:01) eluate yielded 4.8 mg of single compound, which was designated as **AI-1**. The 20 mg eluate obtained using toluene:EtOAc (90:10) system yielded pure crystals (10.5 mg) labeled as **AI-2**.

The two major eluates i.e. toluene:EtOAc (80:20) and toluene: EtOAc (60:40) from column fraction were taken for column purification. The toluene:EtOAc (80:20) yielded two compounds designated as **AI-3** and **AI-4**. Compound **AI-4** (5 mg) was obtained in pure form where as **AI-3** was impure and hence was purified by re-column chromatography using silica gel (#230-400). Yield of purified **AI-3** was found to be 6.8 mg. The 600 mg of toluene:EtOAc (60:40) eluate was re-chromatographed using silica gel (# 230-400) and the hexane:EtOAc (35:65) eluate yielded 15 mg of pure compound designated as **AI-5**.

The final major eluate i.e. 100% EtOAc from **AIM-C** was re-chromatographed using silica gel (# 230-400), which did not yield any major compound other than β-sitosterol glucoside.

AI-1

White amorphous solid; m.p. 138-140 °C; IR (ν_{max}): 3372, 1685, 1590, 1517, 1439 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 7.64 (1H, d, *J* = 16.0 Hz, 7-H), 7.43 (2H, d, *J* = 8.5 Hz, 6-H), 6.86 (2H, d, *J* = 8.5Hz, 5-H), 6.30 (1H, d, *J* = 16.0 Hz, 8-H), 3.78 (3H, s, 10-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 167.9 (C-9), 157.6 (C-8), 144.5 (C-7), 129.9 (C-2), 129.9 (C-6), 115.8 (C-5), 115.8 (C-3) and 51.6 (C-10),; ESI-MS: *m/z* found for $C_{10}H_{10}O_3$, 177 $[M-H]^-$.

AI-2

Colourless crystals; m.p.158-161°C; IR (ν_{max}): 3471, 1684, 1617, 1529 and 1446, cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.59 (1H, d, *J* = 16.0 Hz, 7-H), 7.09 (1H, d, *J* = 2.0 Hz, 5-H), 7.02 (1H, dd, *J* = 2.0 & 8.5 Hz, 2-H), 6.80 (1H, d, *J* = 8.0 Hz, 3-H), 6.27 (1H, d, *J* = 16.0 Hz, 8-H) and 3.80 (3H, s, 10-H); ^{13}C NMR (CD_3OD, 125 MHz): δ 169.7 (C-9), 146.9 (C-7), 129.9 (C-2), 129.9 (C-6), 115.1 (C-8), 115.8 (C-5), 115.8 (C-3) and 52.0 (C-10); ESI-MS: *m/z* found for $C_{10}H_{10}O_4$, 193 $[M-H]^-$.

AI-3

Colourless crystals; m.p. 205-209 °C; IR (ν_{max}): 3482, 3042-2772, 1679 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz): δ 7.22 (2H, s, 1-H), 3.87 (6H, s, 3,4-H); ^{13}C NMR (CD_3OD, 125 MHz): δ 170.0 (C-7), 148.9 (C-3), 144.8 (C-4), 122.0 (C-1), 108.4 (C-2), 56.8 (C-8.9); ESI-MS: *m/z* found for $C_9H_{10}O_5$, 197 $[M-H]^-$.

AI-4

White amorphous powder; m.p. 210-213 °C; IR (ν_{max}): 3487, 3050-2900 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.64 (1H, dd, *J* = 8.5, 2.0 Hz, 6-H), 7.56 (1H, d, *J* = 2.0 Hz, 2-H), 6.91 (1H, d, *J* = 8.5 Hz, 5-H), 3.94 (3H, s, 8-H) and 3.17 (OH, br s, 4-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 168.5 (C-7), 150.4 (C-3), 146.5 (C-4), 121.9 (C-1), 124.4 (C-6), 114.2 (C-5), 112.2 (C-2) and 55.9 (C-8); ESI-MS: *m/z* $[M-H]^-$ found for $C_8H_8O_4$, 167.

