SYNTHESIS, CHARACTERISATION AND BIOLOGICAL EVALUATION OF DALFAMPRIDINE GENOTOXIC IMPURITIES

Dissertation submitted to

THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY

CHENNAI- 600 032

In partial fulfillment for the award of Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL CHEMISTRY

Submitted by

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KOMARAPALAYAM – 638 183

TAMIL NADU

MAY – 2018

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"SYNTHESIS, CHARACTERISATION AND BIOLOGICAL EVALUATION OF DALFAMPRIDINE GENOTOXIC IMPURITIES"**, submitted by the Student bearing **Reg. No: 261615205** to **" The Tamil Nadu Dr. M.G.R. Medical University – Chennai "**, in partial fulfillment for the award of Degree of **Master of Pharmacy in Pharmaceutical Chemistry** was evaluated by us during the examination held on ……..

Internal Examiner **External Examiner**

CERTIFICATE

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Place : Komarapalayam
Date :

Dr. R. Sambathkumar M.Pharm., Ph.D.,
Professor & Principal,
J.K.K.Nattraja College of Pharmacy,
Komarapalayam – 638 183.

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DECLARATION

I do hereby declare that the dissertation **"SYNTHESIS, CHARACTERISATION AND BIOLOGICAL EVALUATION OF DALFAMPRIDINE GENOTOXIC IMPURITIES"**, submitted to **"The Tamil Nadu Dr. M.G.R. Medical University – Chennai"**, in partial fulfillment and requirement of university rules and regulation for the award of Degree of **Master of Pharmacy in Pharmaceutical Chemistry**, is a bonafide research work has been carried out by me during the academic year 2017-2018, under the guidance and supervision of **Dr. M. Vijayabaskaran, M. Pharm., PhD.,** Professor & Head, Department of Pharmaceutical Chemistry, J.K.K. Nattraja College of Pharmacy, Komarapalayam.

I further declare that this research work is original and this dissertation has not been submitted previously for the award of any other degree, diploma, associate ship and fellowship or any other similar title. The information furnished in this dissertation is genuine to the best of my knowledge.

Place : Komarapalayam

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Abbreviations

ABBREVIATIONS

ABBREVIATIONS	FULL FORMS
°C	Degree centigrade
Gm	Gram
Hr	Hour
Mol.Wt.	Molecular Weight
Rf	Retention factor
TLC	Thin layer chromatography
%	Percent
Ar	Aromatic
Cm	Centimeter
Ali	Aliphatic
HCl	Hydrochloric Acid
IP	Indian Pharmacopoeia
L	Litre
NMR	Nuclear magnetic resonance
LC-MS	Liquid chromatography mass spectroscopy
HPLC	High performance liquid chromatography
1H	Proton
CDCl3	Deuterated Chloroform

ABBREVIATIONS	FULL FORMS
DMSO	Dimethyl sulfoxide
Nm	Nano meter
Min	Minute
Mg	Milli gram
Kg	Kilo gram
No.	Number
API	Active pharmaceutical ingredient
PGI	Potential genotoxic impurities
MS	Multiple Sclerosis
Zn	Zinc
RT	Room Temperature
MeOH	Methanol
DCM	Di Chloro Methane
MIC	Minimum inhibitory concentration
MHA	Muller Hinton Agar
ATCC	American Type Culture Collection
NCL	New Castle University Culture Laboratory
NCCLS	National Committee for Clinical Laboratory Standards

INTRODUCTION

1.1. INTRODUCTION TO MEDICINAL CHEMISTRY

Medicinal chemistry is a science having its roots in all branches of chemistry and biology. It involves isolation, characterization and synthesis of new compounds that can be used for prophylaxis and curing of diseases. Medicinal chemistry thus forms the chemical basis of therapeutics.

The subject of medicinal chemistry explains the design and production of compounds that can be used for the prevention, treatment or cure of human and animal diseases. Medicinal chemistry includes the study of already existing drugs, of their biological properties and their structure-activity relationships [1]. Medicinal chemistry was defined by IUPAC specified commission as it concerns the discovery, the development, the identification and the interpretation of the mode of action of biologically active compounds at the molecular level

Medicinal chemistry is an interdisciplinary science. It has been stated that "Medicinal chemistry concern the discovery, the development, the identification and interpretation of the mode of action of biologically active Compounds at the molecular level". Evidently it touches all branches of Chemistry and biology. Medicinal chemistry is also treated under the terms pharmaceutical chemistry, molecular pharmacology, bio-organic chemistry, and selective toxicity.

The focus is mainly on organic medicinal substances. The organic drugs may be of natural or synthetic origin. The synthetic drugs have resulted by simple or more modification of the structures of the natural drugs, or by pure synthesis. The other areas of collaboration in medicinal chemistry includes biology, computer aided drug design (CADD), 3-D QSAR, X-ray crystallography, metabolism, pharmacokinetics, legal and regulatory affairs, clinical franchise management, pharmaceutics and process research chemistry.

Chemists and pharmacologist concerned with the synthesis and evaluation of new compounds. The correlation of structure and activity in such summaries stimulates the visualization of new molecular structure and leads to the synthesis and testing of new compounds

The practice of medicinal chemistry is devoted to the discovery, development of new agents for treating diseases. Most of the activity in this is devoted to new natural or synthetic organic compounds. The process of establishing a new drug is exceedingly complex and involves the talents of people from a variety of disciplines, including chemistry, biochemistry, physiology, pharmacology, pharmaceutics and medicine. Medicinal chemistry is concerned mainly with the organic analytical and biologic interface of chemistry and biology. There is a considerable overlap with chemical aspects of this process. Thus it occupies a strategic position at the other disciplines.

1.2 GENOTOXIC IMPURITIES

Impurities are the unwanted chemicals developed during formulation or that remain with the active pharmaceutical ingredients (APIs). The presence of these small amount of unwanted chemicals may influence the safety and efficacy of the pharmaceutical products **(2).**

As per ICH guideline impurities are classified in to three types- organic, inorganic and process based impurities.

Organic Impurities:

Impurities arises during manufacturing process and/or during storage of the drug substance. These impurities include

- Starting or intermediate impurities,
- By-products impurities,
- Degraded products impurities,
- Enantiomeric impurities.

Inorganic impurities:

These type of impurities raised by manufacturing processes which are used in bulk drugs formulation. This impurities are normally known and identified. It includes

- Heavy metal impurities,
- Material impurities (filter aids, charcoal)
- Residual solvent impurities.

Process Based Impurities:

Impurity produced due to environmental defect, factor defect, mutual interaction among ingredients, functional group reaction degradation. **(3, 4)**

Various regulatory authorities like ICH, United States FDA, Canadian Drug and Health Agency are valued to the purity and safety requirements and the identification of impurities in Active Pharmaceutical Ingredient's. Qualification of the impurities is the process of obtaining and evaluating data that establishes biological safety and purity of an individual impurity. Chromatographic and Spectroscopic techniques is used to identify the impurities, either alone or in combination with other techniques. TLC, HPLC, HPTLC, AAS also used for detecting and characterizing impurities in active pharmaceutical ingredients.

Impurity profiling is the process of acquiring and evaluating data that establishes biological safety of an individual impurity. Impurity is considered as any other organic material, besides the drug substance, or ingredients, arise out of synthesis or unwanted chemicals that remains with API's. The control of impurities is currently a critical issue to the pharmaceutical industry.

Safety and efficacy of pharmaceuticals are fundamental issues of importance in drug therapy. The safety of a drug is determined by its pharmacological and toxicological profile of a drug as well as the adverse effects caused by the impurities in bulk dosage forms.

Identification of impurities is done by TLC, HPLC. The advent of hyphenated techniques has revolutionized impurity profiling, by not only separation but structural elucidation of impurities as well. The most exploited techniques, for impurity profiling of drugs are LC-MS-MS, LC-NMR, LC-NMR- MS, GC-MS, and LC-MS.

Geotaxis impurities are chemical compounds that can produce genetic mutations and chromosomal rearrangements and can act as carcinogenic compounds in target cell. The compounds can cause damage to DNA cells by alkylation or other interactions that can lead to mutation of the genetic codes. Impurities are generated during manufacturing of active pharmaceutical ingredients from the source of starting material, reagents, solvents, intermediates, catalysts and reaction by-products. Particularly, potential genotoxic impurities (PGI) shows structural alert functionality and causes genetic mutations, chromosomal breakage, chromosomal rearrangements and may cause cancer in human being **(5).**

The International Conference on Harmonisation published guidelines in the late 1990s on pharmaceutical products and impurities of drug substances. In the guidelines, genotoxicity tests designed for detecting compounds that induce genetic damage directly or indirectly by invitro and invivo.

However, several important issues have not been addressed in the guidelines, for example, the acceptable levels of impurities in drugs during development as well as the control of genotoxic impurities. The series of thresholds described in ICH Q3A(R) that trigger reporting, identification, and qualification requirements. As per Threshold of Toxicological Concern value is equal or more than that of 1.5 µg/day intake of a genotoxic impurity is considered as a acceptable risk for most pharmaceuticals.

Thresholds	Maximum daily dose	
	≤2 g/day	**>2 g/day**
Reporting threshold	0.05%	0.03%
Identification threshold	0.10% or 1.0 mg per day intake (whichever is lower)	0.05%
Qualification threshold	0.15% or 1.0 mg per day intake (whichever is lower)	0.05%

Table No 1: Threshold of toxicological concern value.

ICH Guidelines for Genotoxic Impurities:

- Impurities in New Drug Substances - Q3A(R2) (Current Step 4 version, dated 25 October 2006)
- Impurities in New Drug Products - Q3B(R2), (Current Step 4 version, dated 2 June 2006)
- Impurities: Guideline for Residual Solvents - Q3C (R3), (Current Step 4 version, parent guideline, 17/7/97, revised November 2005)

The qualification threshold is 0.15% or 0.05%, respectively, if the maximum daily dose of the drug is lower or higher than 2 grams. The Q3A guideline also introduces the concept of TDI (Total Daily Intake) of the impurity, which is expressed in mg/person/day, rather than in concentration. The structure of impurities exceeding the identification threshold (0.10% or 0.05% depending on the daily dose of the drug) must be elucidated. With the identification of the structure it is possible to assess the analytical response factor of the impurity. According to Q3A the qualification threshold in terms of TDI corresponds to 1 mg/person/day of the impurity.

Genotoxic impurity study thresholds levels are given below:

Max daily dose	Qualification Threshold	Identification Threshold	Reporting Threshold
≤2g/day	0.15%/1.0mg/day (lowest)	0.10%/1.0mg/day (lowest)	0.05%
>2g/day	0.05%	0.05%	0.03%

Table No 2: Thresholds levels as per ICH daily dose.

1.3 DALFAMPRIDINE

Dalfampridine otherwise called as 4-Aminopyridine , 4-AP, fampridine. Dalfampridine is the first drug approved in the United States by USFDA to improve walking in patients with multiple sclerosis . Fampridine is also marketed as Ampyra in the United States by Acorda Therapeutics and as Fampyra in Europe. In Canada, the medication as been approved for use by Health Canada since February 10, 2012.

4-Aminopyridine is non-selectively block K+ channel, which is actively blocking a wide variety of potassium channels with different state dependences. Therefore, in search for clues for the structural determinants of K+ channels that are important for the state dependences of drug-channel interactions, 4-AP serves as a useful tool. Potassium channel blocker used to help multiple sclerosis patients walk . This is the first drug that was specifically approved to help with mobility in these patients.

Dalfampridine has to improve visual function and motor skills and relieve fatigue in patients with multiple sclerosis (MS). 4-AP is most effective in patients with the chronic progressive form of MS, in patients who are temperature sensitive, and in patients who have MS for longer than three years. Side effects of dalfampridine is shown to be less than 5 % in all studies like dizziness, nervousness and nausea and the incidence of adverse effects. Potassium channel blockade reverses this effect.

It is very effective avicide and bird repellent and is highly toxic to human; it strongly excites central nervous system. It was undergoing Phase III clinical trials as of 2008 **(6).**

Currently, the McDonald criteria constitute the most widely used diagnostic modality because they focus on clinical, laboratory, and radiologic data of MS lesions and their dissemination in time and space **(7).** More than 90 % of patients with MS report difficulty in walking **(8).** An oral medication, dalfampridine is quickly and completely absorbed in the gastrointestinal (GI) tract. A slight and clinically insignificant increase in the Cmax of dalfampridine is noted when it is taken with food; therefore, this drug may be taken without regard to meals.

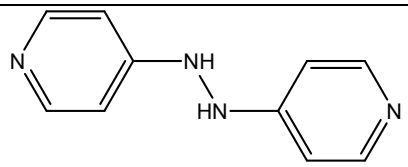
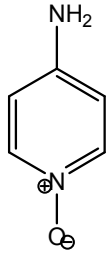
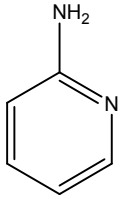
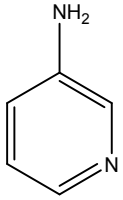
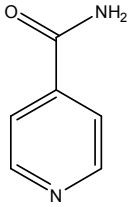
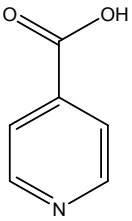
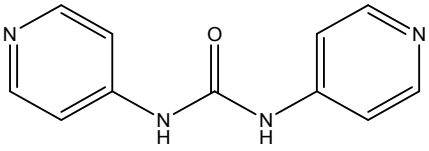
Cells of the immune system, such as B and T lymphocytes and macrophages, express voltage activated K+ channels also sensitive to 4-AP . In addition, 4-AP blocks different lymphocytic functions, including mitogen induced cell proliferation **(9)** and killing by natural killer (NK) cells and cytotoxic T cells. However, the concentration of 4-AP required to inhibit cell functions may be 5-10 fold higher than that required to block K+ channels during voltage clamp experiments Part of this effect may result from a decrease in the channel blocking potency of 4-AP in the presence of serum contained in the culture medium.

The FDA's approval of dalfampridine was based on the results of one phase 2 and two phase 3 randomized, double-blind, placebo-controlled, parallel-group clinical trials (MS-F202 and MS-F203) **(10)**. During the study period, 300 patients were randomly assigned to receive either dalfampridine 10 mg or placebo twice daily. After the 14-week treatment period, patients were observed for an additional four weeks. Multiple sclerosis (MS) is a neurological disorder that affects approximately 400,000 people in the U.S. and 2.5 million people worldwide, with women twice as likely to be affected as

men **(11,12).** MS is manifested by mental and physical symptoms characteristic of the illness.

The FDA granted dalfampridine orphan drug status, which will provide market exclusivity for the drug for 7 years **(13).** Dalfampridine is a potassium channel-blocker that enhances conduction in focally demyelinated axons, improves synaptic transmission and potentiates muscle contraction **(14)**. The clinical use of dalfampridine is found to improve walking in patients with multiple sclerosis (MS) as demonstrated by an increase in walking speed. It has shown efficacy in patients with all four major types of MS **(15)**. Dalfampridine is mostly unbound to plasma protein (97–99 %) and the apparent volume of distribution is 2.6 L/kg. Studies with human liver microsomes indicated that CYP2E1 was primarily accountable for the 3hydroxylatoin of dalfampridine. The common adverse effects in patients administered dalfampridine include urinary tract infections, insomnia, dizziness, headache, nausea, weakness, back pain, ataxia and visual disturbances**(16)**.

During the synthesis of Dalfampridine some impurities formed such as 1,2-Di(pyridine-4-yl)hydrazine, 4-Aminopyridine-N-oxide, 2-Aminopyridine, 3-Aminopyridine, Pyridine-4-carboxamide, Pyridine-4-carboxylic acid, (1, 3-Di (8yridine-4-yl) urea, 4-Amino-3-Chloro-Pyridine, 4-Cyanopyridine, 4-Amino-3,5-Dichloro Pyridine, N-(4-Pyridyl)-2-Hydroxybutyramide) are identified PGIs which is tabulated as follows:

S.NO	CHEMICAL NAME	STRUCTURE
1.	1,2-Di(pyridine-4-yl) hydrazine	
2.	4-Aminopyridine-N-oxide	
3.	2-Aminopyridine	
4.	3-Aminopyridine	
5.	Pyridine-4-carboxamide	
6.	Pyridine-4-carboxylic acid	
7.	(1, 3-Di (pyridin-4-yl) urea	

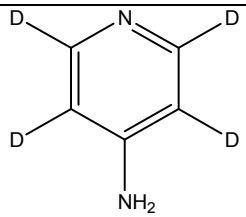
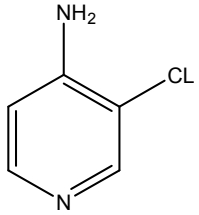
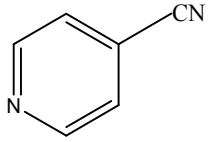
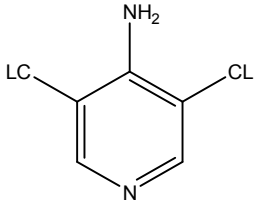
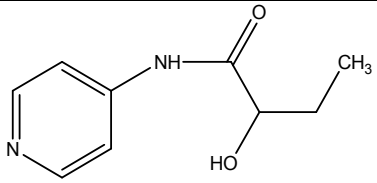
S.NO	CHEMICAL NAME	STRUCTURE
8	Fampridine-d4	
9	4-Amino-3-Chloro-Pyridine	
10	4-Cyanopyridine	
11	4-Amino-3,5-Dichloro Pyridine	
12	N-(4-Pyridyl)-2-Hydroxybutyramide)	

Table No 3: Dalfampridine genotoxic impurities.

MODE OF ACTION OF ANTIBACTERIAL AGENTS:

Antibacterial drugs interfere chemically with the synthesis of function of vital components of bacteria. The cellular structure and functions of eukaryotic cells of the human body. These differences provide us with selective toxicity of chemotherapeutic agents against bacteria.

Antimicrobial drugs may either kill microorganisms outright or simply prevent their growth. There are various ways in which these agents exhibit their antimicrobial activity.

They may inhibit :

(1) Cell-wall synthesis

(2) Protein synthesis

(3) Nucleic acid synthesis

(4) Enzymatic activity

(5) Folate metabolism

(6) Damage cytoplasmic membrane

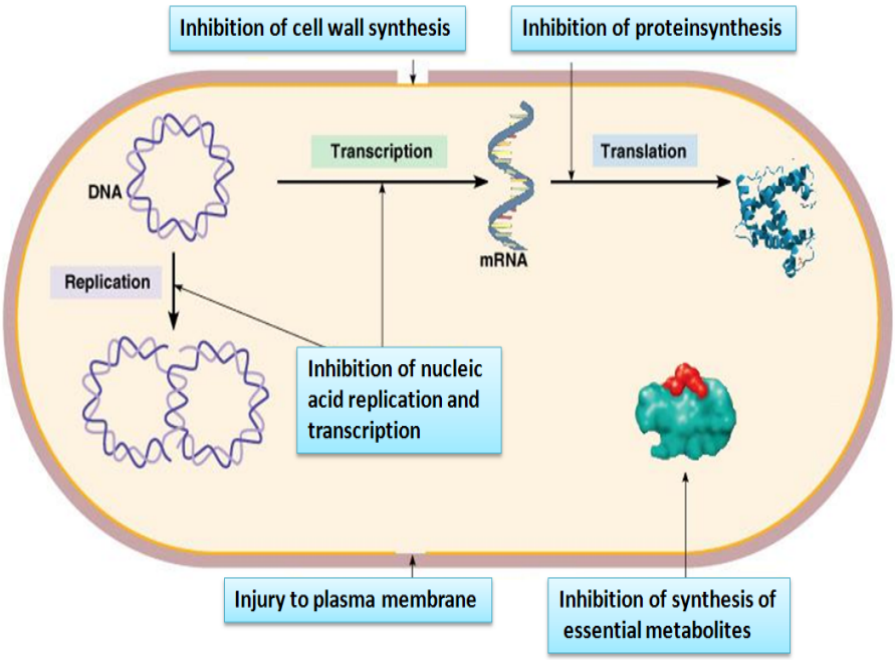


Fig No 01: Common Mechanisam of Bacterial Growth Inhibition

SCREENING OF ANTI BACTERIAL ACTIVITY

The condition which must be fulfilled before screening the antibacterial activity.The organism and drug substance to be evaluated should be contacted intimately. The microorganism's growth should be maintained under conditions like incubation period, temperature, nutrient media, etc. Throughout the study conditions were maintained.

Preparation of different media:

The growth media required for evaluation of antibacterial activity was prepared as per the procedure reported in Himedia manual, as follows:

1. Muller Hinton Agar:

It mainly contains following content,

Ingredients	**Quantity (Gm/L)**
Beef heart infusion,	2.00
Casein acid hydrolysate	17.50
Starch, soluble	1.50
Agar	17.00

Procedure:

Suspend 38 grams in 1000ml distilled water. Mix well and boil the media to dissolve completely. autoclaving at 15 lbs pressure (121^0C) for 15min sterilisation has done. Pour the media after mixing thoroughly.

2. Tryptic soya bean broth:

It mainly contains following content

Ingredients	**Quantity (Gm/L)**
Casein (enzymic hydrolysate)	18.00
Papaic digest of soybean	2.00
Sodium chloride	6.00
Dipotassium phosphate	3.50
Dextrose	3.50

Procedure:

Suspend 30grams of Tryptic soya bean broth powder in one litre of distilled water. Medium was boiled to dissolve completely. Autoclaving at 15lbs pressure (1210C) for 15 minutes.

Methods used for primary and secondary screening:

Primary Screening Method: The test and control drugs were diluted using sterile methanol in 1000 microgram per ml (µg/ml), 500.0 microgram per ml , 250.0 microgram per ml from which 100 µl of solution taken in the cups to obtain a concentration of 100 microgram per ml, 75 microgram per ml and 50 microgram per ml respectively. A methanol control was also run along with test and the reference drug. The promised result from the synthesized molecules was tested by primary screening for all microorganisms by second set dilution.

Secondary Screening Method: The promised results from the synthesized molecules in primary screening were diluted using sterile methanol as 100 microgram per ml, 75 microgram per ml, 50 microgram per ml from which 100 µl of solution taken in the cups to obtain concentration of 0.8 microgram per ml, 0.4 microgram per ml, 0.2 microgram per ml respectively.

CHEMISTRY

4-Aminopyridine (4-AP, fampridine, dalfampridine) is an organic compound with the chemical formula $C_5H_4N\text{-}NH_2$ and molecular weight 94.04 gm/mole. It is chemically pyridine-4-amine, used primarily as a research tool for subtypes of potassium channels. It is use to manage some of the symptoms of multiple sclerosis and is indicated for symptomatic improvement of walking in adults with several variations of the disease.