AI-5

Greenish amorphous powder; m.p. 100-103 °C; $[\alpha]^{25}_{D}$ +10.2 (*c* 0.1, MeOH); IR (ν_{max}): 1760.69, 3550.31 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz): δ 5.64-5.54 (1H, m, 10,11-H), 3.95 (1H, dd, *J* = 5.0 & 15.0 Hz, 9-H), 3.80 (1H, t, *J* = 5.0 Hz, 12-H), 3.31 (1H, br s, 13-H), 2.18 (2H, br s, 2-H), 1.28-1.33 & 1.44-1.60 (22H, m, 3-16-H), 0.80 (3H, t, *J* = 6.3 Hz, 18-H); ^{13}C NMR (CD_3OD, 125 MHz): δ 177.7 (C-1), 136.6 (C-11), 131.1 (C-10), 76.5 (C-12), 75.8 (C-13), 73.0 (C-9), 38.3 (C-8), 35.0 (C-2), 34.9 (C-2), 33.5 (C-14), 33.1 (C-16), 30.5 (C-4), 30.4 (C-5), 30.2 (C-6), 26.6 (C-7), 26.1 (C-3), 23.7 (C-17) and 14.4 (C-18); ESI-MS: *m/z* found for $C_{18}H_{34}O_5$, 329 $[M-H]^-$.

5.8. IN-VITRO SCREENING USING LPS-INDUCED TNF-α PRODUCTION MODEL

5.8.1. In-vitro screening using mouse macrophages cell line RAW 264.7

5.8.1.1. Cells and cell culture

Mouse macrophages cell line RAW 264.7 obtained from the Cell bank of National Center for Cell Sciences, Pune (Maharashtra, India) was cultured in DMEM supplemented with 10% heat inactivated FBS and 1% antibiotic solution in a humidified atmosphere of 5% CO_2 at 37 °C and passaged every 1–2 days to maintain logarithmic growth.

5.8.1.2. Measurement of TNF-α protein production using ELISA

The effect of **EEPA**, **PA-1**, **PA-2**, **PA-3**, **PA-4**, **AIM**, **AIM-C**, **AI-1**, **AI-2**, **AI-3**, **AI-4** and **AI-5** on the production of TNF-α was measured by ELISA assay. For ELISA, 10,000 RAW 264.7 cells per well were seeded on 96-well plate over-night. Cells were pre-incubated with samples for 1 h, and then stimulated with 1 μg/ml of LPS for another 6 h. The cytokine concentration was calculated according to the standard curve using recombinant cytokine in each ELISA kit.

5.9. MEASUREMENT OF RELATIVE mRNA LEVELS OF TNF-α USING QUANTITATIVE RT-PCR

RAW 264.7 cells (2.5×10^5 cells/well) were seeded in 24-well plates over night and were treated with **EEPA**, **PA-1**, **PA-2**, **PA-3**, **PA-4** and standard prednisolone for 24 h. LPS (1 μg/ml) was added to sample wells and incubated for 6 h followed by the isolation of RNA for quantification of expression levels using qRT-PCR.

5.9.1. Isolation of total RNA

The total RNA from the LPS treated-RAW264.7 cells was prepared by adding TRI Reagent according to manufacturer's protocol. The concentration of RNA content was determined by measuring the absorbance at 260 and 280 nm using UV-Vis

Spectrophotometer. Purity of RNA was determined with A_{260}/A_{280} ratio (1.8–2.0 was considered pure). The total RNA solution was stored at –20 °C until used. During RNA isolation and analysis, RNase free environment was maintained and all the solutions used were RNase free.

5.9.2. cDNA Synthesis

One microgram of total RNA was taken and cDNAs were synthesized using Verso cDNA Synthesis kit. RNA (1 μg) sample was further added to reaction mixture containing final volumes of 1X cDNA synthesis buffer, 500 μM of dNTP Mix, 500 ng of Anchored Oligo-dT primers, 1 μl of RT Enhancer (to remove contaminating DNA), Verso Enzyme Mix (includes reverse transcriptase) and the final volume was adjusted with water (molecular biology grade). Reverse transcription cycling program was set at the temperature 42 ºC in 1 cycle of 30 min and at 95 °C for 2 min for reaction termination using MJ Mini Thermal Cycler.

5.9.3. Quantitative RT- PCR analysis

Primer sequences for analysis of TNF-α and GAPDH mRNA were taken from recent published work (Wang *et al*., 2012) and are described in Table 5.2. Quantitative RT-PCR was performed in a 10 μl reaction volume containing 2X SYBR FAST Master Mix, 400 nM primers and appropriately diluted cDNA template. PCR was carried out for 30 cycles under these incubation conditions (a 25 sec denaturation time at 94 °C, an annealing time of 30 sec at 60 °C, an extension time of 60 sec at 72 °C and final extension of 10 min at 72 °C at the end of 30 cycles). The amount of mRNA for all the target genes were normalized against the housekeeping gene GAPDH in the corresponding samples. Quantification of the samples was carried out with Sequence Detection CFX Manager 3.0 analysis software (Bio-Rad Laboratories, Inc.).

Table 5.2: Primers used for TNF-α and GAPDH

mRNA	Primers	Sequence (5'-3')	Size (bp)
TNF-α	Sense	CCGCTCGTTGCCAATAGTGATG	235
	Antisense	CATGCCGTTGGCCAGGAGGG	
GAPDH	Sense	AGTGGCAAAGTGGAGATT	83
	Antisense	GTGGAGTCATACTGGAACA	

5.10. CHEMICAL SYNTHESIS OF CYCLIC DIPEPTIDES

To a stirred solution of *trans*-4-hydroxy-L-proline methyl ester hydrochloride (216 mg, 1.19 mM) and triethylamine (200 µl, 1.43 mM) in dichloromethane (CH_2Cl_2) (6.0 ml) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (389 mg, 2.03 mM) and *N*-(*tert*-butoxycarbonyl)-L-leucine (128 mg, 1.20 mM) at 0 °C. The mixture was stirred at 0 °C for 3 h and then at room temperature for 10 h. CH_2Cl_2 and brine were added and the organic layer was washed with 0.2 M hydrochloric acid (HCl), saturated sodium bi-carbonate ($NaHCO_3$) and brine. The separated CH_2Cl_2 layer was dried over anhydrous Na_2SO_4 and concentrated to give a crude product. This was subjected to CC with increasing polarities of $CHCl_3$-MeOH to give the protected dipeptide (290 mg, 81%, eluted with $CHCl_3$-MeOH 10:1) as oil.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ 0.95 (3H, d, *J*=6.4 Hz), 0.98 (3H, d, *J*=6.4 Hz), 1.41 (9H, s), 1.46 (1H, m), 1.55 (1H, m), 1.75 (1H, m), 2.00 (1H, m), 2.35 (1H, m), 3.19 (1H, brs, O*H*), 3.68 (1H, dd, *J*=11.0, 3.6 Hz), 3.73 (3H, s, OCH_3), 4.02 (1H, brd, *J*=11.0 Hz), 4.41 (1H, m), 4.54 (1H, brs, N*H*), 4.67 (1H, t, *J*=8.4 Hz), 5.17 (1H, d, *J*=8.4 Hz).