Molecular Structure :

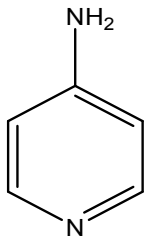


Fig No 02: Structure of Dalfampridine

Chemical Formula	: $C_5H_6N_2$
Molecular Mass	: 94.1164 gm/mole
Physical State	: Solid
Colour	: Colourless
Melting Point	: 155 to 158 °C
Boiling Point	: 273 °C
Solubility	:Water, polar organic solvents.

MECHANISAM OF ACTION

Voltage-gated potassium channels represent open and close in response to changes in the transmembrane potential, and help regulate ionic potassium currents. Axons are progressively demyelinated which exposes potassium channels in multiple sclerosis. As a result, there is a leak of potassium ions which results in the repolarization of cells and a decrease in neuronal excitability.

The overall impact is the impairment of neuromuscular transmission as it is harder to trigger an action potential. Despite characterization of dalfampridine a lipid-soluble compound that readily crosses the blood–brain barrier.The mechanism of action of dalfampridine has not been fully elucidated because the specific potassium channels that are blocked during therapeutic response have not been identified. However, the putative mechanism of action responsible for the therapeutic effects in MS is the restoration of conduction via blockade of the potassium channels that become exposed during demyelination. Dalfampridine act as a potassium channel blocker. Dalfampridine block to voltage-gated potassium channels which leads to prolong the action potential and in CNS to maintain the transmembrane potential.

Dalfampridine act to stimulate conduction in demyelinated axons that are exposed in MS patients.Dalfampridine facilitates neuromuscular and synaptic transmission by relieving conduction blocks in demyelinated axons in multiple sclerosis.MS patients who nonresponded to dalfampridine had an average increase in walking speed is approximately 25% lesser improvements than dalfampridine responders.

REVIEW OF LITERATURE

Corneliu C. Luca et al (2017) Studied that disease-related gait dysfunction causes extensive disability for persons with Parkinson's disease (PD), with no effective therapies currently available. The potassium channel blocker dalfampridine has been used in multiple neurological conditions and improves walking in persons with multiple sclerosis. D-ER is well tolerated in PD patients, however it did not show significant benefit for gait impairment **(18)**.

Aakash Deep et al (2016) revealed that Multiple sclerosis (MS) is a neurodegenerative disease of the central nervous system (CNS), causes irreversible disability in young adults but the cause and cure were unknown and it involves two arms and causes demyelination and neuro degeneration. Since, the treatment strategies for MS have increased rapidly but still proper knowledge regarding the nature of MS cleared the way for several more specific, more effective, and more comfortable therapies. Here, because of the stimulating recent developments about oral treatment for MS, the current state of approved and future therapy options were summarized here. In particular, we highlight oral treatment options in MS and it acts at the central and peripheral nervous systems, enhances conduction in demyelinated axons, and improves the walking ability of MS patients. Moreover number of clinical trials has evaluated the safety and efficacy of fampridine in MS patients and it represents a major advance in symptomatic therapy for MS **(19).**

Ajay Kumara et al (2016) A sensitive and selective HPLC method was developed for identification and quantification of five Potential genotoxic impurities (PGIs) viz. Impurity-I, Impurity-II, Impurity-III, Impurity IV and Impurity-V in Dalfampridine (Drug substance). The method utilizes Zorbax silica column (250 mm x 4.6 mm, 5.0 µm) with UV detector in HILIC (Hydrophilic Interaction Liquid Chromatography) mode for quantitation of five PGIs. It has been validated as per International Council for Harmonisation

(ICH) guidelines and is able to quantitate all PGIs at 75 ppm with respect to 20 mg/mL of sample concentration. It is linear in the range of 22.5 , 112.5 ppm for all PGIs, which matches the range of LOQ-150% of estimated permitted level (75 ppm). Its accuracy was established in the range from 88.14-107.65% for these PGIs. The correlation coefficient of each impurity was >0.999. It is a good quality control tool for quantitation of PGIs in Dalfampridine at low level **(20)**

Poonam P. Patil et al (2015) revealed that impurities are nothing but the unidentified, unintended substance present along with desired substance. The newer regulations of US FDA, MHRA intends for the requirements of impurities rather than purity of pharmaceuticals. The impurity profiling of pharmaceuticals can be done by using various analytical methods like UV, HPLC, LC-MS, GC-MS, SCFC etc. Mostly RP-HPLC method commonly adopted for the qualification as well as quantification of impurities. The present review is an attempt made in the respect of highlighting the some important methods, quality guidelines and applications of impurity profiling **(21).**

Angela Applebee et al (2015) Dalfampridine extended-release (ER) tablets 10 mg BID have been approved for use in improving walking in people with multiple sclerosis (MS). This subgroup analysis evaluated the effects of dalfampridine ER 5 and 10 mg BID on distance walked, as assessed using the 6-minute walk (6MW) test. Effect was compared with standard and control placebo **(22)**.

Alessandra Lugaresi et al (2015) Studied that fampridine 10 mg twice daily, improves walking ability in approximately one-third and walking speed in about 25% of patients, independently from disease course, compared with placebo; it also improves leg strength. Treatment is generally well tolerated, although there is a dose-dependent increased risk of seizures, especially with dosages >10mg twice daily **(23).**

Andrew R et al (2013) was developed to maintain drug plasma levels within an arrow therapeutic window and assessed for its ability to improve walking in MS. The putative mechanism of action of dalfampridine-ER is restoration of axonal conduction via blockade of the potassium channels that become exposed during axonal demyelination. Two pivotal phase III clinical trials demonstrated that dalfampridine-ER 10-mg tablets administered twice daily improved walking speed and patient-reported perceptions of walking in some patients. Dalfampridine-ER was generally well tolerated, and at the approved dose, risk of seizure was neither elevated relative to placebo nor higher than the rate in the MS population. Dalfampridine-ER was approved in the United States for the treatment of walking in patients with MS as demonstrated by an increase in walking speed. It is the first pharmacologic therapy for this indication and has been incorporated into clinical management of MS **(24).**

Chunli Liu et al (2013) synthesised of substituted pyridines has drawn the attention of many chemists due to their importance as building blocks for biologically active compounds and materials. This mini-review focuses on recent developments relating to the synthesis of substituted pyridines from pyridine N-oxides, along with their interesting mechanism aspects. New developments including alkenylation, alkynylation, alkylation, arylation, amination and cyanation **(25).**

Jana Lizrova Preiningerova et al (2013) Prolonged-release fampridine is a potassium channel blocker that improves conductivity of signal on demyelinated axons in central nervous system. Fampridine PR has been approved to improve speed of walking in patients with multiple sclerosis. This statement provides a brief summary of data on fampridine PR and recommendations on practical use of the medication in clinical practice, prediction, and evaluation of response to treatment and patient management. A prolonged-release fampridine has been developed to improve the

pharmacokinetics and to reduce the side effects associated with IR-4AP. Fampridine PR has a reduced peak plasma levels and a prolonged half-life that allows administration every 12 h **(26)**.

Patrick P et al (2013) studied dalfampridine (Ampyra) is indicated to improve walking in patients with multiple sclerosis (MS) and was found to be effective in 35%, 43% of individuals with MS in clinical trials. Dalfampridine may increase seizure risk, particularly in patients with renal impairment. A U.S. managed care expert consensus panel agreed that patient access to dalfampridine is best managed by a prior authorization (PA) in accordance with the FDA-approved labeling. To ensure safe and appropriate dalfampridine use, a health plan developed and implemented a 2-phase point-of-sale PA program. The study indicates that a dalfampridine PA program potentially improved safety and minimized dalfampridine costs. A PA program is effective in selecting appropriate utilisers for initial theraphy. Addition of care management may further optimize use by encouraging adherence and tracking patient response **(27)**.

Abolghasem Jouyban et al (2012) described the structural alerts to predict potential genotoxic activity for identified impurities is now well established. However, the concordance between such alerts and biologically relevant genotoxic potential could be highly imperfect. Structural alerts are defined as molecular functionalities that are known to cause toxicity, and their presence in a molecular structure alerts the investigator to the potential toxicities of the test chemical. In silico toxicology is the application of computer technologies to analyze existing data, model, and predict the toxicological activity of a substance. In sequence, toxicologically based QSARs are mathematical equations used as a predictive technique to estimate the toxicity of new chemicals **(28).**

King AM et al (2012) 4-Aminopyridine (4-AP) selectively blocks voltage-gated potassium channels, prolongs the action potential, increases

calcium influx, and subsequently, enhances interneuronal and neuromuscular synaptic transmission. This medication has been studied and used in many disease processes hallmarked by poor neuronal transmission in both the central and peripheral nervous systems including: multiple sclerosis (MS), spinal cord injuries (SCI), botulism, Lambert-Eaton syndrome, and myasthenia gravis. It has also been postulated as a potential treatment of verapamil toxicity and reversal agent for anesthesia-induced neuromuscular blockade. To date, there have been limited reports of either intentional or accidental 4-AP toxicity in humans. Both a case of a patient with 4-AP toxicity and review of the literature are discussed, highlighting commonalities observed in overdose (**29)**.

Michael D et al (2012) studied Dalfampridine theoretically works to improve conduction and enhance walking by inhibiting potassium channels in the axonal membrane and by prolonging action potentials in demyelinated neurons. The efficacy of dalfampridine has been reported in 2 Phase III clinical trials in patients with MS. When comparing dalfampridine 10 mg twice daily with placebo, these studies found a statistically significant improvement in walking(42.9% vs9.3% and35% vs8%; $P < 0.001$). However, clinical trials and post marketing surveillance have found a statistically significant increased risk of seizures with dalfampridine **(30)**.

Masamitsu Honma et al (2012) described to Major Safety Issues of ICH Guideline for Genotoxic Impurities is DNA reactive substances which can be detected by Ames assay. Application of Threshold of Toxicological Concern (TTC) to control genotoxic impurities. Risk assessment for patients and healthy volunteers during clinical development. Evaluation of genotoxicity of impurities using the Structure Activity Relationship (SAR). Risk mitigation considering exposure duration and hazard characterization.DNA reactive substances that have a potential to directly cause DNA damage when present at low levels leading to mutations and therefore, potentially causing cancer. This type of mutagenic carcinogen is usually detected in a bacterial reverse mutation

assay. Non-mutagenic typically have threshold mechanisms and usually do not pose carcinogenic risk in humans at the level ordinarily present as impurities **(31)**.

Renu Solanki et al (2012) studied Pharmaceuticals impurities are the unwanted chemicals that remain or are generated during the formulation of medicines. Impurity profiling helps in detection, identification and quantification of various types of impurities as well as residual solvents in bulk drugs and in pharmaceutical formulations. It is a best way to characterize quality and stability of bulk drugs and pharmaceutical formulations. ICH, USFDA, Canadian Drug and Health Agencies are emphasizing on the purity requirements and on identification of impurities in active pharmaceutical ingredients as presence of impurities even in small amounts may influence the efficacy and safety of the pharmaceutical products. Thus enlightening the need of impurity profiling of drug substances in pharmaceutical research this review focuses on various analytical methods for identification as well as quantification of impurities present in the pharmaceuticals **(32)**.

Dunn J et al (2011) described that multiple sclerosis (MS) can cause progressive walking impairment that contributes to disability, loss of independence, and reduced quality of life. Dalfampridine a voltage-dependent potassium channel blocker, has been shown to improve walking in patients with MS, as demonstrated by an increase in walking speed. Voltage-gated potassium channels represent a family of related proteins that span cell membranes, open and close in response to changes in the transmembrane potential, and help regulate ionic potassium currents. The extended-release formulation of dalfampridine has been shown in clinical trials to improve walking speed in approximately one third of MS patients with ambulatory impairment. The putative mechanism of action of dalfampridine is restoration of action potential conduction via blockade of an as yet uncharacterized subset of potassium channels in demyelinated axons **(33)**.

Fabio Garavaglia et al (2011) A process for the preparation of Dalfampridine, starting from 4-pyridinecarbonitrile using a one-pot procedure said process is carried out with no need for isolating intermediates and is particularly advantageous as far as environment, yields, productivity and purity of the resulting product are concerned, both in the reaction mixture and in the isolated crystal **(34).**

Ayre1 A et al (2011) described impurity profiling is the process of acquiring and evaluating data that establishes biological safety of an individual impurity. Thus, revealing its need and scope in pharmaceutical research. There is no clear definition for impurity in the pharmaceutical world. Impurity profiling includes identification, structure elucidation and quantitative determination of impurities and degradation products in bulk drug materials and pharmaceutical formulations. Identification of impurities is done by variety of Chromatographic and Spectroscopic techniques, either alone or in combination with other techniques. The advent of hyphenated techniques has revolutionized impurity profiling, by not only separation but structural identification of impurities as well. The present review covers various aspects related to the analytical method development for impurity profiling of an active pharmaceutical ingredient **(35)**.

FDA (2008) The Food and Drug Administration (FDA) is announcing the availability of a draft guidance for industry entitled‘‘Genotoxic and Carcinogenic Impurities in Drug Substances and Products: Recommended Approaches.’’ This draft guidance is intended to inform pharmaceutical manufacturers of the agency’s thinking regarding genotoxic and carcinogenic impurities in drug substances and drug products, including biologic products that are regulated by the Center for Drug Evaluation and Research (CDER), and to provide recommendations on how to evaluate the safety of these impurities during clinical development and for marketing applications. This draft guidance, when finalized, will clarify FDA’s additional testing and

exposure threshold recommendations for situations in which genotoxic or carcinogenic impurities are present. This draft guidance addresses synthetic impurities and degradants in drug substances, but does not otherwise address the genotoxicity or carcinogenicity of actual drug substances or intended drug product ingredients. This draft guidance also applies to known starting materials or anticipated reaction products **(36)**.

Sanjay B. Bari et al (2007) Described that ICH, USFDA, Canadian Drug and Health Agency are emphasizing on the purity requirements and the identification of impurities in Active Pharmaceutical Ingredient's (API's). Qualification of the impurities is the process of acquiring and evaluating data that establishes biological safety of an individual impurity. Conventional Liquid Chromatography, particularly, HPLC has been exploited widely in field of impurity profiling; the wide range of detectors, and stationary phases along with its sensitivity and costeffective separation have attributed to its varied applications. Headspace GC is one of the most preferred techniques for identification of residual solvents. The advent of hyphenated techniques has revolutionized impurity profiling, by not only separation but structural identification of impurities as well **(37)**.

Do B& Goulay et al (2006) 3,4-Diaminopyridine is used to treat some symptoms met in Lambert Eaton myasthenia syndrome. It was shown efficient to reduce a form of variable muscle weakness and fatigability typical of the disease and correlated to a block of acetylcholine release. A high performance liquid chromatographic method for the determination of 3,4-diaminopyridine in the presence of related substances and its degradation products is described. The method is based on the use of a C18 bonded phase column and a mobile phase composed of 10 volumes of acetonitrile and 90 volumes of an aqueous solution containing 1 g L)1 sodium octane sulfonate and 0.77 g L)1 ammonium acetate. The pH of the aqueous solution was adjusted to 1.9 with trifluoracetic acid. All peaks are eluted in <40 min. The method was demonstrated to be

precise, accurate and specific even though a major part of the drug is decomposed. The results indicate that the proposed method could be used in stability assays **(38)**.

Hayes K.C et al (2004) studied that 4-AP is a potassium channel-blocker that enhances conduction in focally demyelinated axons, improves synaptic transmission and potentiates muscle contraction. This is the first drug approved in the United States by USFDA to improve walking in patients with multiple sclerosis and is chemically known as 4-aminopyridine.The pharmacokinetics and safety of sustained-release 4-aminopyridine, a potassium channel blocker, in subjects with chronic, incomplete spinal cord injury (SCI).In subjects with chronic, incomplete SCI, Fampridine-SR was slowly absorbed and eliminated, which will allow Fampridine-SR to be administered in a convenient twice-daily manner. Fampridine-SR was well tolerated at dosages from 25 to 60 mg twice daily **(39)**.

Donnelly RF et al, (2004) Determined the chemical stability of 4-AP capsules containing 10 mg of active ingredient. Ten-milligram capsules were prepared from 4-AP obtained from 2 suppliers, with either lactose or microcrystalline. The hard gelatin capsules were stored in amber snap top prescription vials at room temperature (20°C to 25°C) with protection from light. Two capsules were collected from each group on days 0, 14, 28, 62, 96, 125, 180, and 365 and stored in a rubber stoppered glass test tube containing desiccant material at 70°C.. Ten-milligram capsules of 4-AP, prepared from material obtained from each supplier and diluted with either lactose or microcrystalline cellulose, retained at least 94 % of the initial content for 365 days when stored in plastic prescription vials at room temperature with protection from light. Extemporaneously prepared 10 mg capsules of 4-AP were considered stable for 365 days when stored in plastic prescription vials at room temperature with protection from light **(40)**.

Trisse L A et al (2002) Studied the chemical stability of 4 aminopyridine 5-mg capsules and 3,4-diaminopyridine 5mg capsules under a variety of storage conditions. Each of the two drug preparations was extemporaneously prepared in hard gelatin capsules; lactose and micronized silica gel were used as excipients. Samples were stored under three conditions: refrigeration at 4°C and protected from light for 6 months, protected from light at room temperature that ranged from 22°C to 24°C for 6 months, and at a temperature of 37°C and protected from light for 1 month. Capsule content weight did not change during the study. Both 4-aminopyridine and 3,4-diaminopyridine exhibited excellent chemical stability under all study conditions. Little or no loss of drug content occurred in either product under refrigeration, at room temperature, and even at the elevated temperature of 37°C. The oral 5 mg capsules of 4-aminopyridine and 3,4-diaminopyridine did not undergo decomposition or other adverse changes within 6 months at refrigerated or room temperature or within 1 month of storage at 37°C **(41)**.

Vladimir N. Bulavka et al (2000) studied the reduction of 4-nitropyridine-N-oxide with iron and aqueous mineral acids. The reduction with iron and hydrochloric acid gives mainly 4-aminopyridine (80-85%), and as by-products 4aminopyridine-N-oxide, 4-pyridone, and 4,4'-azopyridine. The reduction with iron and 25-30% sulphuric acid proceeds slowly, but the yield of the desired 4-aminopyridine is better. The isolation of the reaction product after reduction, subsequent neutralization with sodium carbonate, and filtration was carried out by two methods. The first method was extraction with ethyl acetate. After removal of the solvent 4aminopyridine was obtained in 85-90% yield. The second method was the concentration of the filtrate on the rotatory evaporator, extraction with ethanol, and after evaporation of ethanol reextraction with hot benzene to give a title compound after cooling (85%). After evaporation of the solvent, crude 4-aminopyridine, if desired, was

acetylated with acetic anhydride to produce 4-acetylaminopyridine in 80-85% yield **(42).**

Choquet D et al (1992) Studied the mechanism by which 4-aminopyridine blocks the delayed rectifier type potassium (K+) channels present on lipopolysaccharide activated murine B lymphocytes was investigated using whole-cell and single channel patch clamp recordings. 4-AP, was super fused for 3-4 min before applying depolarizing pulses to activate the channel. During the first pulse after application of 4-AP above 50 M, the current inactivated faster, as compared with the control, but its peak was only reduced at high concentrations of 4-AP (Kd = 3.1 mM). After washing out the drug, the current elicited by the first voltage step was still markedly reduced, as compared with the control one, and displayed very slow activation and inactivation kinetics; this suggests that the K+ channels move from a blocked to an unblocked state slowly during the depolarizing pulse **(43)**.

Casteel SW, BR. Thomas et al (1990) Determined appropriate diagnostic samples in acute poisoning cases. Tissues from rats dosed with 4aminopyridine were extracted with methylene chloride. Extracts were analyzed by high performance liquid chromatography using an isocratic solvent system of acetonitrile and aqueous solution (15 : 85 v/v) consisting of 0.015 M sodium salt of 1-heptane-sulfonic acid, 0.002 M tetra methyl ammonium bromide, and 0.01 M sodium di-hydrogen phosphate adjusted to pH 3.0 with phosphoric acid **(44)**.

AIM AND OBJECTIVES

4-Aminopyridines are important intermediates for chemicals, pharmaceuticals and agrochemical industry. Several 4-aminopyridine derivatives are known to be important intermediates for the preparation of various herbicides, antibacterial, antiviral drugs and dyes. For example, 4-aminopyridine, as fampridine, is used for the treatment of multiple sclerosis. It has been found that fampridine improve impulse conduction in nerve fibers in which the insulating layer, called myelin, has been damaged. 4-Aminopyridine is also used as an intermediate for antineoplastic, anticoagulant, anti-inflammatory, antispasmodic and antiasthmatic drugs and as additives for foodstuffs. 3 - aminopyridine and 2-aminopyridine derivatives are used as an intermediate for fungicides, herbicides and several drugs.

Dalfampridine used for the treatment of multiple sclerosis so, the drug availability in the finished dosage form is very important, owing the importance of drug availability the percentage of impurities should be within the limit as per ICH guidelines. International Conference on Harmonization (ICH) formulated guidelines regarding the control of impurities. Various regulatory authorities like ICH, USFDA, Canadian Drug and Health Agency are emphasizing on the purity requirements and the identification of impurities in Active Pharmaceutical Ingredient's (API's).

Importance is drawn to identify and characterize the level of genotoxic impurities in dalfampridine, the present study was aimed to perform identification, synthesis and characterization and biological evaluvation of genotoxic dalfampridine impurities and its characterization done by the analytical techniques such as the LC-MS, HPLC, and NMR spectroscopy. Finally evaluvation of its anti bacterial activity done by using agar well diffusion method with some bacterial strains.

OBJECTIVES

Hence the main objectives of present project are:

01. To Synthesize the targeted molecules

02. To Characterize synthesized molecules by the LC-MS spectroscopy

03. To achieve highly pure impurities by Purification of the synthesized molecule by chromatographic techniques like column and automated combi flash.