A solution of the protected dipeptide (289 mg, 0.806 mM) in CH_2Cl_2 (3.0 ml) and trifluoroacetic acid (TFA) (0.4 ml, 5.22 mM) was stirred at room temperature for 2.5 h. The solvent and TFA were removed on a rotary evaporator and the residue was dissolved in

toluene (10 ml). The solution was heated at 70 °C for 2 h. The reaction mixture was subjected to CC without workup. Elution with $CHCl_3$-MeOH (12:1) afforded the cyclic dipeptide (**PA-4**) (159 mg, 87%) as a colorless solid. Crystallization from MeOH gave an analytical sample as white needles, melting point 174-176 °C (lit. 178–179 °C), $[\alpha]^{25}_D$ –129.5 (*c* =1.0, MeOH) (lit. –148.4 (H_2O)) (Ienaga *et al*., 1987). The ^1H and ^{13}C NMR spectra were superimposable with those of literature report (Sajeli Begum *et al*., 2014).

5.11. CHEMICAL SYNTHESIS OF METHYL CAFFEATE (AI-2)

Caffeic acid (2.5 gm, 13.873 mmol) was dissolved in dry methanol (150 ml) and conc. H_2SO_4 (2.5 ml, 96%, v/v) was added. Reaction mixture was stirred at room temperature for 12 h and then evaporated to approximately 50 ml. To the mixture, ice-cold saturated solution of $NaHCO_3$ (150 ml) was added and after short stirring, it was extracted with ethyl acetate (3 x 75 ml). Organic layers were combined, dried over anhydrous Na_2SO_4 and evaporated to afford **AI-2** (700 mg) (Džubák *et al*., 2006).

AI-2 was further purified by CC and was eluted with 100% hexane slowly increasing the polarity and finally **AI-2** was found to be eluted with EtOAc:Hexane (15:85) in pure form (Yield = 400 mg). The authenticity of **AI-2** was confirmed by co-TLC [EtOAc:Hexane (1:1), R_f 0.55] and co-injection in HPLC (eluted at same retention time as that of isolated **AI-2**) with the natural **AI-2**.

5.12. IN-VIVO SCREENING OF EEPA, PA-4 AND AI-2 FOR THE ESTIMATION OF LPS-INDUCED TNF-α PRODUCTION

5.12.1. Experimental Protocol

Animals	Wistar rat
Sex	Female
Weight range of animals	220-250gm
Inducing agent	LPS (E. coli serotype 0111:B4)
Vehicle and Preparation	Sterile saline, 0.3 mg of LPS was dissolved in 1 ml of sterile saline
Dose	0.3 mg/kg
Dose volume	1 ml/kg
Administration Route	Intraperitoneal injection
Test compound solubility	0.5% Sodium methyl cellulose
Dose of test compound	Based on respective acute toxicity data
Route of administration of test compound	Oral
Standard drug used	Prednisolone
Vehicle	0.5% Sodium methyl cellulose
Dose	10 mg/kg
Number of rats in each group	5

5.12.2. Animals

Inbred female Wistar albino rats of 8-10 weeks old weighing between 150-250 g were used for studying TNF-α inhibition effect. The animals were stabilized for one week. They were maintained on standard pellet diet and water *ad libitum* throughout the experiments, animals were processed according to the stipulation of the Institutional Animal Care and Use

Committee (IACUC). Approval from the Institutional Animal Ethics Committee, BITS-Pilani Hyderabad Campus was taken prior to the experimental work. The animals were randomly divided into groups of five animals each and kept in separate cages. None of the animals were sacrificed throughout the study. All procedures were conducted according to the guidelines of the committee for the purpose of Controls and Supervision of Experiments on Animals, India.

5.12.3. Procedure

Rats were fasted 18 h prior to oral dosing, and allowed free access to water throughout the experiment. Each treatment group consisted of five animals. Test compounds were prepared as a suspension in a vehicle consisting of 0.5% methylcellulose, 0.025% Tween 20. The **EEPA**, **PA-4**, **AI-2** or vehicle was administered by oral gavage in a volume of 10 ml/kg. LPS was administered after 60 min of test compounds as intraperitonial injection at a dose of 0.3 mg/kg in sterile saline, a dose previously determined to be optimal. Blood was collected in eppendorf tubes via retro-orbital puncture 90 min after LPS injection, a time point corresponding to maximal TNF-α production. Plasma was separated by centrifugating blood sample at 4 °C for 10 min at 4000 rpm and stored at –80 °C until it was assayed for TNF-α by ELISA (Burnette *et al*., 2009).

5.13. MEASUREMENT OF RELATIVE mRNA LEVELS OF IL-1β AND IL-6 USING QUANTITATIVE RT-PCR

The procedure remains same as described in section 5.9. The primer sequences for analysis of IL-1β, IL-6 and GAPDH mRNA were taken from recent published work (Wang *et al*., 2012) and are described in Table 5.3.

Table 5.3: Primers used for IL-1β, IL-6 and GAPDH

mRNA	Primers	Sequence (5'-3')	Size (bp)
IL-1β	Sense	GCACTACAGGCTCCGAGATGAA	145
	Antisense	GTCGTTGCTTGGTTCTCCTTGT	
IL-6	Sense	CTTGGGACTGATGCTGGTGACA	118
	Antisense	GCCTCCGACTTGTGAAGTGGTA	
GAPDH	Sense	AGTGGCAAAGTGGAGATT	83
	Antisense	GTGGAGTCATACTGGAACA	

5.14. MEASUREMENT OF NITRIC OXIDE PRODUCTION

Nitrite, which is the end-point of NO generation by activated macrophage, was measured by a colorimetric assay. Cells (5×10^5 cells/ml) were seeded in 24-well plates and treated with different concentrations of **EEPA**, **PA-1**, **PA-2**, **PA-3** and **PA-4** for 5 h, and then incubated in the presence of LPS (1 μg/ml) for 24 h. Nitrite accumulation in the culture medium was measured as an indicator of NO production. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid), the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm was measured using a Multi detection reader. Fresh culture medium was used as a blank, the net NO levels were calculated by subtracting the control values obtained without LPS and drug alone.

5.15. MTT ASSAY FOR TESTING CELL VIABILITY

Cells were cultured at a density of 5000 cells RAW 264.7 per well in flat-bottomed 96-well plates and supplemented with various concentrations of **EEPA** (100-1 μg/ml) and **PA-1** to **PA-4** (100-1 μM/ml) after 1 h of incubation LPS (1 μg/ml) was added. LPS incubation was for 24 h, and then 20 μl of MTT reagent dissolved at 5 mg/ml in phosphate buffer saline (PBS) was added to each well and incubated for 3 h at 37 °C. After 3 h, media was removed by aspiration and in each well, 50 μl of DMSO was added to dissolve formazan crystal. The extent of the reduction of MTT was quantified by measurement of absorbance at 570 nm using multimode plate reader.

CHAPTER 6
SUMMARY AND CONCLUSION

Based on the thorough literature review, one microbial source i.e. ***Pseudomonas* bacteria** and one plant source i.e. ***Abutilon indicum*** were selected for the discovery of the lead molecules inhibiting pro-inflammatory cytokines.

In the first part of the study, a bacterial strain isolated from the rhizospheres region of groundnut crop using King's B media was identified as ***Pseudomonas aeruginosa*** 16S ribosomal RNA through molecular characterization by 16S rDNA sequencing and amplification using universal forward and reverse primers, and genomic DNA as template. The strain showed 97% similarity to *P. aeruginosa* (Accession number: HM067869.1) when compared to the reported species of database. A freshly grown *P. aeruginosa* culture broth mixed with ethyl acetate was centrifuged and the supernatant was extracted with ethyl acetate, which was then lyophilized to get a dry residue, designated as **EEPA**.

Four compounds designated as, **PA-1**, **PA-2**, **PA-3** and **PA-4** were isolated from **EEPA** by column chromatographic separation. Their structures were extensively elucidated by spectral analysis and were determined as cyclo(L-Proline-Glycine) (**PA-1**), cyclo(L-Proline-L-Phenylalanine) (**PA-2**), cyclo(*trans*-4-hydroxy-L-Proline-L-Phenylalanine) (**PA-3**) and cyclo(*trans*-4-hydroxy-L-Proline-L-Leucine) (**PA-4**). These cyclic dipeptides are reported for the first time from the culture broth of *P. aeruginosa*.