04. Analysis of products by HPLC for its purity

05. Product confirmation by the NMR spectroscopy.

06. Evaluvation of anti bacterial activity by using agar well diffusion method.

PLAN OF WORK

MATERIAL & METHODS

- All the chemicals used for current work were laboratory Grade and purchased from approved vendors.
- All reactions were carried out in dry glassware under an atmosphere of dry nitrogen.
- Glass wares used for all the reactions were of borosilicate quality.
- Rotavapor-B.U.CHI is a device used in R&D for the efficient and removal of solvents from samples by evaporation
- All the reactions every step were monitored by using thin layer chromatography.
- The suitable mobile phases (solvent system for TLC) as applicable were developed using silica Gel-G ready plates. Pre-coated aluminium silica plates were used with suitable mobile phase.
- Progress of Reaction and monitoring for each stage is performing by LCMS.
- Mass of all synthesized compounds can be determined by using Thermo- DSQ-Trace and Advantage Max, Agilent 6130 LC/MS single quad mass spectrometer.
- Separation and purification of synthesized impurities carried out by using column chromatography and automated chromatography COMBI FLASH Rf device.
- The purity of all synthesized compounds identified by HPLC Agilent-1200 with PDA detector.
- Melting points for synthesized molecules were recorded by METTLER TOLEDO apparatus.

- All the proton NMR spectra by using Varian Mercury NMR Spectrophotometer at 400MHz frequency at PADM Laboratory. The solvent used to record was $CDCl_3$ and DMSO.
- All the compounds considered for biological evaluation were prepared by standard methods as outlined in scheme.
- The media used for biological evaluation was purchased from Hi Media and media were prepared according to manual.
- Biological evaluation was done under Laminar Air Flow.
- The biological evolutions for all synthesized compounds were carried out at J.K.K. Nattraja College of Pharmacy.

SEPERATION AND PURIFICATION:

The separation of Dalfampridine and its potential genotoxic impurities was critical because of similar polarity and chemical structures. Baseline separation of impurities and Dalfampridine were given the priority.

Teledyne ISCO has developed this advance automated chromatography Combi Flash Rf system that uses RFID (Radio Frequency Identification) technology. This system can automatically set the parameters for purification runs and fraction collection.

Separartion was performed by using CHROMABOND FLASH RS-40 SiOH 40-63 micron prepacked catridges (columns)

IDENTIFICATION AND CHARACTERISATION

Synthesized potential genotoxic impurities were identified and characterized by following methods such as:

- TLC-Thin layer chromatography
- LCMS-MS spectroscopy
- HPLC-High Performance liquid chromatography
- H-NMR-proton nuclear magnetic resonance spectroscopy
- Melting point determination.

Thin layer chromatography:

TLC is a technique in which the stationary phase is a subdivided solid and which is spread over on aluminium rigid plate. A liquid i.e. mobile phase is allowed to move above the surface of the plate. This technique is normally used for the identification of the organic compounds with variable Rf values. The same method is also applied to study the progress of the reaction during onsite monitoring and to confirm the approximate purity of the desired product.

Pre-coated aluminium silica plates were used with suitable mobile phase. Once after TLC development the spots were observed and detected by placing the developed TLC plate in UV cabinet.

Mass spectroscopy:

The mass spectroscopy technique is useful to provide information regarding the atomic and molecular weight, structure of the compound, its mechanism, kinetic study of the reaction and the mixture analysis. The mass technique involves bombarding the electrons and converted to highly energetic molecular ions are also called positively charged ions, that can be easily break up into their smaller ions (fragment ions) and sorting them in gas phase, spectra is obtained according to mass/charge ratio. The Mass spectra of all synthesized compounds were carried out in Thermo- DSQ-Trace and Advantage Max, Agilent 6130 LC-MS single quad mass spectrometer.

HPLC spectroscopy:

The HPLC method is useful to identify the purity of all synthesized compounds with its different retention times helps to confirm the product from raw material or starting compounds. The HPLC for all compounds were carried out in Agilent-1200 with PDA detector.

Nuclear magnetic resonance spectroscopy:

The interaction between molecule and applied electromagnetic forces is observed by subjecting a molecule simultaneously to two magnetic forces, one is stationary and other variable radio frequency. At a combination of electromagnetic fields some of the energy is absorbed by the molecule and absorption of the electromagnetic field was observed as a change in signals developed by the radio frequency detector. This technique is useful in detecting the structure of the molecule. The NMR spectra for the synthesized compounds were recorded out in Varian Mercury NMR Spectrophotometer and Agilent at 400MHz frequency. Varien software used to record the spectrum, named as "Vnmr J" 3.2 version which is supported by Lynux Red Hat. The solvent used to record was $CDCl_3$ and DMSO.

Melting point determination:

Melting point determination of a compound is one way to test if the substance is pure or impure. Melting point study is the one of the valuable criteria for purity determination of an organic compounds. The pure crystalline compound will be having definite and sharp melting point. The melting point for all the newly synthesized molecules were carried out using METTLER TOLEDO melting point apparatus.

SYNTHESIZING PLAN FOR DALFAMPRIDINE GENOTOXIC IMPURITIES:

Dalfampridine Genotoxic impurities listed in United States Pharmacopoeia **(45)** and few potential and non pharmacopoeial impurities were selected for the synthesis as per the literature review and listed below**.**

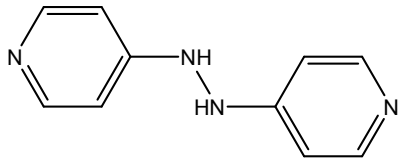
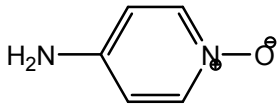
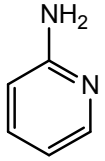
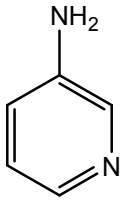
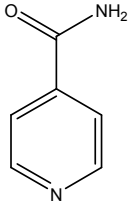
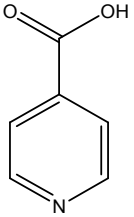
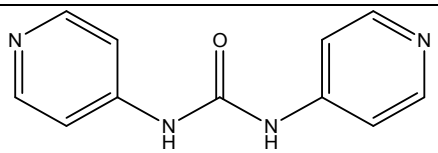
S. NO	IMPURITY CODE	CHEMICAL NAME	STRUCTURE
1.	IMP - DAL 1	1,2-Di(pyridine-4-yl) hydrazine	
2.	IMP – DAL 2	4-Aminopyridine-N-oxide	
3.	IMP – DAL 3	2-Aminopyridine	
4.	IMP – DAL 4	3-Aminopyridine	
5.	IMP – DAL 5	Pyridine-4-carboxamide	
6.	IMP – DAL 6	Pyridine-4-carboxylic acid	
7.	IMP – DAL 7	(1, 3-Di (pyridin-4-yl) urea	

Table No 04: Plan to synthesis of Dalfampridine impurities

METHODOLOGY

SCHEME TO SYNTHESIS IMP-DAL 1 (1,2-di(pyridin-4-yl)hydrazine):

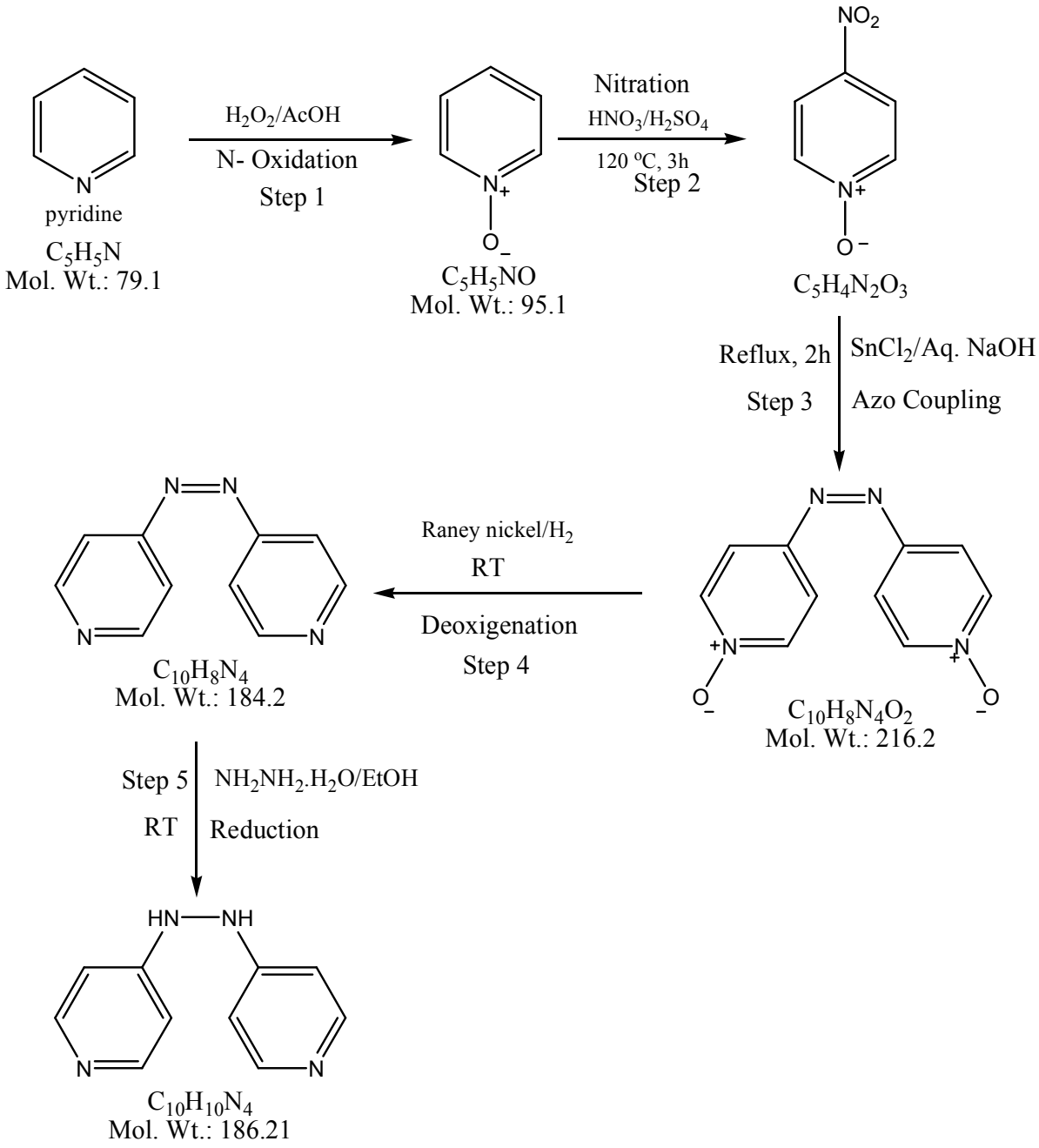


Fig No 03: Synthetic scheme of IMP-DAL 1. (1, 2-di(pyridin-4-yl)hydrazine).

STEP 1: Oxidation of Pyridine:

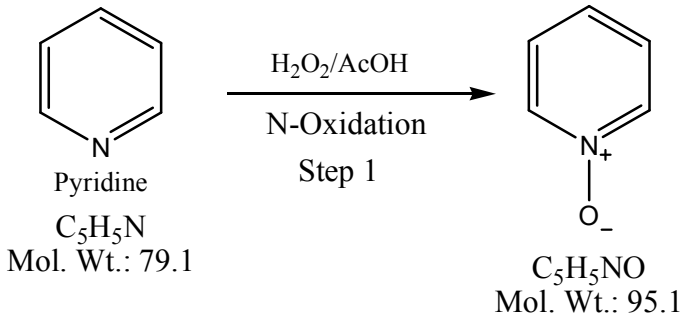


Fig No 04: Step 1 Oxidation of IMP-DAL 1

Pyridine is oxidized into pyridine N oxide which on treatment with H_2O_2/ AcOH.10 grams of Pyridine was taken in the single neck RB flask, to this 30 ml of acetic acid was added and stirred for ten minutes. 50 ml of hydrogen peroxide was added and closed the flask with stopper. The reaction mixture was stirred over night.

Workup:

After completion of the reaction, removed the excess acetic acid and peroxide by concentrating in the rotavapor and dried completely. Three times added a toluene and removed the solvent in rotavapor to expel the traces of water. Product Yield is 10 gm.

STEP 2: Nitration of Pyridine N- Oxide:

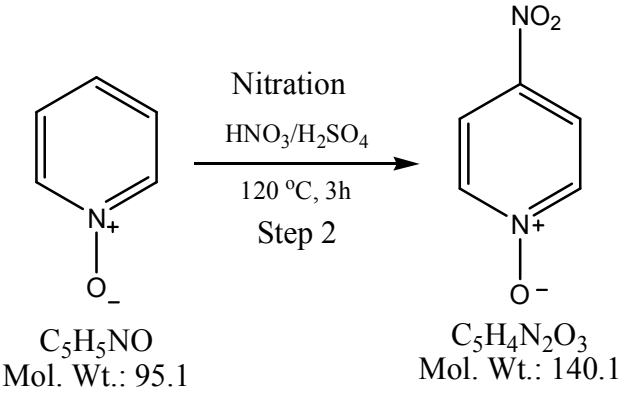


Fig No 05: Step 2 Nitration of IMP-DAL 1

Preparation of the nitrating mixture:

10ml of fuming HNO_3 are filled in a 250 mL round bottom flask with magnetic stir bar. Slowly and in portions 10ml of conc. H_2SO_4 are added under stirring and cooling in an ice bath. The nitrating mixture is brought to a temperature of 20 °C.

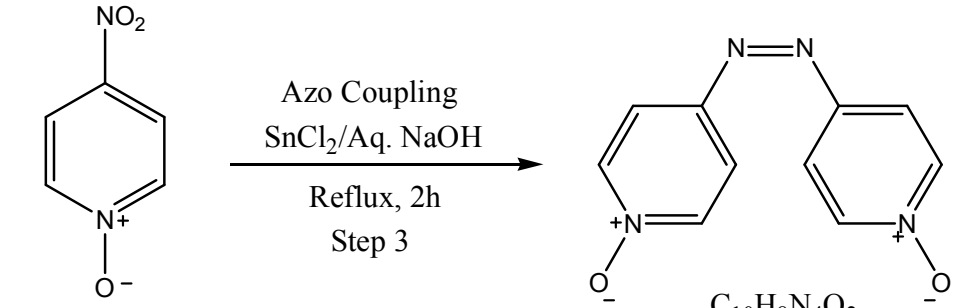
10gms of pyridine-N-oxide are filled in the reaction flask and heated to 60°C. The nitrating mixture is transferred into an addition funnel and added drop wise within 30 minutes under stirring without further heating. Thereby the internal temperature drops to about 40°C. Afterwards the reaction mixture is refluxed for 3 hours to 125-130°C internal temperature.

Work up:

After cooling down to room temperature the reaction mixture is poured in a 1 L beaker containing 150g finely crunched ice. Then about 170 mL of a saturated sodium bicarbonate solution are added carefully in portions until a pH-value of 7 - 8 is reached. A yellow crystalline solid precipitates, consisting of product and sodium sulfate. Crude yield is 5.5 gm.

To the yellow crude product acetone is added and the insoluble white salt is separated over a Buechner funnel. The solvent is evaporated from the filtrate at a rotary evaporator, the remaining yellow product is dried in a desiccator. Product Yield is 3.5 gm.

STEP 3: Azo Coupling of 4-Nitro Pyridine N- Oxide:



$C_5H_4N_2O_3$
Mol. Wt.: 140.1

$C_{10}H_8N_4O_2$
Mol. Wt.: 216.2

Fig No 06: Step 3 Azo Coupling of IMP-DAL 1

Three-neck round-bottomed flask, fitted with a stirrer and a reflux condenser, is placed on a steam cone. In the flask are placed 3.5 gm of 4-nitro pyridine -N- oxide is dissolved in 200 ml of 10% Aq. NaOH . To the mixture is added 10 gm of $SnCl_2$, the stirrer is started, and the mixture is refluxed for 1 hour at 100°C. The initial addition of the reactants resulted in a cloudy yellow solution with a black deposit on the bottom of the flask (metallic tin). At the end of two hours, the solution was a vivid red with dark red oil on the surface.

Workup:

The reaction mass was concentrated by removing sodium hydroxide. The reaction mass quenched in 20 ml of water and extracted with 100 ml of DCM and concentrated. Product Yield is 2gm.

STEP 4: Deoxygenation:

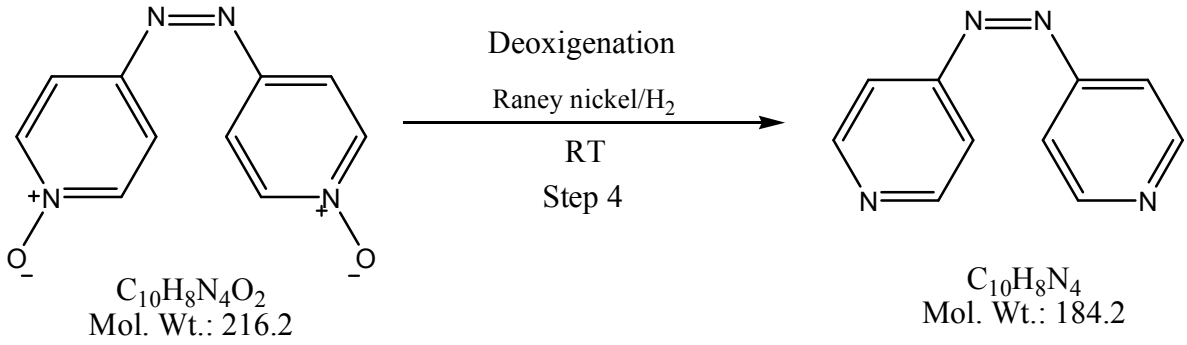


Fig No 07: Step 4 Deoxygenation of IMP-DAL 1

The catalytic reduction over Raney nickel at atmospheric temperature and pressure was suitable for this reaction. The catalytic reduction of 2gm of step 3 product in 100 ml of MeOH, with 1 mole equivalent of Raney nickel added. The reaction is carried under hydrogen pressure. The reaction is stopped after 3-4 hrs reduction. The reaction is monitored by TLC and LC-MS.

Workup:

The reaction mass is filtered off by using high vacuum remove Raney nickel. The filtrate is concentrated to remove methanol by using rotary evaporator. Product Yield is 1.5 gm.

STEP 5: Reduction:

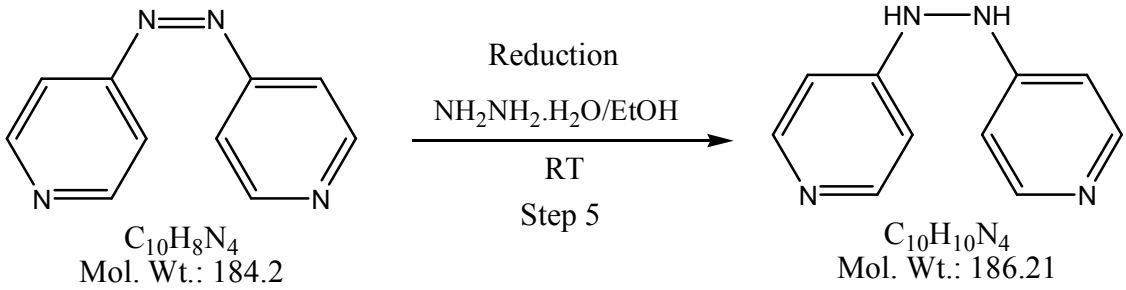


Fig No 08: Step 5 Reduction of IMP-DAL 1

1.5gms of step 4 product was dissolved in 30 ml of ethanol and 0.3ml of hydrazine hydrate was added. The reaction was carried out to color change from deep orange to brown color. The reaction mass was monitored by TLC and LC-MS.

Workup:

The reaction mass was concentrated by removing ethanol and unreacted hydrazine hydrate .The reaction mass quenched in 20 ml of water and extracted with 100 ml of DCM and concentrated. Product Yield is1gm.

Synthesized final mass of the compound was separated and purified by automated combiflash RF and purity of compound is analyzed by HPLC.

SCHEME TO SYNTHESIS IMP-DAL 2 (4- Amino pyridine N- Oxide)

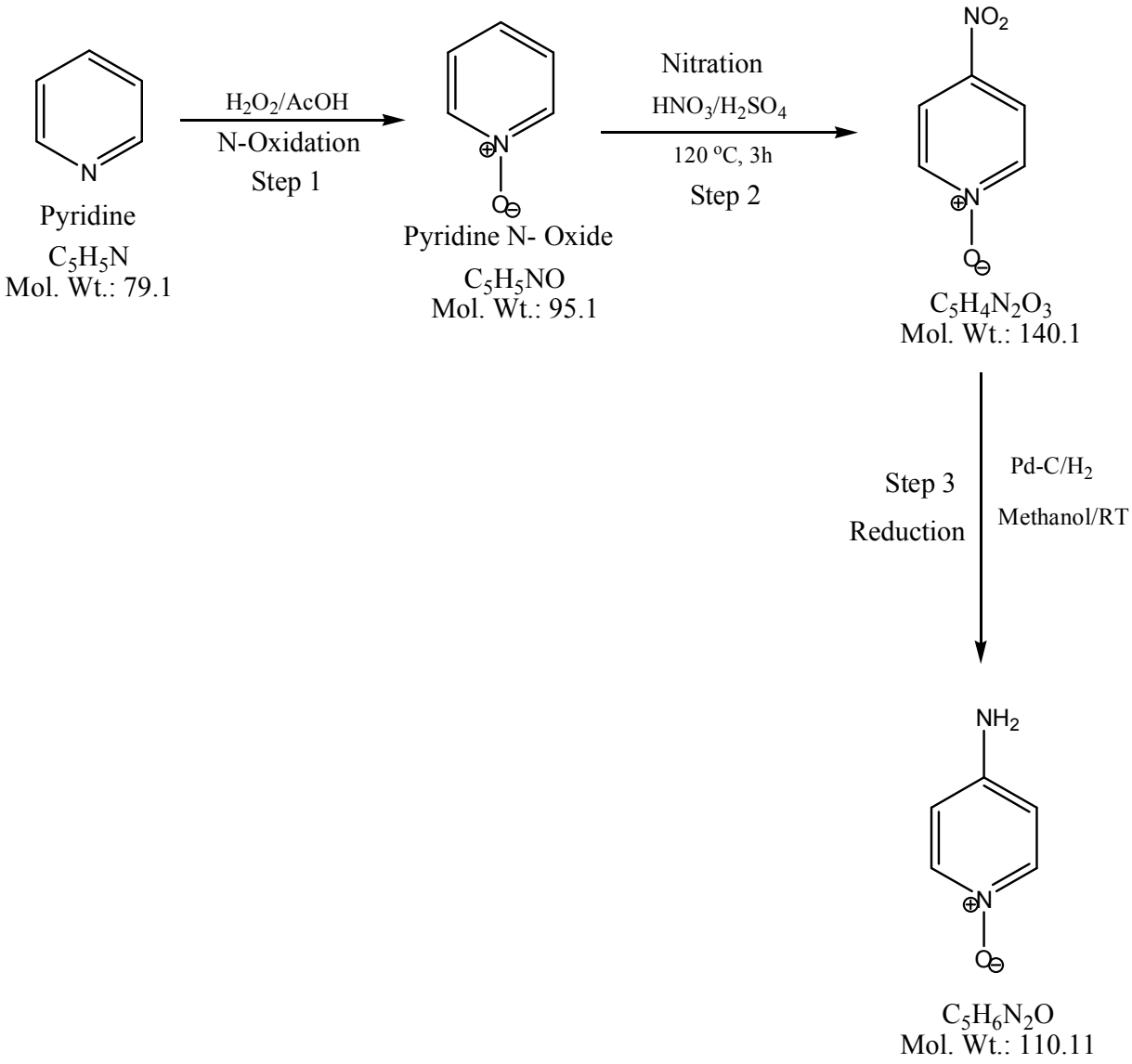


Fig No 09: Synthetic Scheme of IMP-DAL 2 (4- Amino Pyridine N- Oxide).