In the second part, *A. indicum* shrub was collected from Jawahar Nagar village, Hyderabad and was authenticated. The aerial parts were dried, milled and subjected for extraction with pet-ether followed by methanol. The crude methanol extract (**AIM**) was fractionated with different solvents of increasing polarities. Chloroform soluble fraction of *A. indicum* (**AIM-C**) showing phenolic constituents upon column chromatography lead to the isolation of five compounds. They were spectroscopically characterized as methyl *trans-p*-coumarate (**AI-1**), methyl caffeate (**AI-2**), syringic acid (**AI-3**), vanillic acid (**AI-4**) and pinellic acid (**AI-5**). All compounds are hereby reported for the first time from *A. indicum*

except vanillic acid.

During the pharmacological screening of **EEPA**, **AIM-C** and all isolated single compounds under in-vitro LPS-induced production of TNF-α using RAW 264.7 cell lines estimated by ELISA kits, all samples demonstrated a significant inhibition effect, except **AIM-C**, **AI-1**, **AI-3**, **AI-4** and **AI-5**.

Results displayed around 74.2% suppression of TNF-α by **EEPA** (100 μg/ml) with an IC_{50} value of 38.79 μg/ml. Among the isolated constituents of **EEPA**, cyclo(Gly-L-Pro) (**PA-1**) was found to be more potent exhibiting 60.2% inhibition (IC_{50} 4.48 μg/ml). Compounds **PA-3** (IC_{50} 14.24 μg/ml), **PA-4** and **PA-2** were found to inhibit TNF-α production to 53.8%, 49.3% and 45.0%, respectively at 100 μM concentration. The reference standard, prednisolone showed 42.8% inhibition at 10 μM concentration. The inhibitory effect of **PA-1** and **PA-3** was found to be similar to the standard drug, prednisolone at 10 μM concentration. Compound **AI-2** showed 55% inhibition at 100 μM concentration.

Parallelly, marked attenuation of mRNA expression of TNF-α in LPS-stimulated mouse macrophages was exhibited by **EEPA**, and **PA-1** ($p<0.001$), which were around 5 fold and 3 fold reduction respectively compared with LPS-control.

Further, in-vivo testing of **EEPA** at various dose levels and chemically synthesized **PA-4** and **AI-2** under rat endotoxin model was carried out. Results showed significant TNF-α reduction ($p < 0.001$) by **EEPA** and **PA-4** with 79.3% (500 mg/kg) and 51% (50 mg/kg), respectively. The less active drug (**PA-4**) under in-vitro TNF-α production assay, showing 51% inhibition under in-vivo model indicated drug like property of proline based cyclic dipeptides. **AI-2** did not show any inhibition at 10 and 50 mg/kg dose levels. The **EEPA** and isolated **PA** compounds were further tested for their inhibitory effect on IL-1β and IL-6 cytokines.

The effect of **EEPA** was more pronounced on IL-1β (7.5 fold reduction) compared to TNF-α and IL-6 (5 fold reduction). The highly functional **PA-1** was found to be potentially active in decreasing the expression of IL-1β (5.3 fold) and TNF-α (3.5 fold). **PA-2** was observed to be most active ($p < 0.001$) in reducing IL-6 compared to other diketopiperazines derivatives.

The study was further extended to measure the inhibitory effect of **EEPA** and **PA** compounds on NO production by Griess method. Reduction of > 50% by **EEPA** at 100 μg/ml concentration was supporting the previously observed cytokine inhibitory effect. Among the cyclic dipeptides, cyclo(Gly-L-Pro) (**PA-1**) exhibited significant control over NO production compared to **PA-2**, **PA-3** and **PA-4**, substantiating its pro-inflammatory cytokine suppressing effect.