STEP 1: Oxidation of Pyridine:

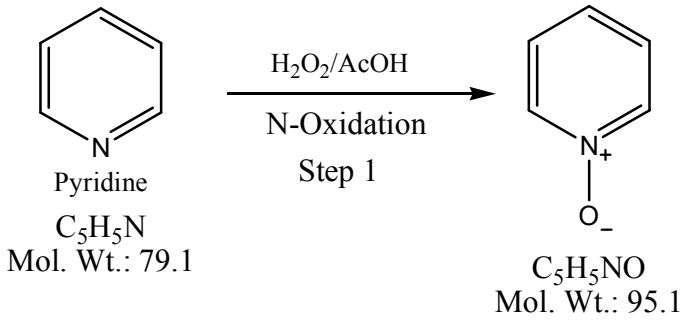


Fig No 10: Step 1 Oxidation of IMP-DAL 2

Pyridine is oxidized into pyridine N oxide which on treatment with H_2O_2/ AcOH.10 grams of Pyridine was taken in the single neck RB flask, to this 30 ml of acetic acid was added and stirred for ten minutes. 50 ml of hydrogen peroxide was added and closed the flask with stopper. The reaction mixture was stirred over night.

Workup:

After completion of the reaction, removed the excess acetic acid and peroxide by concentraring in the rotavapor and dried completely. Three times added a toluene and removed the solvent in rotavapor to expel the traces of water. Product Yield is 10 gm.

STEP 2: Nitration Of Pyridine N – Oxide:

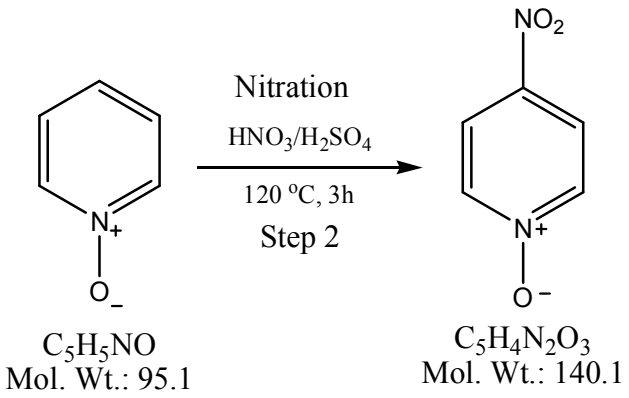


Fig No 11: Step 2 Nitration of IMP-DAL 2

Preparation of the nitrating mixture: 10ml of fuming HNO_3 are filled in a 250 mL round bottom flask with magnetic stir bar. Slowly and in portions 10ml of conc. H_2SO_4 are added under stirring and cooling in an ice bath. The nitrating mixture is brought to a temperature of 20 °C.

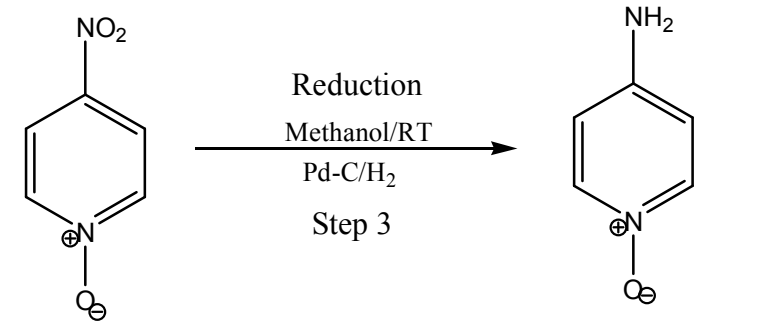
10gms of pyridine-N-oxide are filled in the reaction flask and heated to 60°C. The nitrating mixture is transferred into an addition funnel and added dropwise within 30 minutes under stirring without further heating. Thereby the internal temperature drops to about 40°C. Afterwards the reaction mixture is refluxed for 3 hours to 125-130°C internal temperature.

Work up

After cooling down to room temperature the reaction mixture is poured in a 1 L beaker containing 150g finely crunched ice. Then about 170 mL of a saturated sodium bicarbonate solution are added carefully in portions until a pH-value of 7 - 8 is reached. A yellow crystalline solid precipitates, consisting of product and sodium sulfate. Crude yield: 5.5 g

To the yellow crude product acetone is added and the insoluble white salt is separated over a Buechner funnel. The solvent is evaporated from the filtrate at a rotary evaporator, the remaining yellow product is dried in a desiccator. Product Yield is 3.5 g.

STEP 3: Reduction:



$C_5H_4N_2O_3$
Mol. Wt.: 140.1

$C_5H_6N_2O$
Mol. Wt.: 110.11

Fig No 12: Step 3 Reduction of IMP-DAL 2

Nitro group rapidly reduced at room temperature to amino group in methanol in the presence of 10% palladium on carbon. 0.5gm of 4-nitro pyridine -N- oxide was dissolved in 30 ml of methanol .100 mg of 10% palladium added in that reaction mass. After added palladium the reaction mass carried under hyderogen pressure condition for overnight. The reaction is monitored by TLC and LC-MS.

Workup

The reaction mass is filtered off by using high vacuum to remove palladium. The filterate is concentrated to remove methanol by using rotary evaporator.Yield:190 mg

SCHEME TO SYNTHESIS IMP-DAL 3 (2 – Amino pyridine)

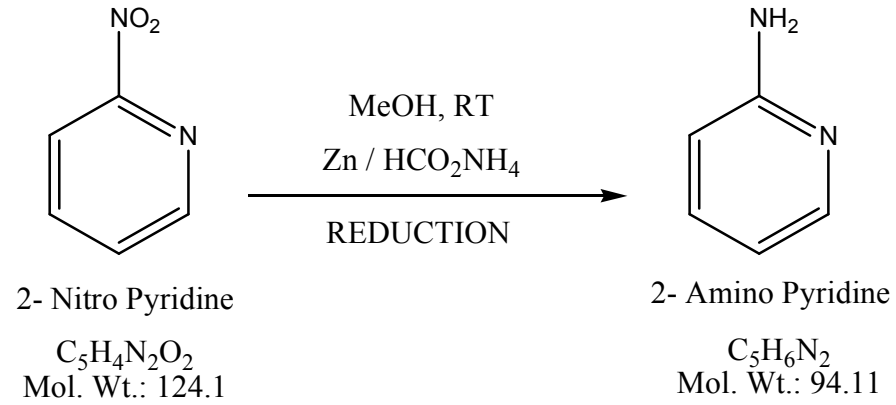


Fig No 13: Synthetic Scheme of IMP-DAL 3

Reduction:

Nitro group rapidly reduced at room temperature to amino group in methanol in the presence of zinc and ammonium formate . 1gm of 2-nitro pyridine was dissolved in 20 ml of methanol. 0.5gm of ammonium formate and 100mg of zinc added in that reaction mass at room temperature. After added zinc the reaction mass carried for overnight. The reaction is monitored by TLC and LC-MS.

Workup

The reaction mass is filtered off by using high vacuum to remove zinc. The filterate is concentrated to remove methanol and unreacted ammonium formate by using rotary evaporator. Product Yield is 0.6gm.

SCHEME TO SYNTHESIS IMP-DAL 4 (3 – Amino pyridine)

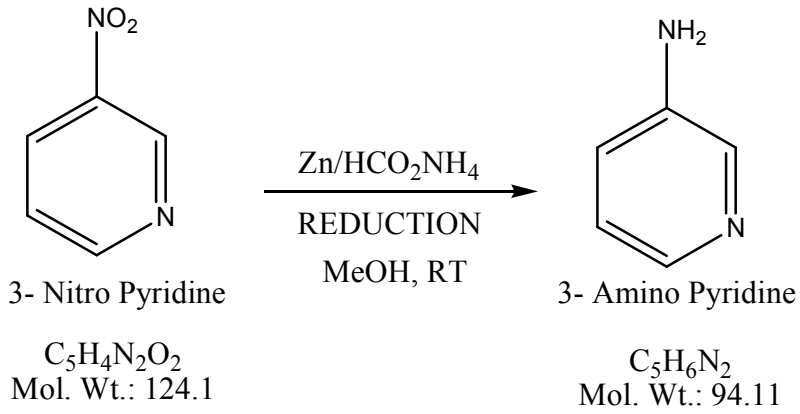


Fig No 14: Synthetic Scheme of IMP-DAL 4

Reduction:

Nitro group rapidly reduced at room temperature to amino group in methanol in the presence of zinc and ammonium formate. 1gm of 3-nitro pyridine was dissolved in 30 ml of methanol. 0.5gm of ammonium formate and 50mg of zinc added in that reaction mass at room temperature. After added zinc the reaction mass carried for overnight. The reaction is monitored by TLC and LC-MS.

Workup

The reaction mass is filtered off by using high vacuum to remove zinc. The filterate is concentrated to remove methanol and unreacted ammonium formate by using rotary evaporator.

Product Yield is 0.5 gm.

SCHEME TO SYNTHESIS IMP-DAL 5 (Pyridine 4 - Carboxamide)

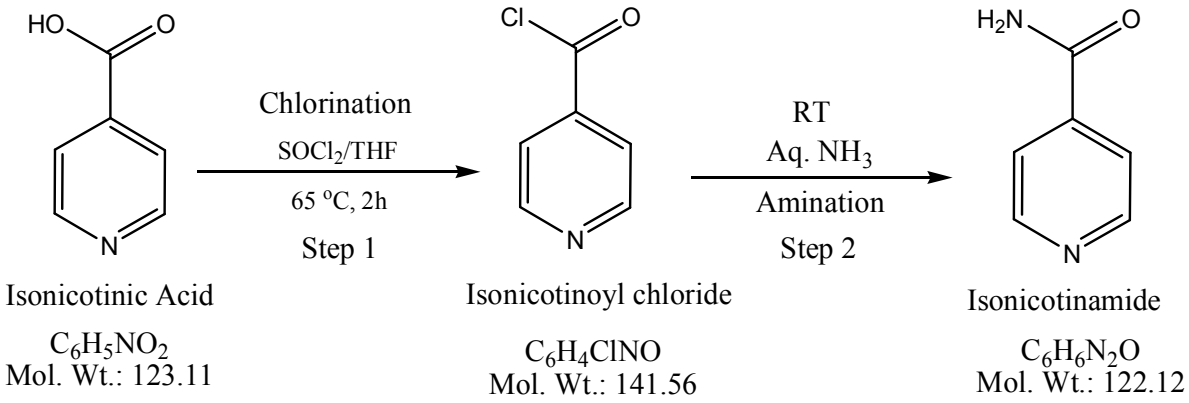


Fig No 15: Synthetic Scheme of IMP-DAL 5

STEP 1: Chlorination of Pyridine 4 – Carboxylic acid:

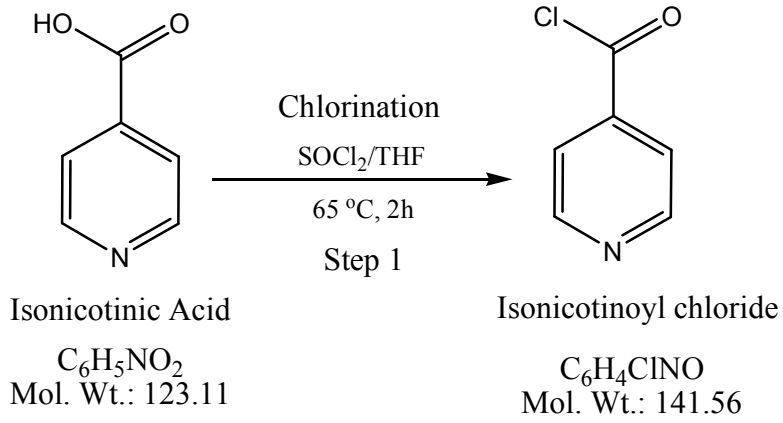


Fig No 16: Step 1 Chlorination of IMP-DAL 5

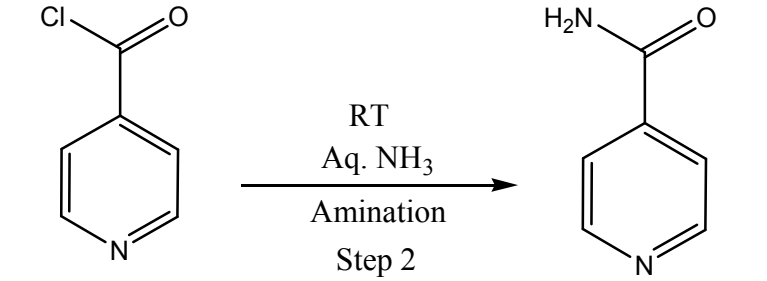
1gm of isonicotinic acid was dissolved in 30ml of tetrahydrocarbon was taken in the single neck RB flask, to this add 5 ml of thionyl chloride. After added thionyl chloride the reaction mixture was refluxed for 2 hours to 65^{o}c. The reaction is monitored by TLC and LC-MS.

Workup

The reaction mass was concentrated by removing sodium tetra hydrofuran. The reaction mass quenched in 20 ml of water , neutralized by

saturated sodium bicarbonate and extracted with 100 ml of DCM and concentrated. Product Yield is 0.5gm

STEP 2: Amination:



Isonicotinoyl chloride

C_6H_4ClNO
Mol. Wt.: 141.56

Isonicotinamide

$C_6H_6N_2O$
Mol. Wt.: 122.12

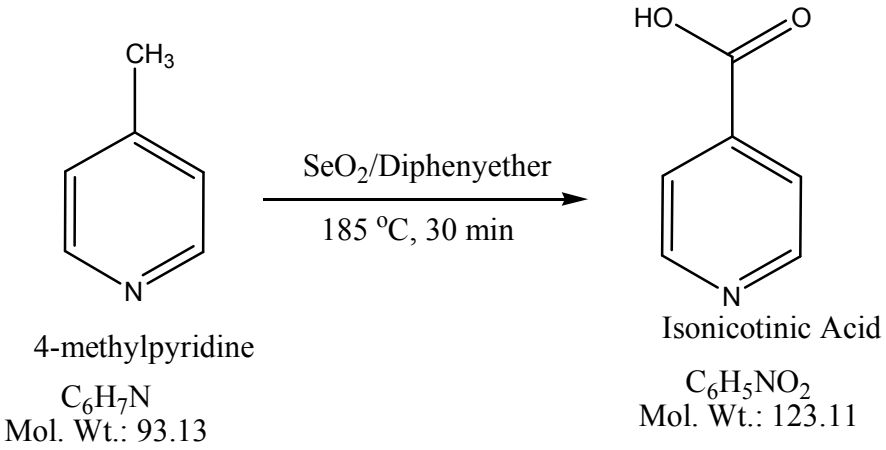
Fig No 17: Step 2 Amination of IMP-DAL 5

The step one 0.5gm of product was dissolved in 30 ml of aqueous ammonia kept in a overnight. The reaction is monitored by TLC and LC-MS.

Workup

After completion of the reaction, the excess ammonia was removed by concentrating in the rotavapor and dried completely. Product Yield is 0.4gm.

SCHEME TO SYNTHESIS IMP-DAL 6 (Pyridine 4 – Carboxylic acid)



4-methylpyridine

C_6H_7N

Mol. Wt.: 93.13

Isonicotinic Acid

$C_6H_5NO_2$

Mol. Wt.: 123.11

Fig No 18: Synthetic Scheme of IMP-DAL 6

Oxidation of 4 – Methyl Pyridine :

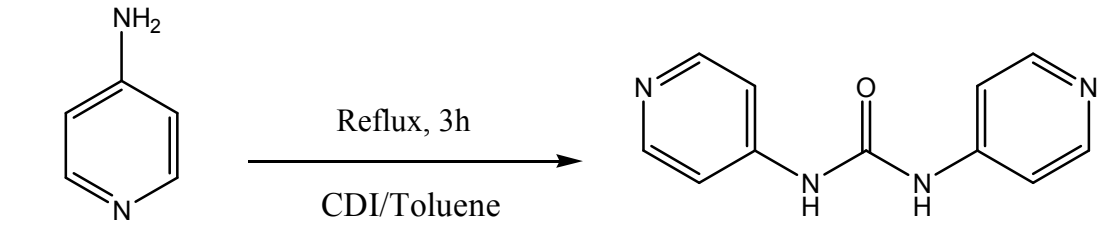
0.5gm of 4-methyl pyridine placed in a three neck RB fitted with condenser and dissolved in 150ml of di-phenyl ether. The solution was heated up to 150°C. After reaching that heat 10 gm of selenium dioxide was added portion wise over a period of 15mins.The reaction temperature was raised up to 185°C for 30 mins and end of the reaction precipitation is formed.

Workup

After completion of reaction diphenyl ether was decanted from the precipitated selenium iso nicotinic acid residue. The residue was washed with 75ml of low boiling ligroin.

The black selenium acid residue was ground to the powder and extracted with 100ml of boiling water. The fractions were formed and cooled in ice bath, rapidly crystals of iso nicotinic acid formed. Product Yield is 0.48gm.

SCHEME TO SYNTHESIS IMP-DAL 7 (1,3 di (pyridine- 4 -yl) urea)



4- Amino Pyridine

$C_5H_6N_2$
Mol. Wt.: 94.11

1,3-di(pyridin-4-yl)urea

$C_{11}H_{10}N_4O$
Mol. Wt.: 214.22

Fig No 19: Synthetic Scheme of IMP-DAL 7

500mg of 4-Aminopyridine was taken in the single neck RB flask, to this 30 ml of toluene was added and stirred for ten minutes. To the mixture is added 0.7gm of 1,1'carbonyl di-imidazole and the mixture is refluxed for 3 hours at 120°C.The reaction is monitored by TLC.

Workup

The reaction mass was concentrated to removing toluene by rotary evaporator and the crude product was recrystallised from hexane wash and diethyl ether. Product Yield is 0.2gm.

LC-MS INSTRUMENT & CHROMATOGRAPHIC DETAILS FOR CHARACTERISATION STUDY

Equipment Details:

LC - Agilent technologies - 1200 Series.

MS - Agilent technologies - 6130 Quadrupole LC/MS.

Chemicals and Reagents:

Formic acid : AR grade

Acetonitrile : HPLC grade

Methanol : HPLC grade

Ethanol : HPLC grade

Water : Milli-Q

Chromatographic Condition:

Column description : C18 3.5µm, 4.6x100mm

Column temperature: 30^{o}

Flow rate : 0.6ml/Minute

Wavelength: 254 nm

Injection volume: 20µm

Runtime: 10 Minutes

Gradient method details:

Time	Solv. B	Solv. C	Solv. D	Flow	Pressure
0.00	**95.0**	**0.0**	**0.0**	**0.600**	**400**
4.00	**5.0**	**0.0**	**0.0**	**0.600**	**400**
7.00	**5.0**	**0.0**	**0.0**	**0.600**	**400**
7.10	**95.0**	**0.0**	**0.0**	**0.600**	**400**
10.00	**95.0**	**0.0**	**0.0**	**0.600**	**400**

Table No 05 : LC-MS gradient details

Lamp: UV Lamp

Detector: DAD

MS Conditions

Method spray chamber: MM-ES+APCI

	Positive	**Negative**
Capillary Voltage(V)	4000	2500
Corona Current(μA)	10.0	10.0
Charging Voltage(V)	2000	2000

	Actual	Set point	Maximum
Drying gas flow (1/min)	12.0	12.0	13.0
Nebulizer pressure (psig)	35.0	35.0	60.0
Drying gas temperature(^{o}C)	249.0	250.0	250.0
Vaporizer temperature(^{o}C)	200	200.0	250.0

Table No 06 : MS Conditions of Dalfampridine

Solution preparation:

Moble phase A [0.1% formic acid buffer]

Add 1 ml of formic acid to 1000 ml of water , mix well and sonicate for 10 mins.

Mobile phase B [100 % acetonitrile**].**

HPLC INSTRUMENT & CHROMATOGRAPHIC DETAILS FOR CHARACTERISATION STUDY

Equipment Details:

HPLC – Agilent technologies -1100 series

Chemicals and Reagents:

Formic acid : AR grade

Acetonitrile : HPLC grade

Water : Milli-Q

CHROMATOGRAPHIC METHOD: CNX- LC

Chromatographic Condition:

Column description : C18 3.5µm, 4.6x100mm

Column temperature: 25^{o} C

Flow rate : 0.6ml/Minute

Wavelength: 254 nm

Injection volume: 20µl

Runtime: 10 Minutes

Gradient method details:

Time	Solv. B	Solv. C	Solv. D	Flow	Pressure
0.00	95.0	0.0	0.0	0.600	400
4.00	5.0	0.0	0.0	0.600	400
7.00	5.0	0.0	0.0	0.600	400
7.10	95.0	0.0	0.0	0.600	400
10.00	**95.0**	**0.0**	**0.0**	**0.600**	**400**

Table No 07 : HPLC Gradient Method Details For CNX- LC

Solution preparation:

Moble phase A [0.1% formic acid buffer]

Add 1 ml of formic acid to 1000 ml of water , mix well and sonicate for 10 mins.

Mobile phase B [100 % acetonitrile**]**

CHROMATOGRAPHIC METHOD: CNX

Chromatographic Condition:

Column description : Shodex C 18 4 D 5 µm

Column temperature: 25^{o} C

Flow rate : 0.8ml/Minute

Wavelength: 254 nm

Injection volume: 5µl

Runtime: 35 Minutes

Gradient method details:

Time	Flow(ml/min)	%A	%B	%C	%D	Max Pressure (bar)
5.00	0.800	50.00	50.00	0.00	0.00	400
20.00	0.800	40.00	60.00	0.00	0.00	400
25.00	0.800	30.00	70.00	0.00	0.00	400
25.00	0.800	70.00	30.00	0.00	0.00	400
35.00	0.800	70.00	30.00	0.00	0.00	400

Table No 08 : HPLC Gradient Method Details For CNX

Solution preparation:

Moble phase A [0.1% formic acid buffer]

Add 1 ml of formic acid to 1000 ml of water , mix well and sonicate for 10 mins.

Mobile phase B [100 % acetonitrile**]**

CHROMATOGRAPHIC METHOD: DALFAMPRIDINE

Chromatographic Condition:

Column description : Shodex C 18 4 D 5 µm

Column temperature: 30° C

Flow rate : 2ml/Minute

Wavelength: 275 nm

Injection volume: 10µl

Runtime: 25 Minutes

Gradient method details:

Time	Flow(ml/min)	%A	%B	%C	%D	Max Pressure (bar)
0.00	2.00	90.00	10.00	0.00	0.00	400
5.00	2.00	80.00	20.00	0.00	0.00	400
15.00	2.00	80.00	20.00	0.00	0.00	400
20.00	2.00	90.00	10.00	0.00	0.00	400
25.00	2.00	90.00	10.00	0.00	0.00	400

Table No 09 : HPLC Gradient Method Details For Dalfampridine

Solution preparation

Mobile phase A : 3.03 g/l of sodium 1-heptanesulfonate, 1.36 g/l of monobasic potassium phosphate, and 1.15 g/l of phosphoric acid in water.

Mobile phase B : 100% Acetonitrile

AUTOMATED COMBI FLASH INSTRUMENT AND CHROMATOGRAPHIC ITS DETAILS FOR PURIFICATION

Equipment:

Combi flash Rf 200

Chemicals and reagents :

Methanol

DCM

Ethyl acetate

Hexane

Chromatographic Condition:

Column description : iLOK – 40g (70 ml) 1 D 26.8 x H127

Column temperature: 25^{o} C

Flow rate : 30ml/Minute1

Wavelength: 210 nm, 254 nm

Runtime: 60 Minutes

Solvent system:

Moble phase A [80 % DCM]

Mobile phase B [20 % Methanol**]**

EVALUATION OF ANTI BACTERIAL ACTIVITY

The condition which must be fulfilled before screening the antibacterial activity. The organism and drug substance to be evaluated should be contacted intimately. The microorganism's growth should be maintained under conditions like incubation period, temperature, nutrient media, etc. Throughout the study conditions were maintained. Sterile environment should be maintained. Several researchers reported different method for evaluation of antibacterial activity. Few of the widely used method were as follows,

1. Agar-streak dilution method
2. Serial-dilution method
3. Agar-diffusion method

From above mentioned technique agar diffusion method was used for screening of antibacterial activity which is described as follows,

Agar Diffusion Method

Following are the techniques which were used for agar diffusion method

1. Agarcup
2. Agarditch
3. Paperdisc

For our study, we used the first method, to assess the minimum inhibitory concentration (MIC).

Non-automated invitro bacterial tests. This method gives accurate, precise results to identify the antimicrobial agent which is required to inhibit the growth of precise microorganisms.

Identification of MIC (Minimal Inhibitory Concentration) by using agar cup method

1. All novel synthesized molecules were tested for antibacterial activity.

2. Necessary controls were used. Like,

- Drug (Standard) control
- Organism control
- Gentamycin (Known antibacterial agent) as reference was used in the present study.
- Muller Hinton Agar (MHA) (Hi Media) was used as nutrient medium for growth of microorganisms.
- The cultures used for testing were obtained from NCL which were equivalent to ATCC cultures.
- All microorganisms were inoculated in Tryptic Soya Bean Broth (Hi Media).
- All the drugs were dissolved in sterile Methanol and DMSO (AR Grade).

3. The dissolved drugs were serially diluted according to NCCLS guidelines.

4. Microbial strain:

Name of Microorganism Used	**Type**	**Strain Used**
A. Staphylococcus-aureus.	(Gram Positive)	ATCC 25923
B. Bacillus-subtilis	(Gram Positive)	ATCC 21332
C. Pseudomonas-aeruginosa.	(Gram Negative)	ATCC 27853
D. Escherichia-coli.	(Gram Negative)	ATCC 25922

5. Sterile molten MHA was poured aseptically under laminar air flow unit into sterile Petri plates containing the test microorganism and was allowed to solidify. After solidification of the media cups/wells were bored using 'T' borer.

6. The serially diluted antibacterial test solution(synthesized compounds) concentrations are 50µg/ml, 75µg/ml and 100 µg/ml were added in the cups and allowed to diffuse in the agar by placing the Petri plates under refrigeration for 10 minutes.

7. At 37^0C all the plates stood incubated for 24 hours.

8. The inhibition zone of the test antibacterial solution and the control drug was measured using Vernier callipers and the MIC was determined.

Preparation of different media:

The growth media required for evaluation of antibacterial activity was prepared as per the procedure reported in Himedia manual, as follows:

1. Muller Hinton Agar:

It mainly contains following content,

Ingredients	**Quantity (Gm/L)**
Beef heart infusion,	2.00
Casein acid hydrolysate	17.50
Starch, soluble	1.50
Agar	17.00

Procedure:

Suspend 38 grams in 1000ml distilled water. Mix well and boil the media to dissolve completely. autoclaving at 15 lbs pressure (121^0C) for 15min sterilisation has done. Pour the media after mixing thoroughly.

2. Tryptic soya bean broth:

It mainly contains following content

Ingredients	**Quantity (Gm/L)**
Casein (enzymic hydrolysate)	18.00
Papaic digest of soybean	2.00
Sodium chloride	6.00
Dipotassium phosphate	3.50
Dextrose	3.50

Procedure:

Suspend 30grams of Tryptic soya bean broth powder in one litre of distilled water. Medium was boiled to dissolve completely. Autoclaving at 15lbs pressure (1210C) for 15 minutes.

Methods used for primary and secondary screening:

Primary screening method, the test and control drugs were diluted using sterile methanol in 1000 microgram per ml (µg/ml), 500.0 microgram per ml , 250.0 microgram per ml from which 100 µl of solution taken in the cups to obtain a concentration of 100 microgram per ml, 75 microgram per ml and 50 microgram per ml respectively. A methanol control was also run along with test and the reference drug. The promised result from the synthesized molecules was tested by primary screening for all microorganisms by second set dilution.

Secondary screening method, The promised results from the synthesized molecules in primary screening were diluted using sterile methanol as 100 microgram per ml, 75 microgram per ml, 50 microgram per ml from which 100 µl of solution taken in the cups to obtain concentration of 0.8 microgram per ml, 0.4 microgram per ml, 0.2 microgram per ml respectively.

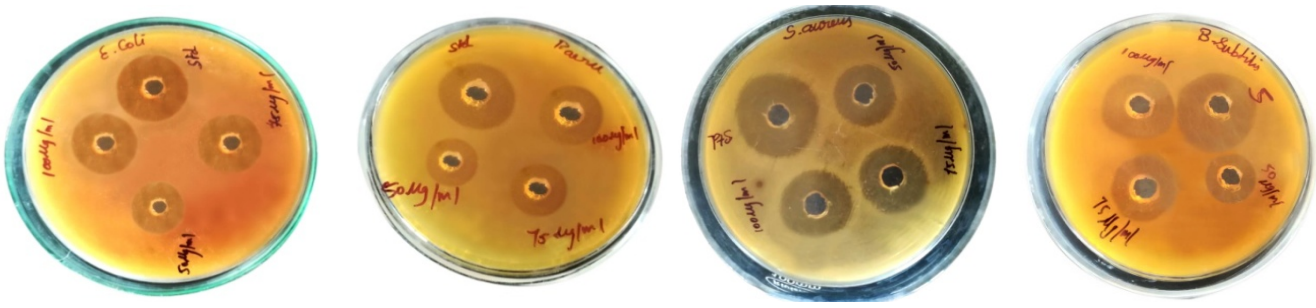


Fig No 20: Photographs showing zone of inhibition of IMP-DAL 1

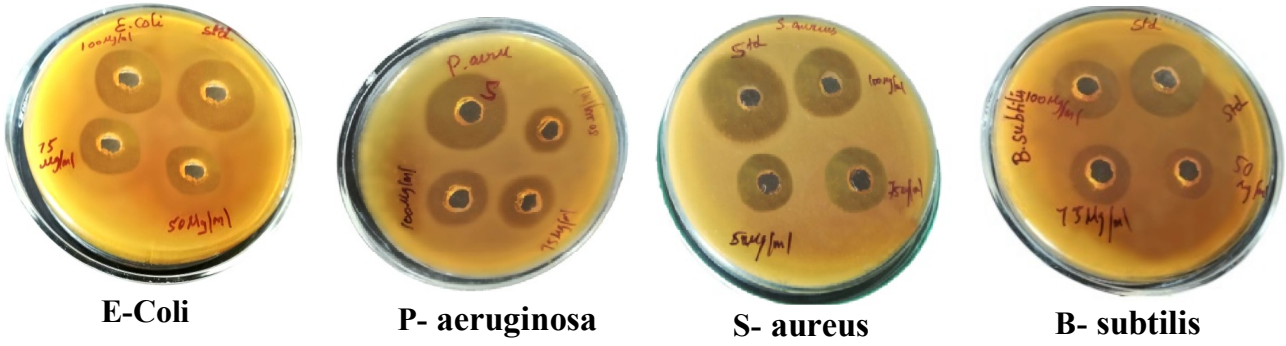


Fig No 21: Photographs showing zone of inhibition of IMP-DAL 2

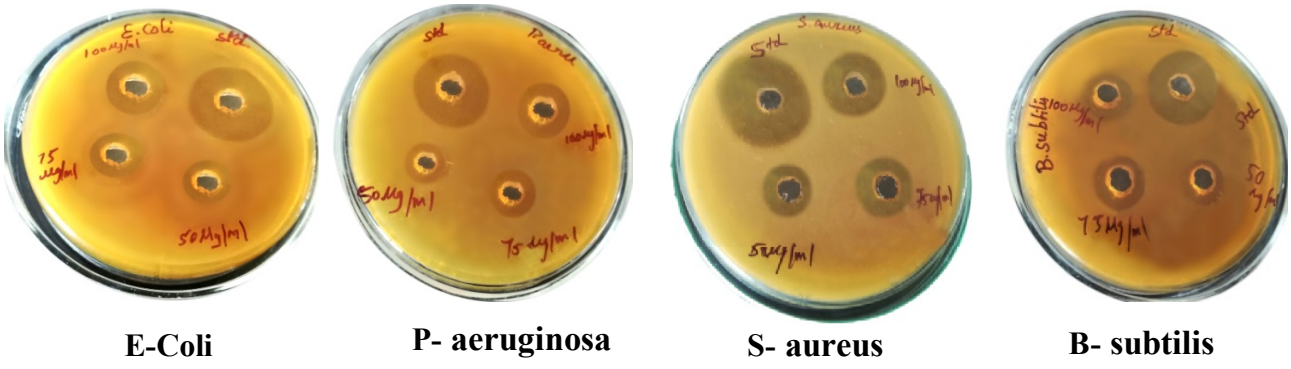


Fig No 22: Photographs showing zone of inhibition of IMP-DAL 3

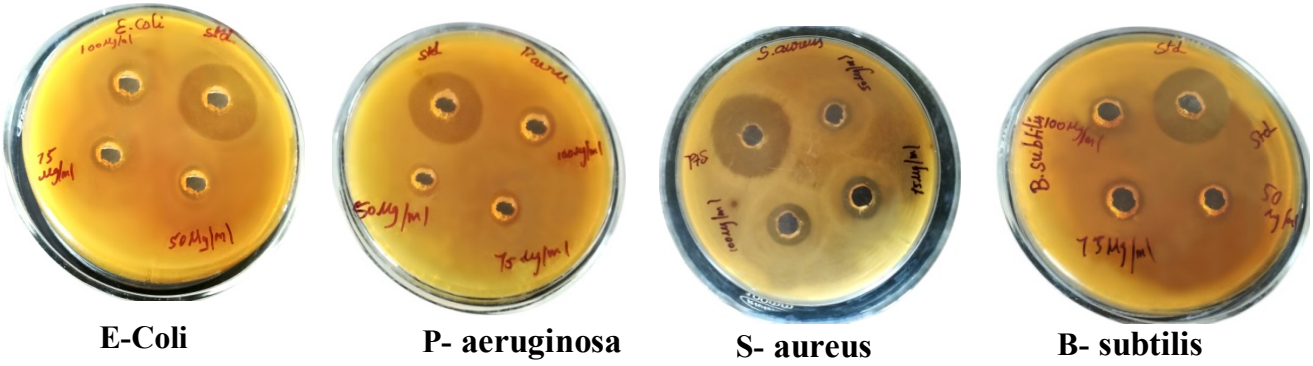


Fig No 23: Photographs showing zone of inhibition of IMP-DAL 4

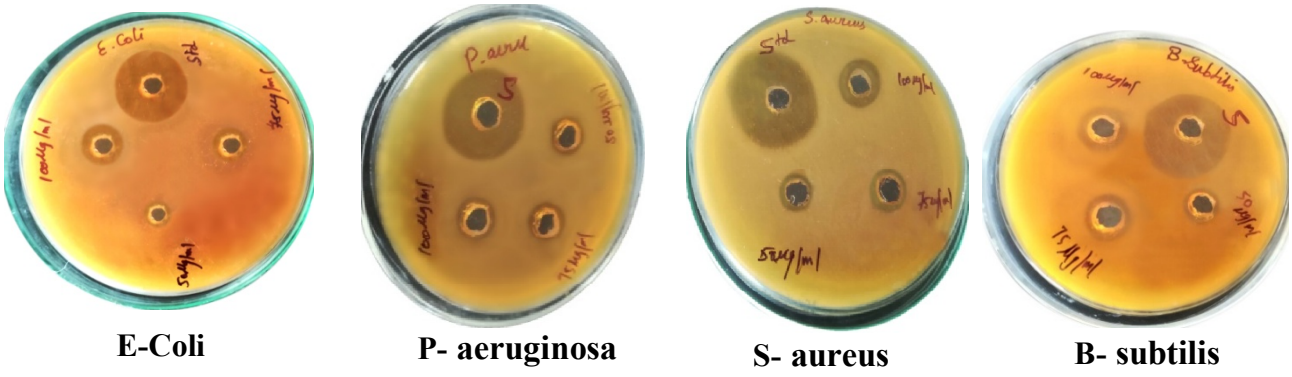


Fig No 24: Photographs showing zone of inhibition of IMP-DAL 5

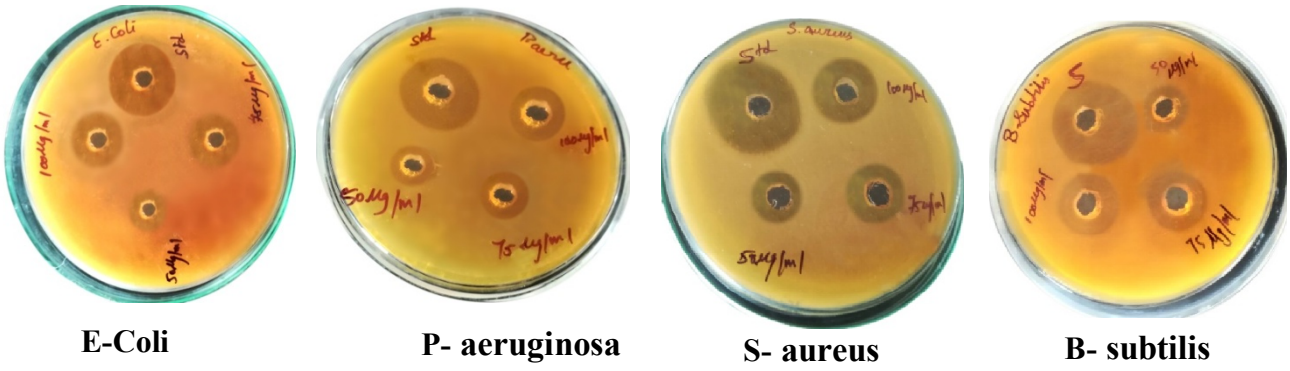


Fig No 25: Photographs showing zone of inhibition of IMP-DAL 6

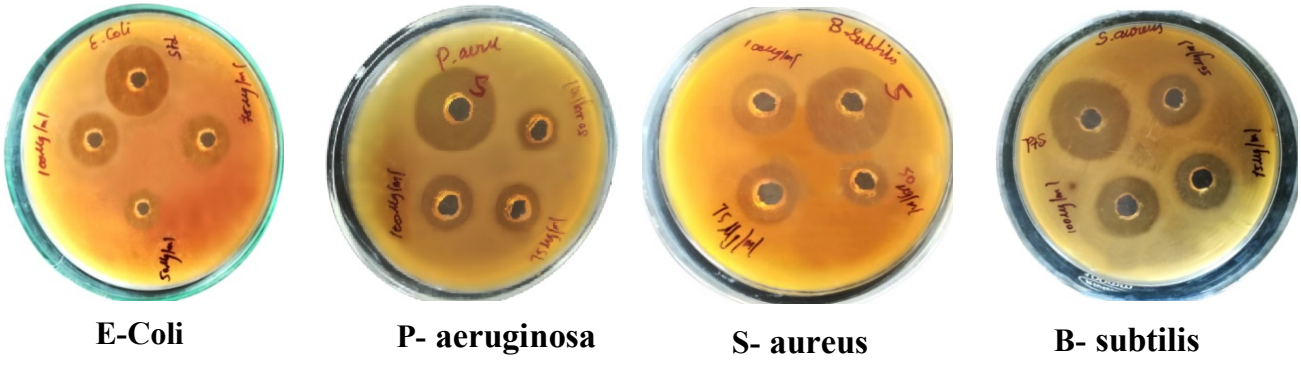
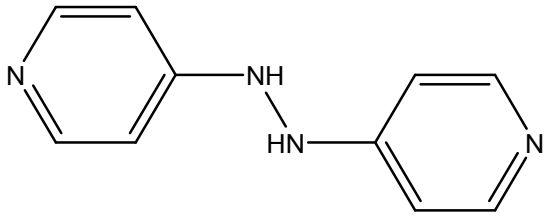


Fig No 26: Photographs showing zone of inhibition of IMP-DAL 7

7.1 RESULTS

IMP-DAL 1



1. Molecular formula : $C_{10}H_{10}N_4$
2. Molecular wt : 186.21
3. Molecular composition : C(64.50%), H(5.41%), N(30.09%).
4. Melting point : 240^{o}C
5. Colour : Brown
6. State : Solid
7. Rf value : 0.16
8. Percentage yield : 60%
9. NMR data : δ 8.39-8.41 (doublet, 4H -Ar), 8.19 - 8.21 (doublet, 4H-Ar), 3.39 (Singlet, 1H, -NH), 3.29 (Singlet, 1H)
10. HPLC purity : 92.15%
11. Mass data : m/z ratio 185.1
12. Solubility : Methanol , DMSO

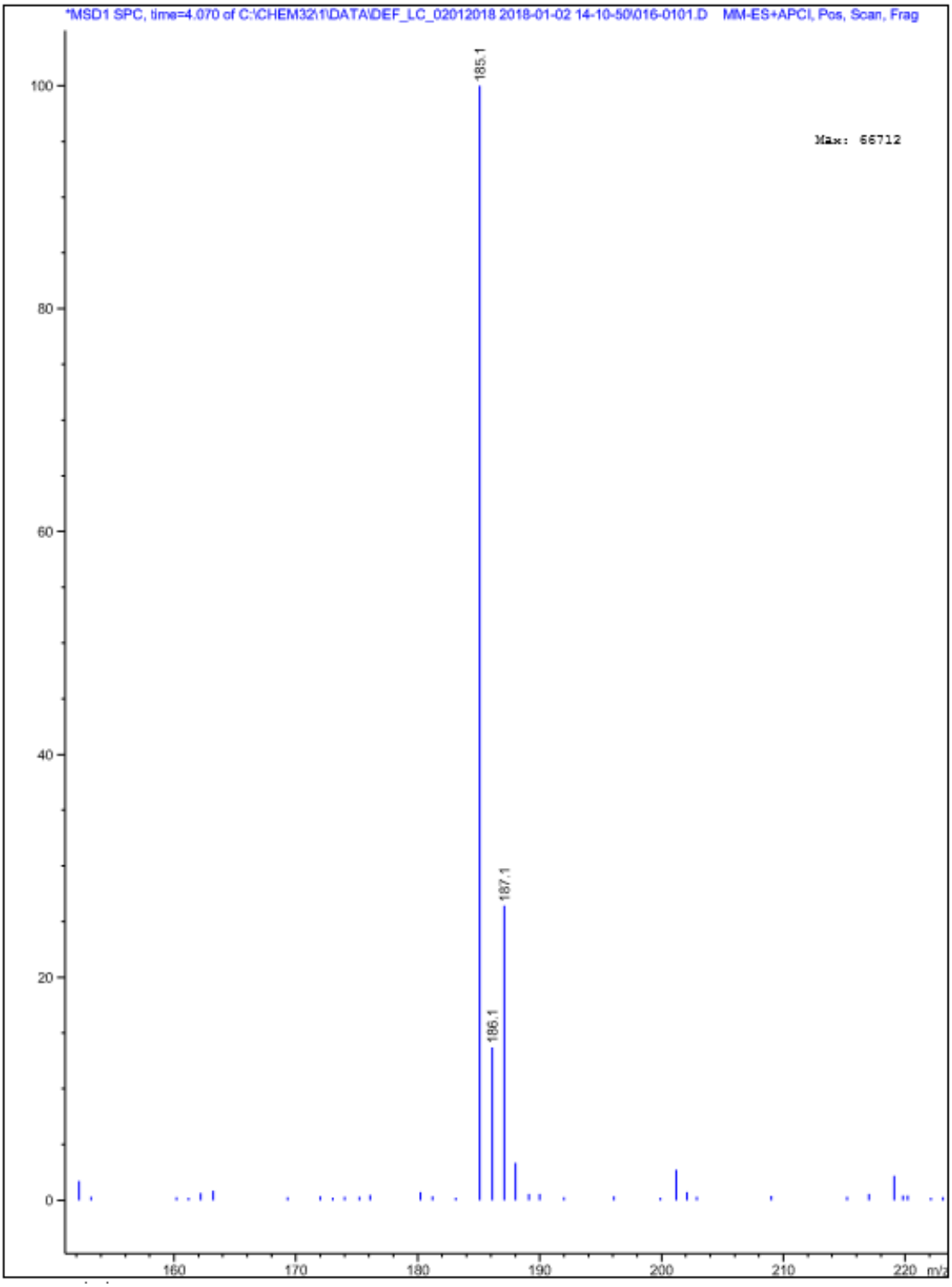
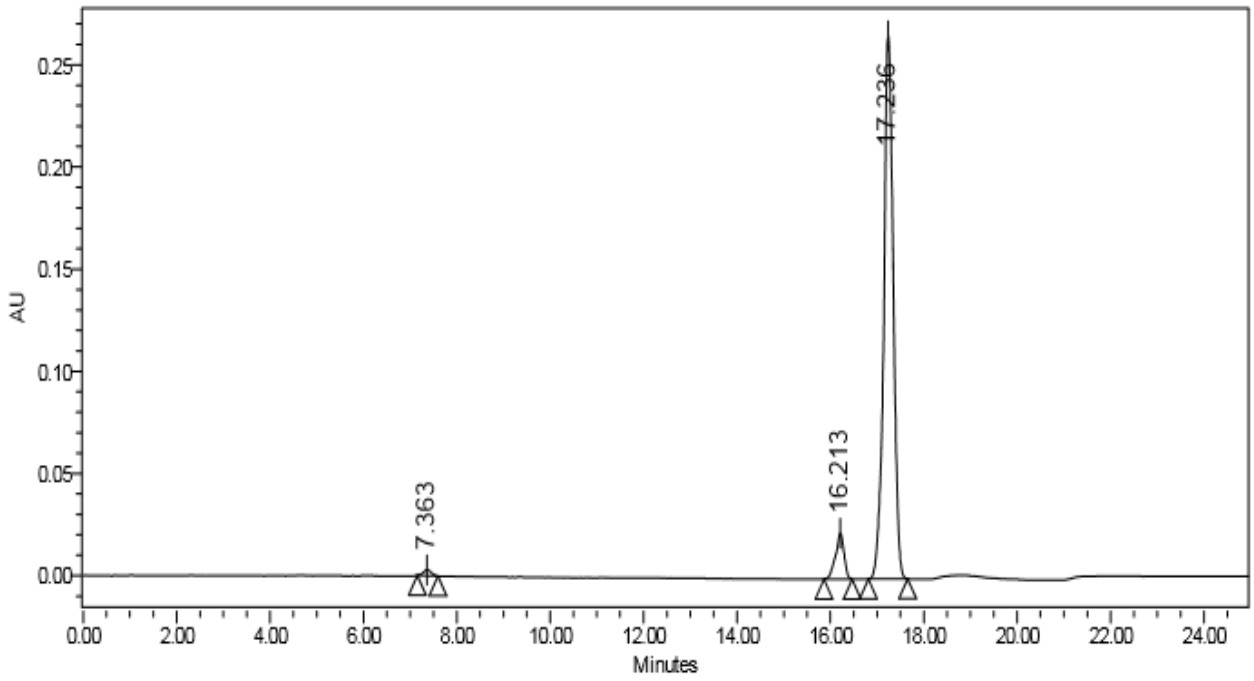