In addition the cytotoxicity of **EEPA** and isolated peptides against LPS-stimulated RAW 264.7 cells proved their less toxic behavior i.e. **EEPA** (8% inhibition at 50 μg/ml) and isolated cyclic dipeptides (3-13% inhibition at 50 μM).

Thus, the outcome of in-vitro and in-vivo experiments disclosed the potent effect of culture broth extract of *P. aeruginosa* and its secondary metabolites in down-regulating a key cytokine, TNF-α. The study also explored the possibility of these cyclic dipeptide molecules to inhibit or control the expression of other cytokines, IL-1β and IL-6. As an outcome of the study, effective small molecular weight molecules as TNF-α inhibitors, which were also found to inhibit IL-1β and IL-6, and NO with preferred less cytotoxicity were identified.

In summary, a potential microbial source *P. aeruginosa,* secreting pro-inflammatory cytokine inhibitory compounds in the culture broth was discovered, based on various cellular models and an acute animal model. Interestingly, all identified cytokine inhibitory compounds were found to be small molecules.

Additionally existence of four 2,5-diketopiperazines derivative in *P. aeruginosa* culture broth and four phenolics in aerial part of *A. indicum* were reported for the first time. The aim for the identification of effective small molecules for treating chronic inflammation derived disease conditions was accomplished through the isolation of cyclo(L-Proline-Glycine) (**PA-1**) and cyclo(L-Proline-L-Phenylalanine) (**PA-2**) which were found to be TNF-α inhibitors (IC_{50} 4.48 μg/ml and 14.24 μg/ml respectively) as well as inhibiting IL-1β and IL-6, and NO production with preferred less cytotoxicity.

CHAPTER 7

FUTURE PERSPECTIVES

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1. In the present work we attempted to isolate low molecular weight lead molecules from two different natural sources effective in the treatment of inflammation derived therapeutic conditions. The ethyl acetate extract of culture broth of *P. aeruginosa* (**EEPA**) as well as the isolated low molecular weight cyclic dipeptides were found to show significant suppressive effect against important pro-inflammatory cytokines. The work can be continued to study the pharmacokinetic properties of these cyclic dipeptides and subjecting to chronic in-vivo studies, which will yield a new class of TNF-α inhibitors.
2. Potential activity and LC-MS map of **EEPA** upholds the possibility of existence of more effective chemical principles besides the isolated cyclic dipeptides in *P. aeruginosa* culture broth, hence further chemical profiling is warranted.

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Khan Rukaiyya Sirajuddin, Senthi Mahibalan, Rao Poorna Chandra, Basha Ameer, Alvala Mallika, Tummuri Dinesh, Hironori Masubuti , Yoshinori Fujimoto & Begum Ahil Sajeli (2014). Cytotoxic constituents of *Abutilon indicum* leaves against U87MG human glioblastoma cells. *Natural product research*, DOI:10.1080/14786419.2014.976643

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OTHER PUBLICATION

Ravi Alvala, Mallika Alvala, Sama Venkatesh, Begum Ahil Sajeli, **Khan Rukaiyya Sirajuddin**, & Reddy Mafhava. (2011). Antiproliferative activity and standardization of *Tecomella undulata* bark extract on K562 cells. *Journal of Ethnopharmacology*, *137*(3), 1353-1359.