Fig No 27: LC-MS Spectrum For Title Compound IMP-DAL 1



Peak Results

	Name	RT	Area	% Area
1		7.363	34028	0.83
2		16.213	286665	7.02
3		17.236	3765214	92.15

Fig No 28: HPLC Spectrum For Title Compound IMP-DAL 1

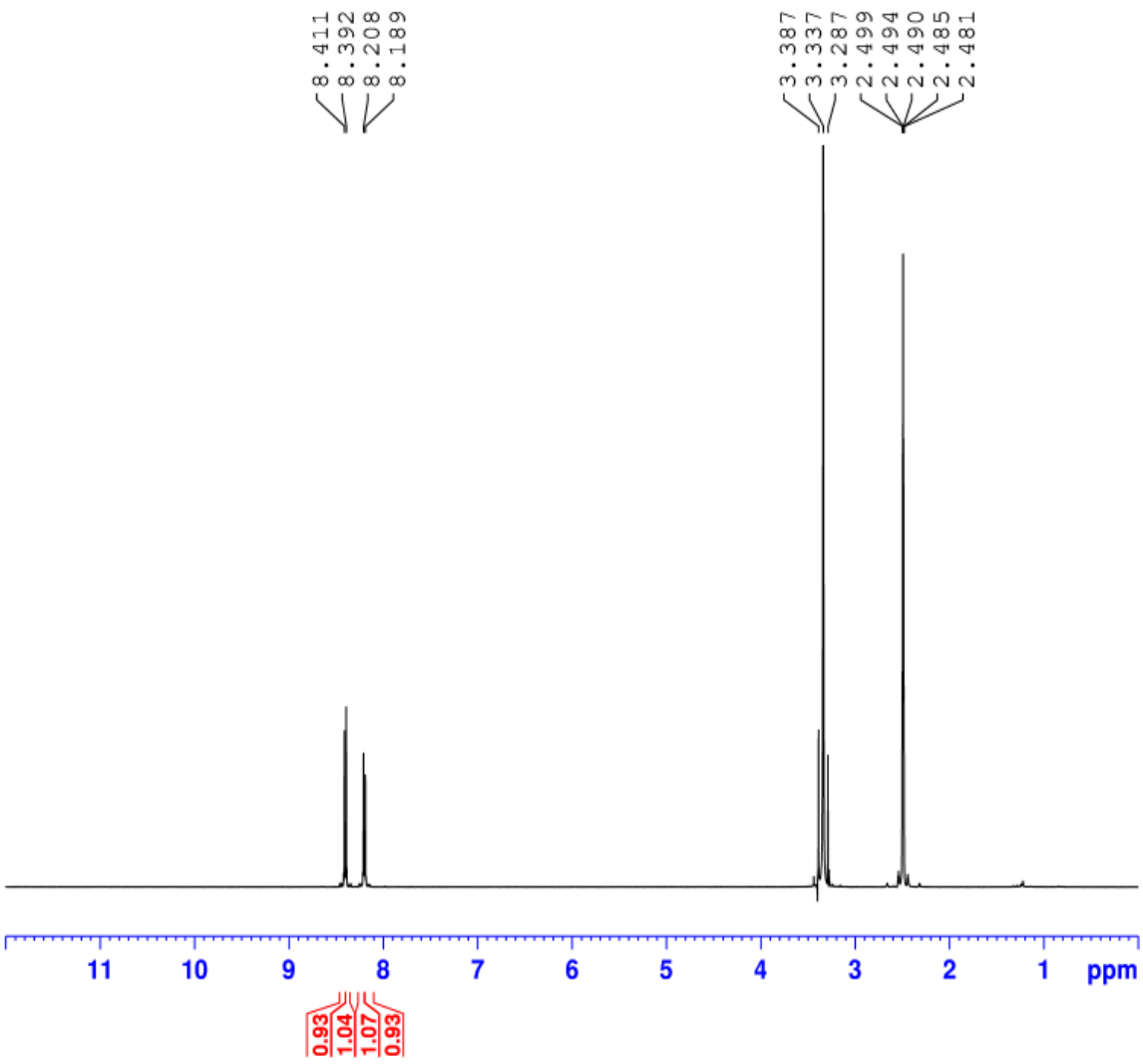
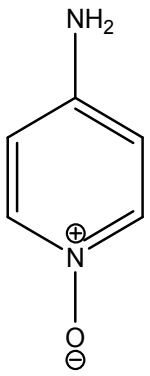


Fig No 29: ^{1}H NMR Spectrum For Title Compound IMP-DAL 1

IMP-DAL 2



1.	Molecular formula	: $C_5H_6N_2O$
2.	Molecular wt	: 110.11
3.	Molecular composition	: C(54.54%), H(5.49%), N(25.44%), O (14.53%).
4.	Melting point	: 180°C - 181°C
5.	Colour	: Brown
6.	State	: Solid
7.	Rf value	: 0.23
8.	Percentage yield	: 48.71%
9.	NMR data	: δ 7.75-7.77 (Singlet, 2 H, -Ar), 8.38-8.40 (doublet, 2H, -Ar), 6.48-6.50 (multiplet, 2H, NH)
10.	HPLC purity	: 94.42%
11.	Mass data	: m/z ratio 111.2
12.	Solubility	: Methanol , DMSO

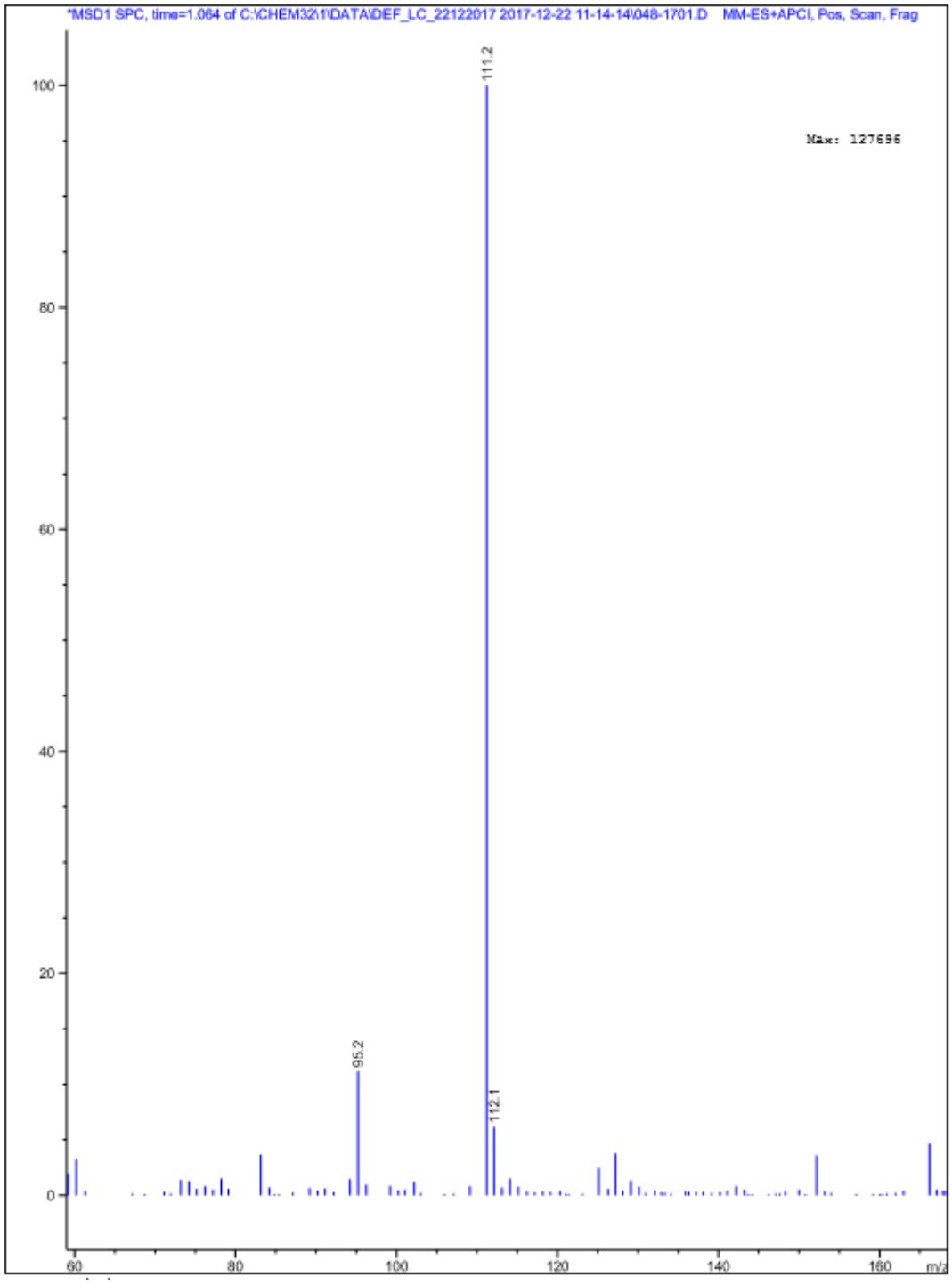
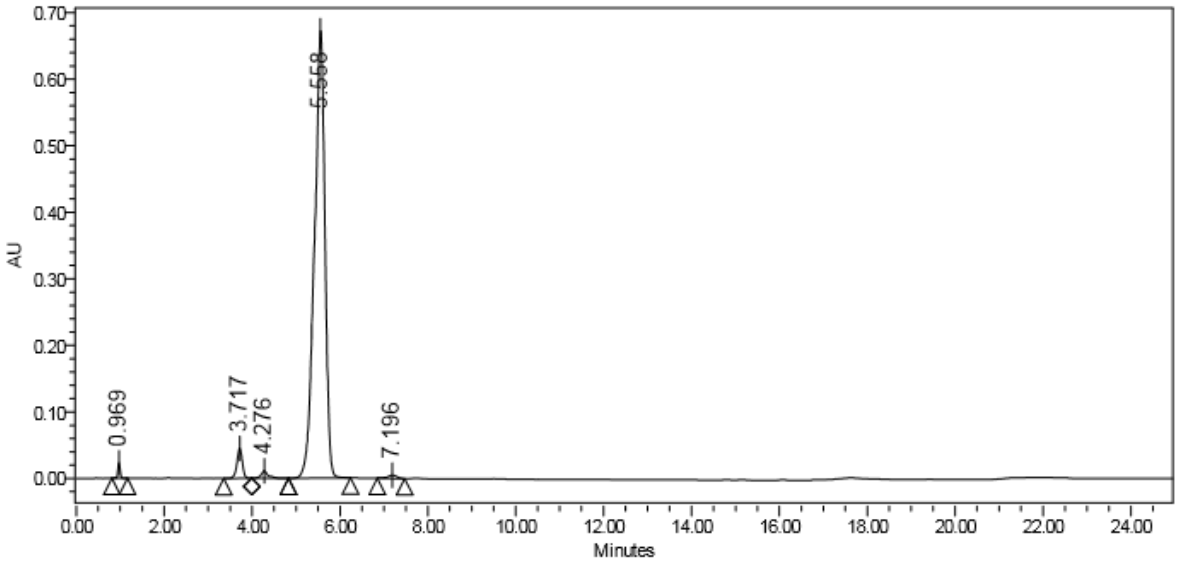


Fig No 30: LC-MS Spectrum For Title Compound IMP-DAL 2



Peak Results

	Name	RT	Area	% Area
1		0.969	83994	0.71
2		3.717	381565	3.23
3		4.276	124018	1.05
4		5.558	11142492	94.42
5		7.196	68813	0.58

Fig No 31: HPLC Spectrum For Title Compound IMP-DAL 2

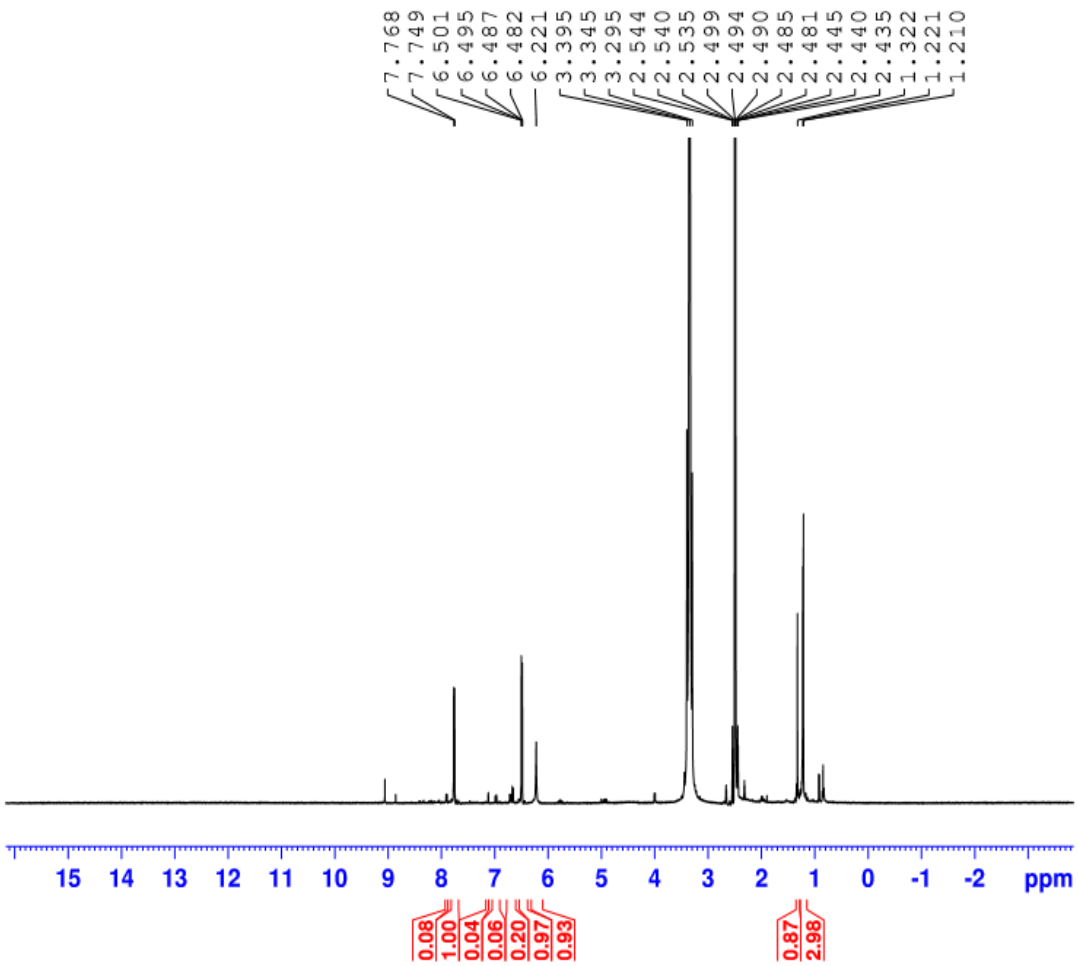
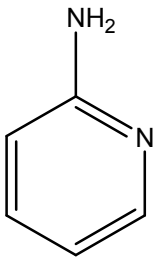


Fig No 32: ^{1}H NMR Spectrum For Title Compound IMP-DAL 2

IMP-DAL 3



1.	Molecular formula	: $C_6H_5N_2$
2.	Molecular wt	: 94.11
3.	Molecular composition	: C(63.81%), H(6.43%), N(29.77%).
4.	Melting point	: 56°C-58°C
5.	Colour	: Off - white
6.	State	: Solid
7.	Rf value	: 0.54
8.	Percentage yield	: 80%
9.	NMR data	: δ 7.86-7.87 (multiplet, 1 H, - Ar), 7.29-7.33 (multiplet, 1 H, -Ar), 6.39 – 6.44 (multiplet, 2 H, -Ar), 5.85(singlet, 2 H, $-NH_2$).
10.	HPLC purity	: 99.93%
11.	Mass data	: m/z ratio 96.4
12.	Solubility	: Methanol

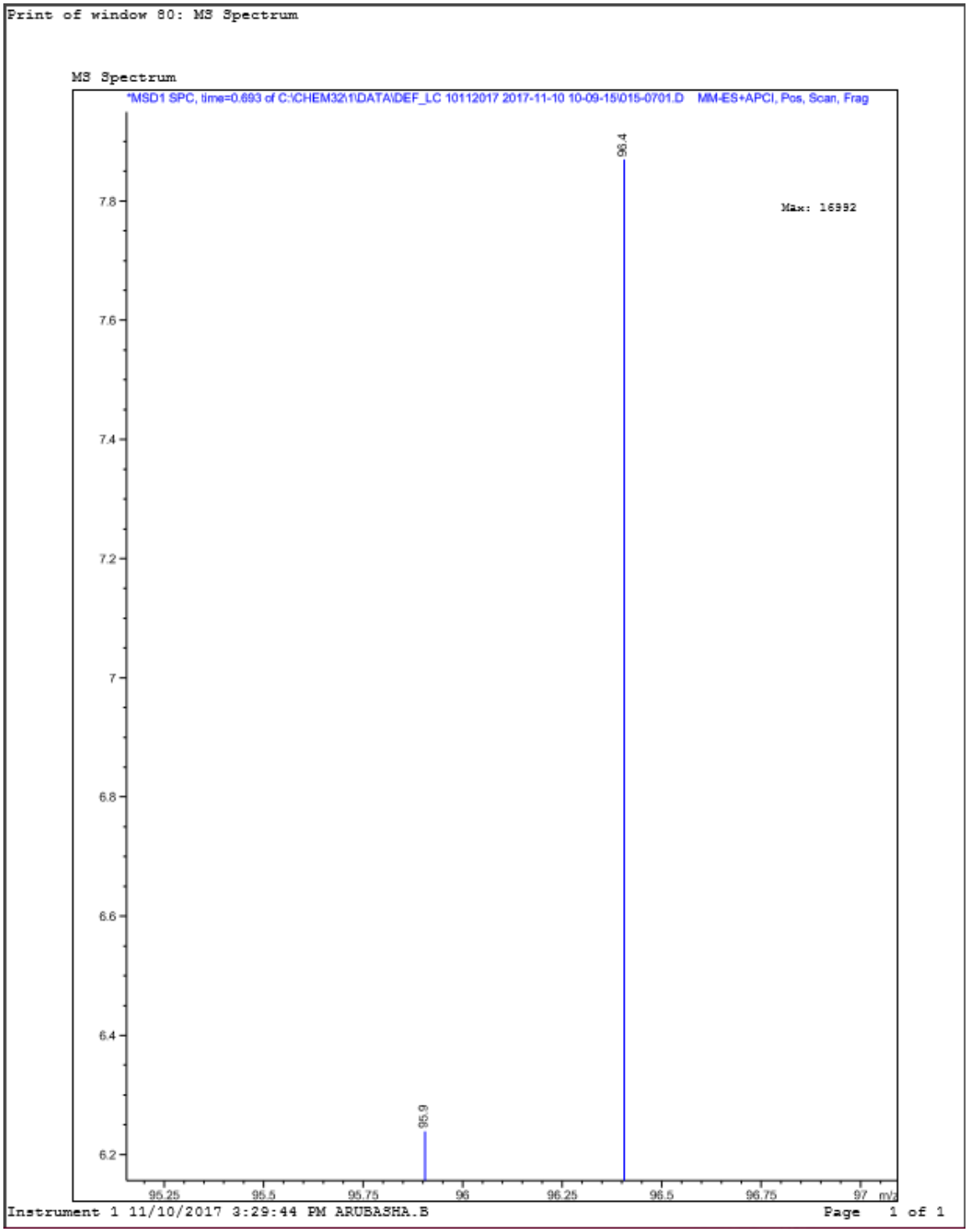


Fig No 33: LCMS Spectrum For Title Compound IMP-DAL 3

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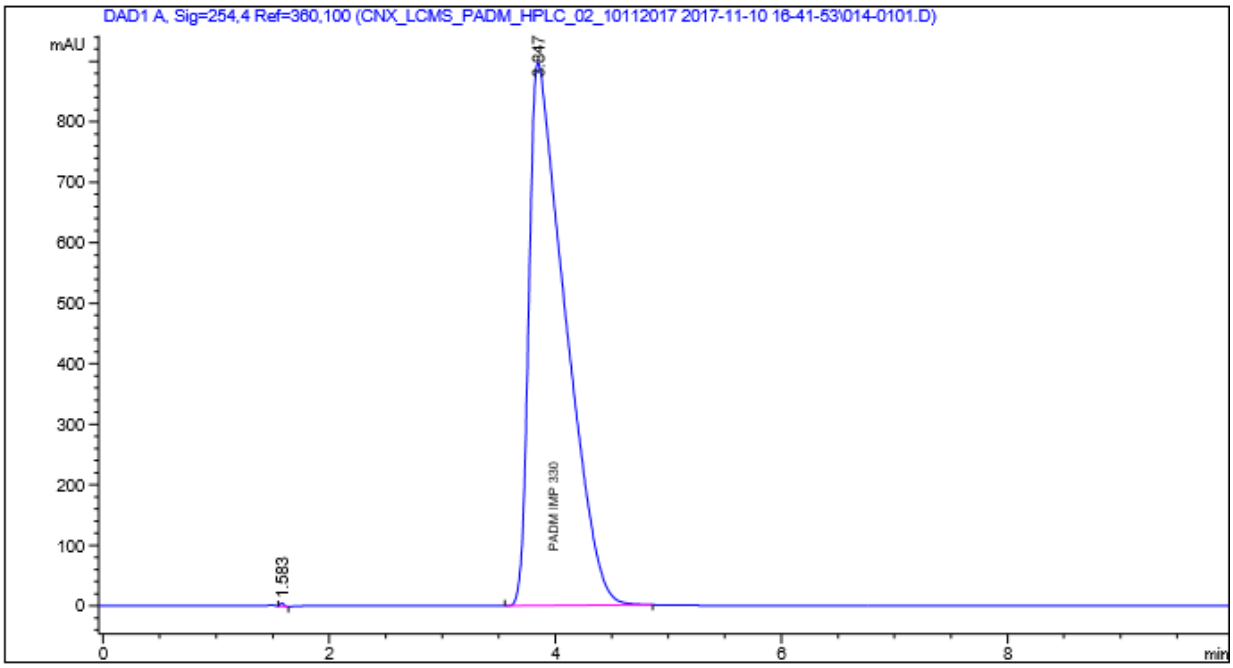
Method Info      : NEW METHOD

Additional Info : Peak(s) manually integrated
```

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=====================================================================
                        Area Percent Report
=====================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=254,4 Ref=360,100

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   1.583 BB    0.0386   12.24079    5.37850   0.0614
   2   3.847 BBA   0.3172 1.99345e4   897.42499  99.9386

Totals :                  1.99467e4   902.80349
```

Fig No 34: HPLC Spectrum For Title Compound IMP-DAL 3

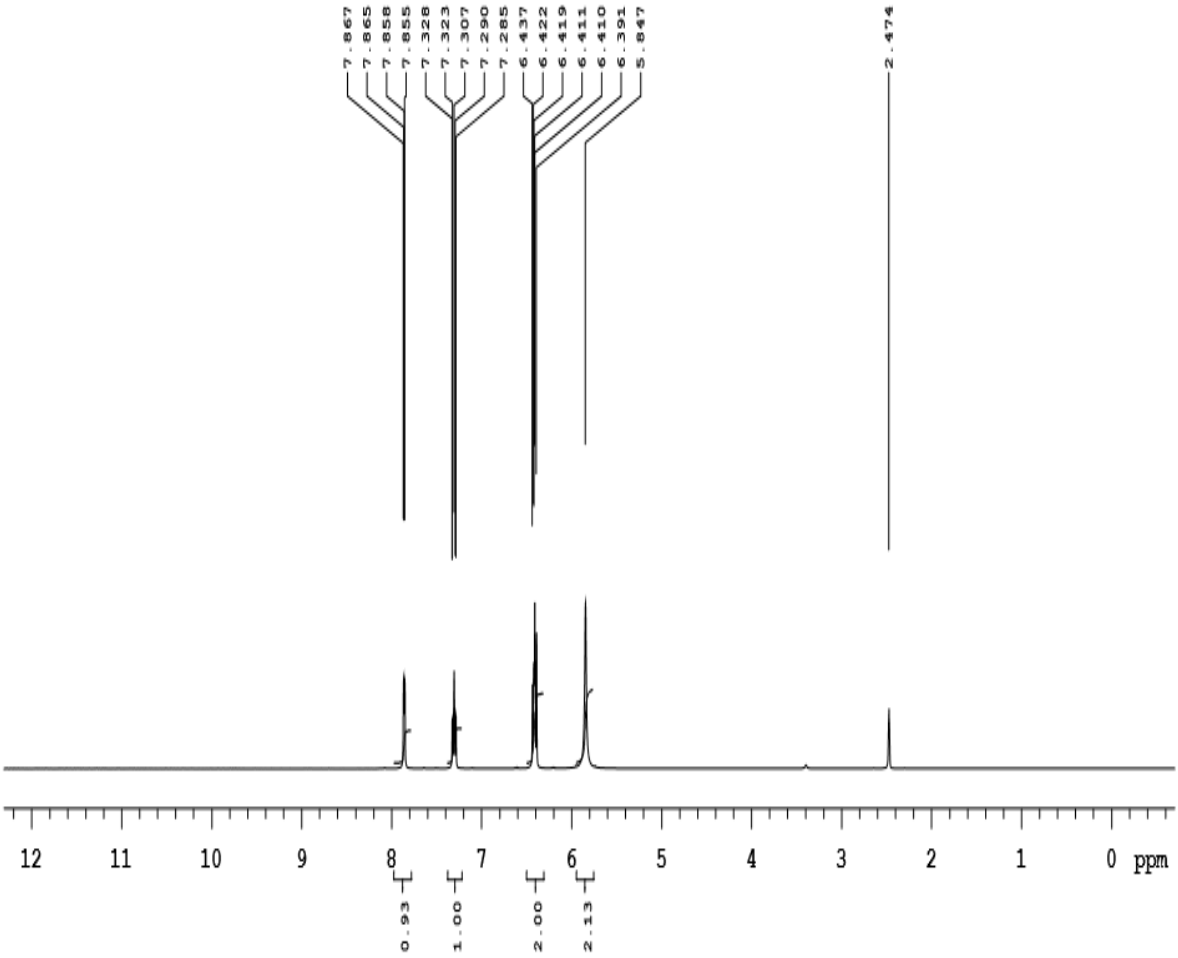
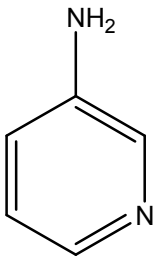


Fig No 35: ^{1}H NMR Spectrum For Title Compound IMP-DAL 3

IMP-DAL 4



1.	Molecular formula	: $C_6H_5N_2$
2.	Molecular wt	: 94.11
3.	Molecular composition	: C(63.81%), H(6.43%), N(29.77%).
4.	Melting point	: 56°C-58°C
5.	Colour	: Brown
6.	State	: Solid
7.	Rf value	: 0.76
8.	Percentage yield	: 66.66%
9.	NMR data	: δ 7.80-8.00 (doublet, 1 H, -Ar), 6.80-7.00 (multiplet, 1 H, -Ar), 6.96-6.99 (multiplet, 1 H, -Ar), 6.86-6.89 (multiplet, 1 H, -Ar), 5.24 (singlet, 2 H, $-NH_2$).
10.	HPLC purity	: 99.05%
11.	Mass data	: m/z ratio 92.8
12.	Solubility	: Methanol

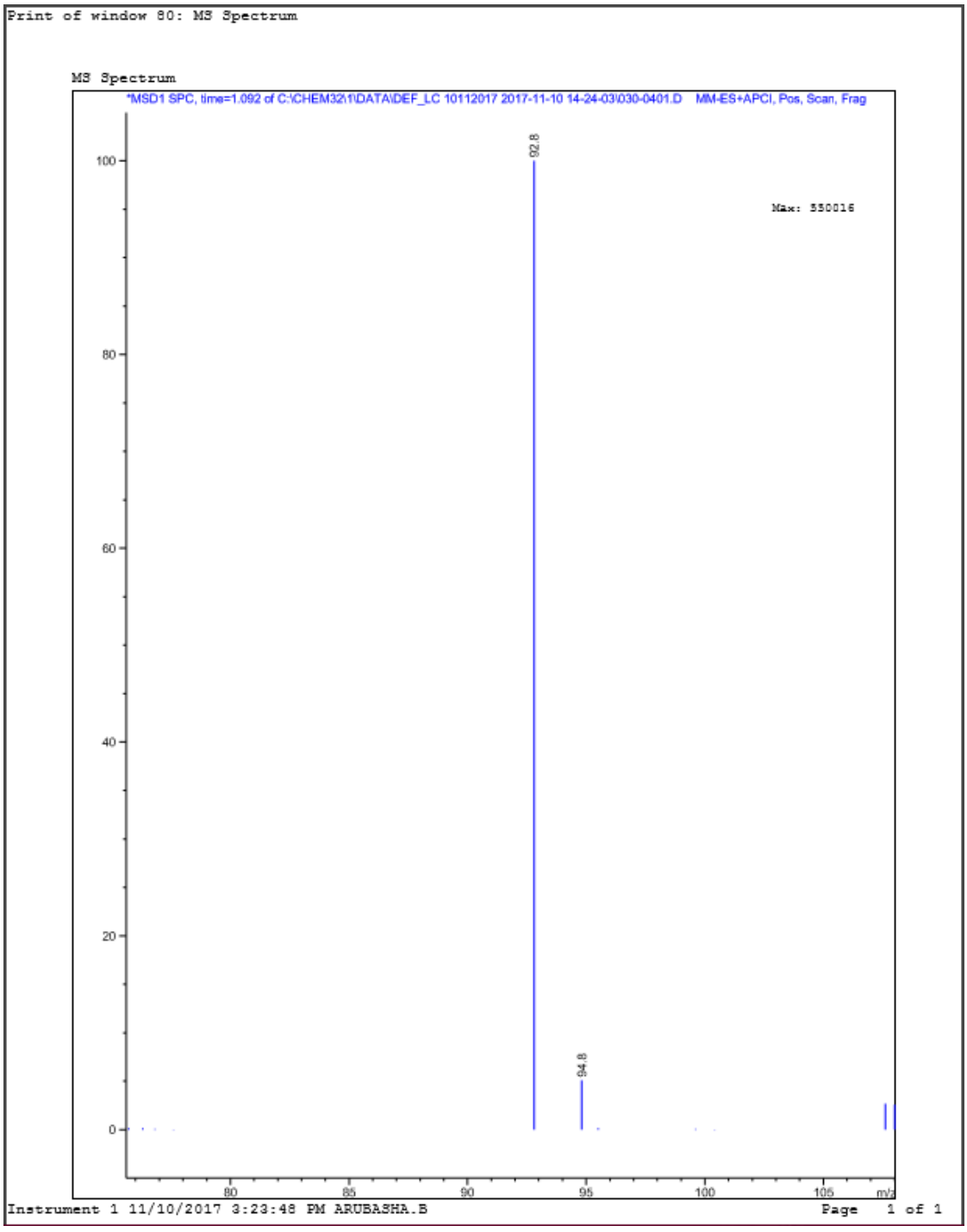
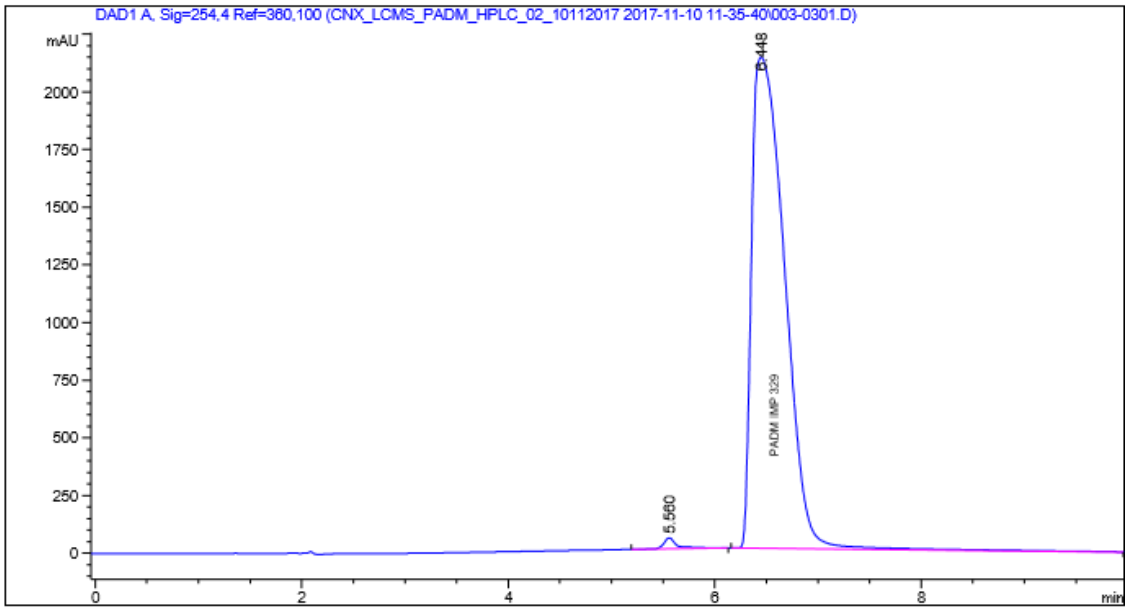


Fig No 36: LC-MS Spectrum For Title Compound IMP-DAL 4

Method Info : NEW METHOD

Additional Info : Peak(s) manually integrated

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==================================================================
                      Area Percent Report
==================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=254,4 Ref=360,100

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   5.560 VB    0.1353  450.83560   48.10327   0.9455
   2   6.448 BBA   0.3568 4.72308e4  2128.93970  99.0545

Totals :                  4.76816e4  2177.04296
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Fig No 37: HPLC Spectrum For Title Compound IMP-DAL 4

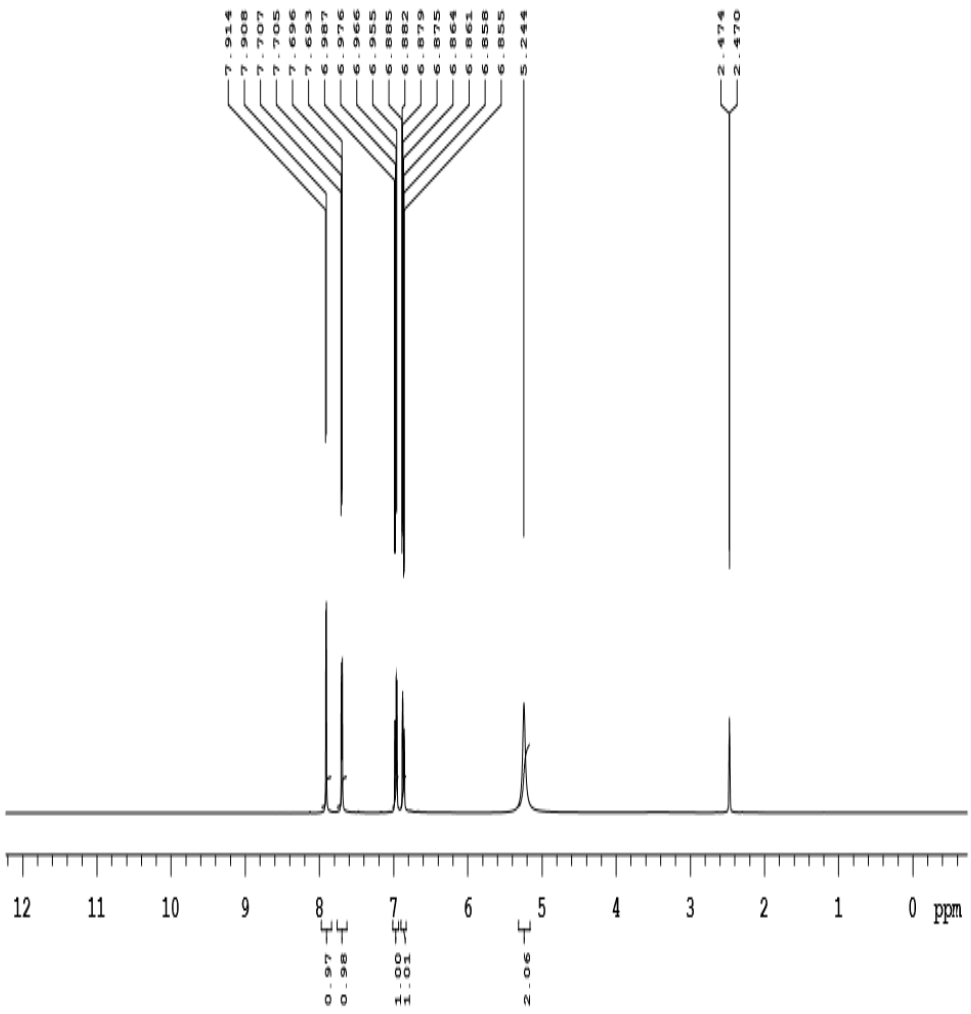
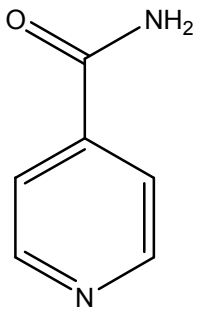


Fig No 38: ^{1}H NMR Spectrum For Title Compound IMP-DAL 4

IMP-DAL 5



1.	Molecular formula	: $C_6H_6N_2O$
2.	Molecular wt	: 122.14 g/mol
3.	Molecular composition	: C(59.01%), H(4.95%), N(22.94%), O(13.10%).
4.	Melting point	: 155°C-157°C
5.	Colour	: White
6.	State	: Solid
7.	Rf value	: 0.73
8.	Percentage yield	: 95%
9.	NMR Data	: δ 8.68-8.70 (multiplet, 2H, -Ar), 8.22 (singlet, 1H, -Ar), 7.72-7.74 (multiplet, 2H, -Ar), 7.71 (singlet, 2H , -CO-NH2)
10.	HPLC purity	: 99.05%
11.	Mass data	: m/z ratio 122.2
12.	Solubility	: Methanol , DMSO

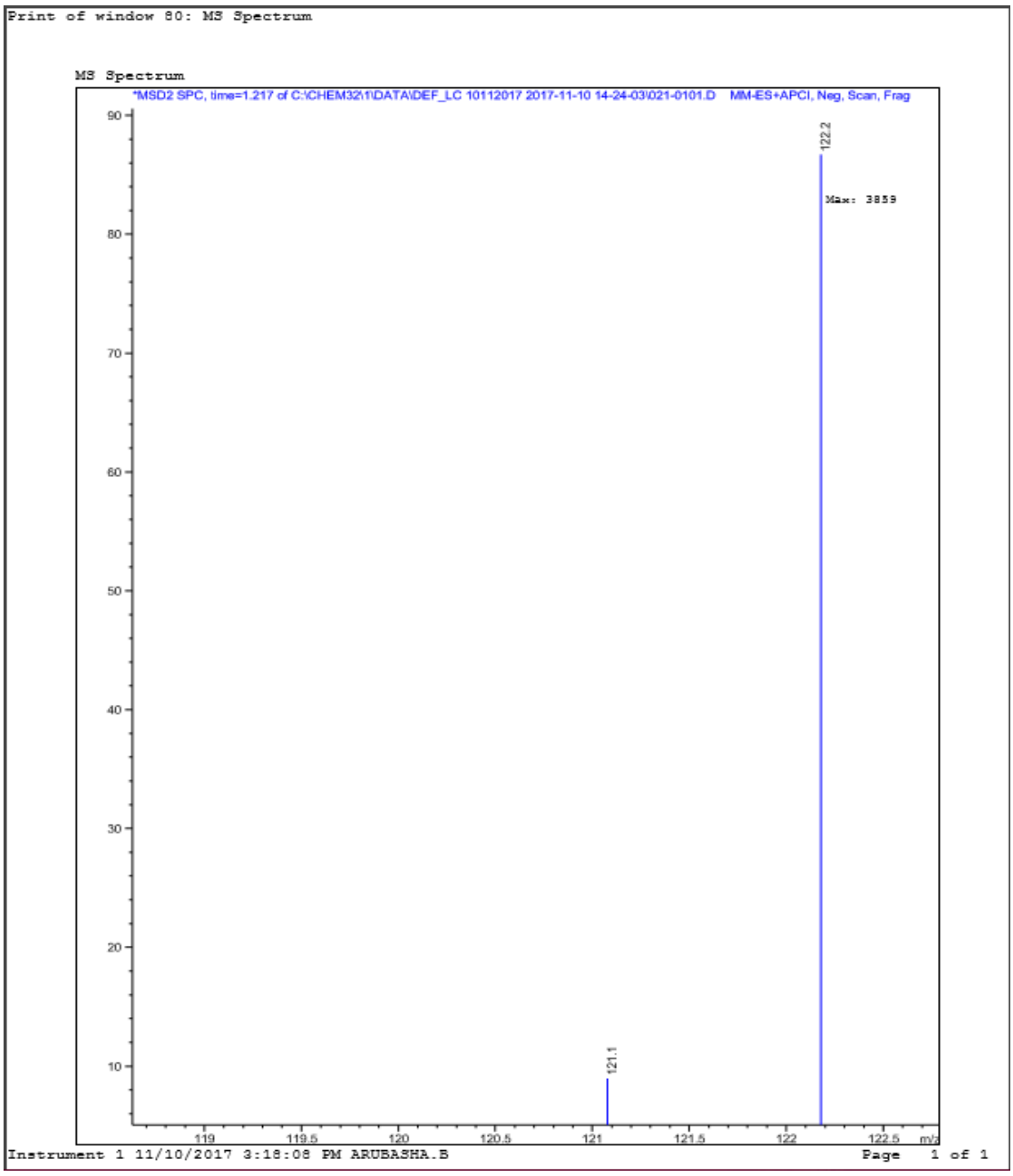
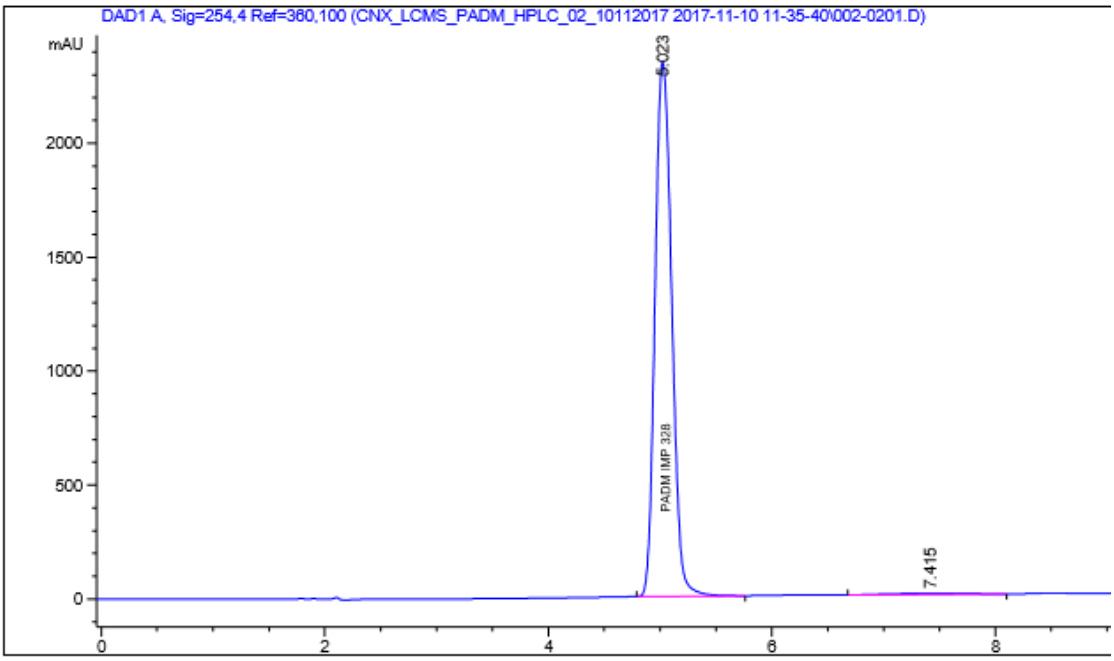