PAPERS PRESENTED AT NATIONAL/INTERNATIONAL CONFERENCES

1. Senthil Mahibalan, Poorna Chandra Rao, **Rukaiyya Sirajuddin Khan**, Ahil Sajeli Begum. Newer cytotoxic leads from *Hedyotis umbellata* against lung and breast cancer. **66th Indian Pharmaceutical Congress**, 23rd to 25th January 2015 at Hyderabad, India.
2. **Rukaiyya Sirajuddin Khan**, Senthil Mahibalan, Ahil Sajeli Begum. Isolation and identification of chemical constituents of *Abutilon indicum*. **International Conference and Exhibition on Pharmacognosy, Phytochemistry and Natural Products**, 21st to 23rd October 2013 at Hyderabad, India.
3. Senthil Mahibalan, S. Maria, T. N. Rohan, **Rukaiyya Sirajuddin Khan**, Ahil Sajeli Begum. Dermal Wound Healing Potential of Alkaloid Enriched Ointment *Evolvulus alsinoides* in Albino rats. **International Symposium on Chemistry and Chemical Biology of Natural Products**, CCBNP-2012, 2 to 4 August, 2012, IICT (CSIR), Hyderabad, India.
4. **Rukaiyya Sirajuddin Khan**, Alvala Mallika, Senthil Mahibalan, Dharmarajan Sriram, Ahil Sajeli Begum. *In vitro* anticancer activity of *Abutilon indicum* leaves on human breast carcinoma cell line (MDA-MB-231). **Current Medicinal Chemistry, 4th International Conference on Drug Discovery and Therapy**, 12th to 15th February 2012 at Dubai, UAE.
5. **Rukaiyya Sirajuddin Khan**, Alvala Mallika, Mahibalan Senthil, Dharmarajan Sriram, Sajeli Begum Ahil. *In vitro* anticancer activity of *Abutilon indicum* leaves on U-87MG, a brain tumor cell line. **XLIV Annual Conference of Indian Pharmacological Society**, 19th to 21st December 2011 at Manipal, India.
6. Mahibalan Senthil, **Rukaiyya Sirajuddin Khan**, Patnaik A., Sajeli Begum Ahil.

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Rukaiyya Sirajuddin Khan completed her Bachelor of Pharmacy from Pune University, Maharashtra in year 2008 and M. Pharm (Medicinal Chemistry) from School of Pharmacy and Technology Management, Narsee Monjee Institute of Management Studies (NMIMS), Mumbai, Maharashtra in year 2010. She worked as research assistant at Chemical Process Research Laboratory (CPRL), USV Pvt. Ltd., Mumbai as a part of internship during M. Pharm. In January 2011 she got admitted for PhD program at Department of Pharmacy, Birla Institute of Technology and Science (BITS-Pilani), Hyderabad Campus, under the supervision of Prof. A. Sajeli Begum. Later in the same year, she was awarded with UGC-MANF Fellowship for five years from UGC, New Delhi. She has published two scientific papers in International journals. She had presented papers at various National and International conferences. She was also awarded with Travel Grant from CSIR and ICMR, New Delhi, in January 2012, to present her research work at "Current Medicinal Chemistry, 4th International Conference on Drug Discovery and Therapy" (12th to 15th February 2012) at Dubai, UAE.

BIOGRAPHY OF Prof. A. SAJELI BEGUM

Dr. A. Sajeli Begum is presently working as Associate Professor in Department of Pharmacy, Birla Institute of Technology and Science, Pilani, Hyderabad Campus. She received her Ph.D. degree in the year 2005 from Banaras Hindu University; Varanasi. She was awarded with Deutscher Akademischer Austausch Dienst (DAAD) fellowship in year 2004 to pursue research at Eberhard Karls Univesrity, Tubingen, in the group of Dr. Roderich Suessmuth. She started her career as an Assistant professor in Department of Pharmaceutics, Institute of technology, Banaras Hindu University, later in 2010 she joined in Birla Institute of Technology and Science, Pilani, Hyderabad Campus. She has been involved in research for the last 15 years and in teaching for 10 years. Her prime research interests are centered on isolation, structure elucidation, chemical transformation, and pharmacological screening of bioactive natural products. She has collaborations at Department of Chemistry and Material Sciences, Tokyo Institute of Technology, Tokyo. She has several National and International publications to her credit. She has also authored a book chapter in "Progress in the Chemistry of Organic Natural Products" published by Springer Wien New York. She is a lifetime member of IPGA, APTI and Fellow of Indian Chemical Society. She has successively completed UGC sponsored projects and is currently running a project sponsored by CSIR. Presently four students are pursuing Ph.D work under her guidance.