Fig No 39: LC-MS Spectrum For Title Compound IMP-DAL 5

Method Info : NEW METHOD

Additional Info : Peak(s) manually integrated



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Area Percent Report

==========

Sorted By : Signal
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 A, Sig=254,4 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.023	BB	0.1648	2.42891e4	2345.46045	99.0578
2	7.415	BB	0.6303	231.03932	4.35961	0.9422
Totals :				2.45201e4	2349.82006	

Fig No 40: HPLC Spectrum For Title Compound IMP-DAL 5

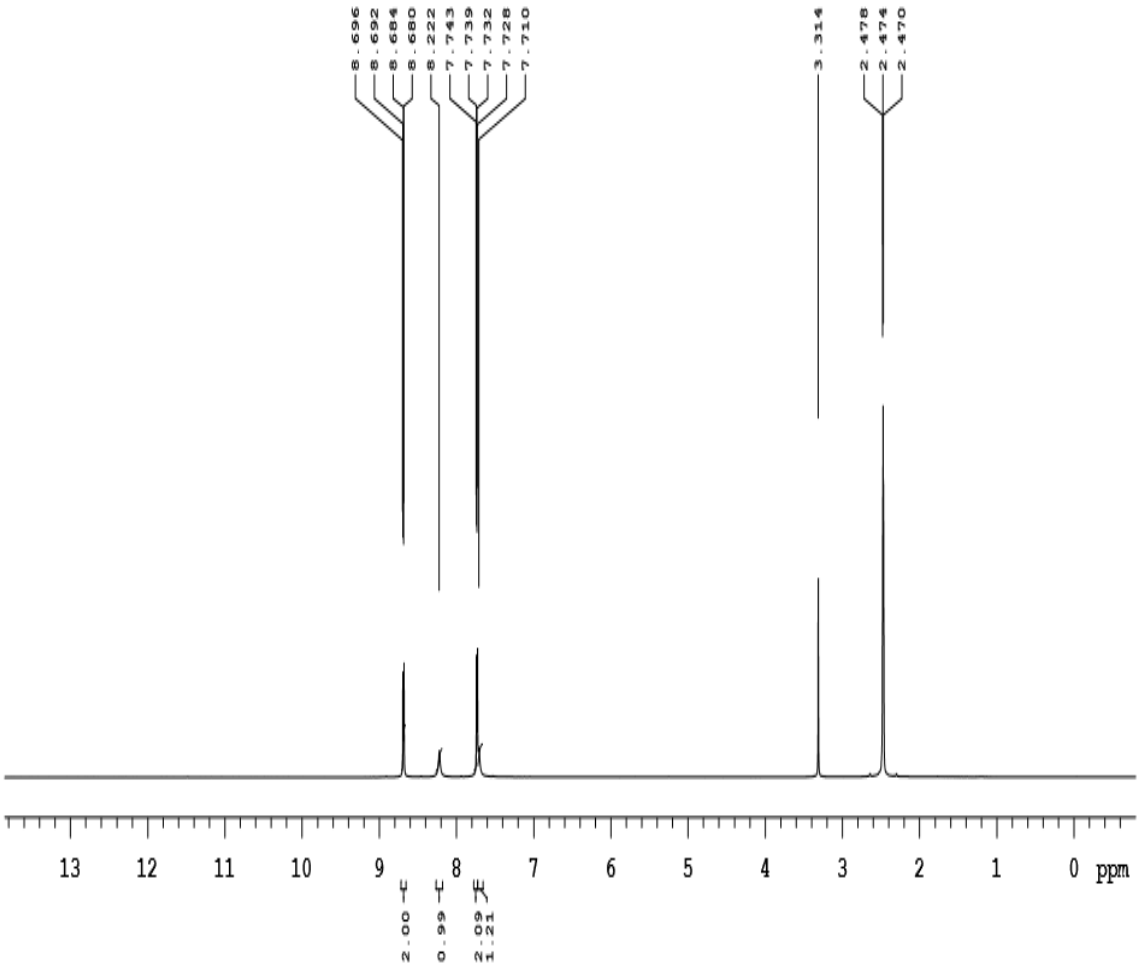
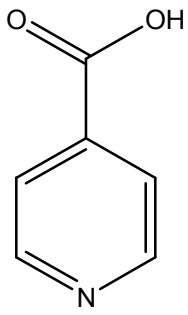


Fig No 41: ^{1}H NMR Spectrum For Title Compound IMP-DAL 5

IMP-DAL 6



1.	Molecular formula	: $C_6H_5NO_2$
2.	Molecular wt	: 123.11
3.	Molecular composition	: C(58.54%), H(4.09%), N(11.38%), O(25.99%).
4.	Melting point	: 310^oC
5.	Colour	: White
6.	State	: Solid
7.	Rf value	: 0.36
8.	Percentage yield	: 70.17%
9.	NMR data	: δ 13.9 (Singlet, 1 H, - COOH), 8.74-8.75 (doublet, 2 H, - Ar), 7.77-7.79 (doublet, 2 H, - Ar).
10.	HPLC purity	: 99.70%
11.	Mass data	: m/z ratio 122.2
12.	Solubility	: Methanol , DMSO

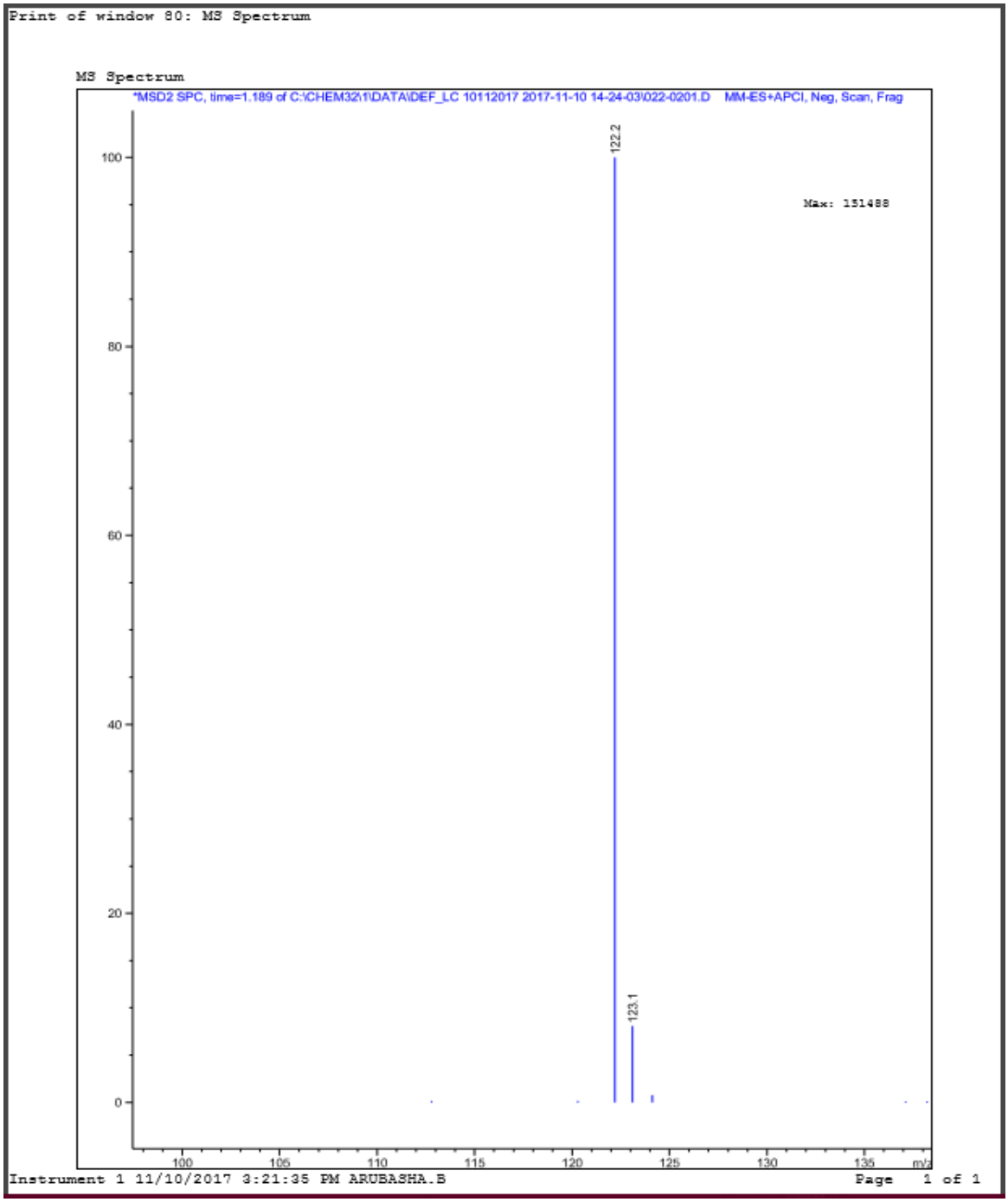
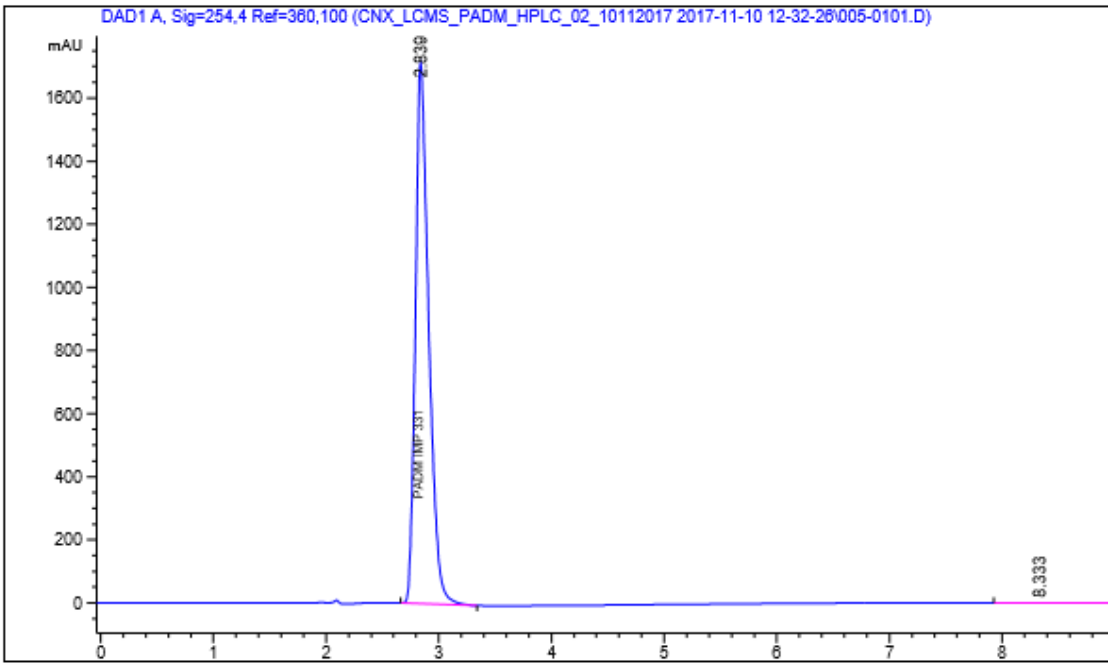


Fig No 42: LC-MS Spectrum For Title Compound IMP-DAL 6

Method Info : NEW METHOD

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==================================================================
                        Area Percent Report
==================================================================

Sorted By             :      Signal
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=254,4 Ref=360,100

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   2.839 BB    0.1216 1.40633e4  1715.98938  99.7031
   2   8.333 BB    0.3087   41.88099    1.98029   0.2969

Totals :                  1.41052e4  1717.96967
```

Fig No 43: HPLC Spectrum For Title Compound IMP-DAL 6

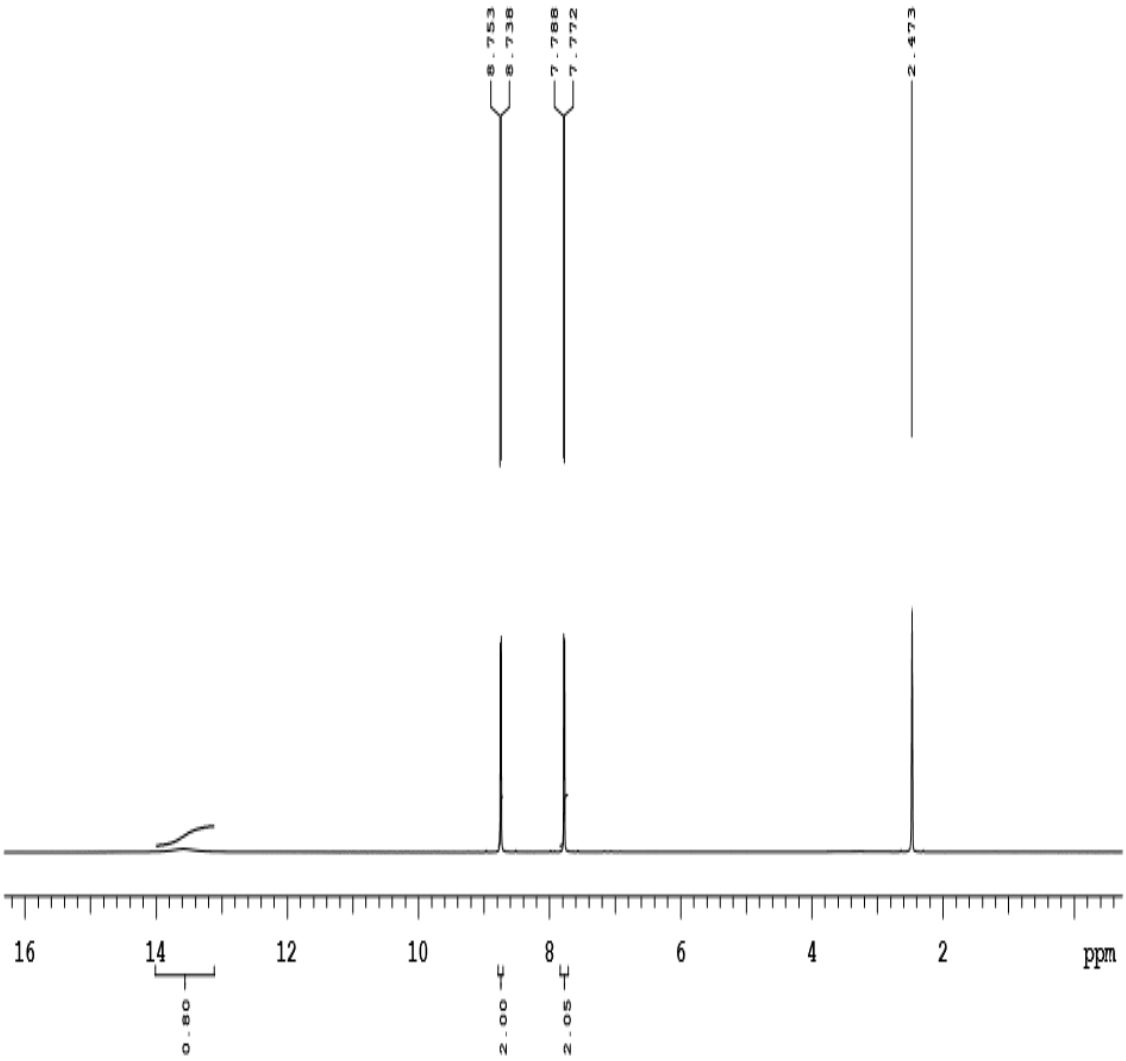
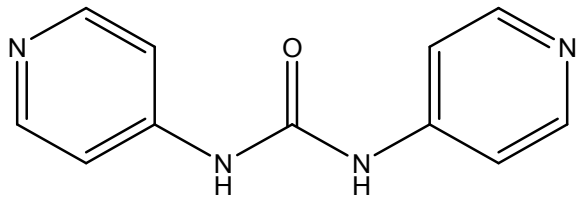


Fig No 44: ^{1}H NMR Spectrum For Title Compound IMP-DAL 6

IMP-DAL 7



1.	Molecular formula	: $C_{11}H_{10}N_4O$
2.	Molecular wt	: 214.22
3.	Molecular composition	: C(61.67%), H(4.71%), N(26.15%), O(7.47%).
4.	Melting point	: 56°C-58°C
5.	Colour	: Off-white
6.	State	: Solid
7.	Rf value	: 0.54
8.	Percentage yield	: 22.22%
9.	NMR data	: δ 9.41(Singlet, 2 H, -CONH), 8.38-8.40 (multiplet, 4 H, -Ar), 7.44-7.78 (multiplet, 4 H , - Ar).
10.	HPLC purity	: 96.62%
11.	Mass data	: m/z ratio 215.2
12.	Solubility	: Water

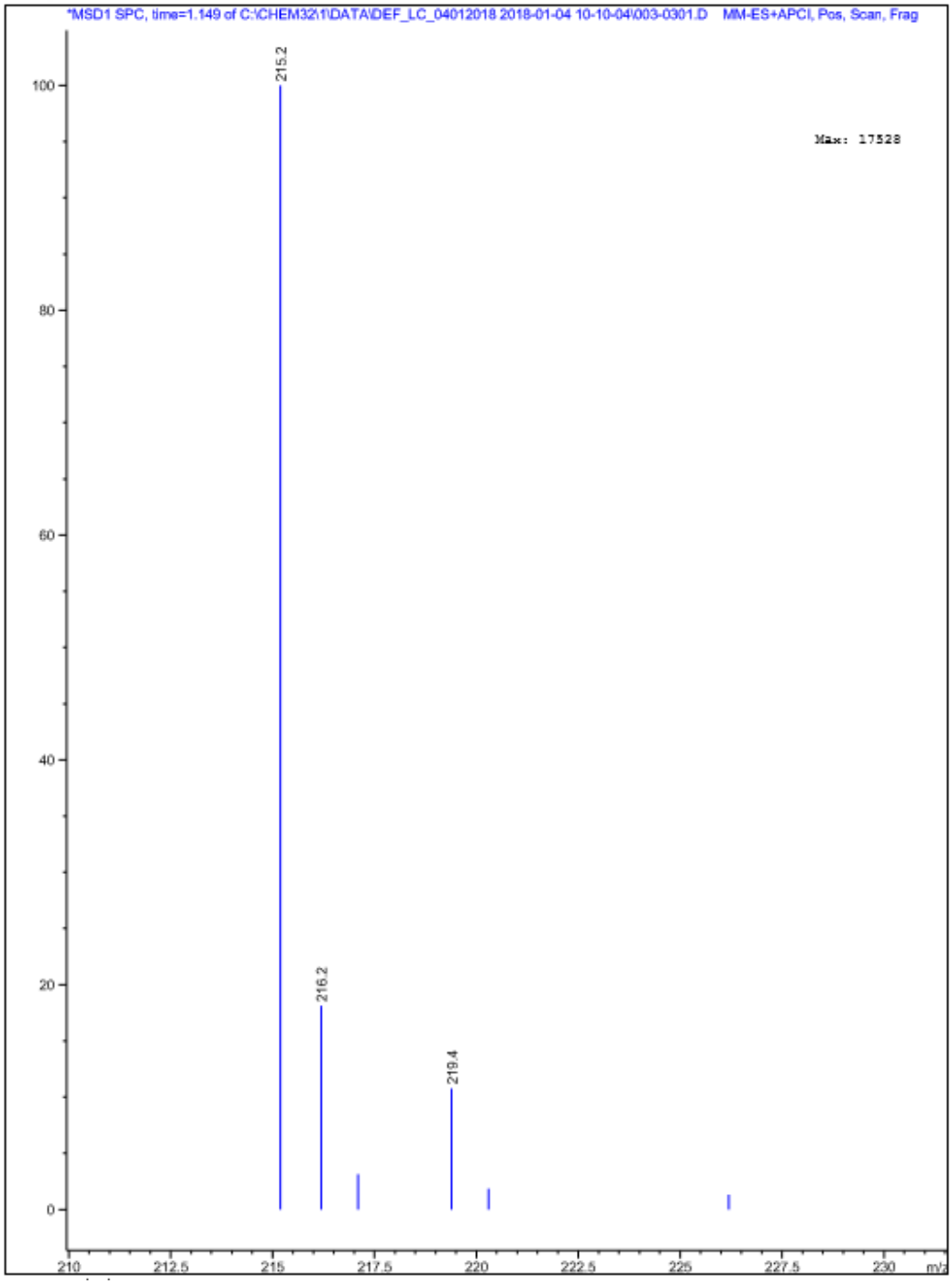
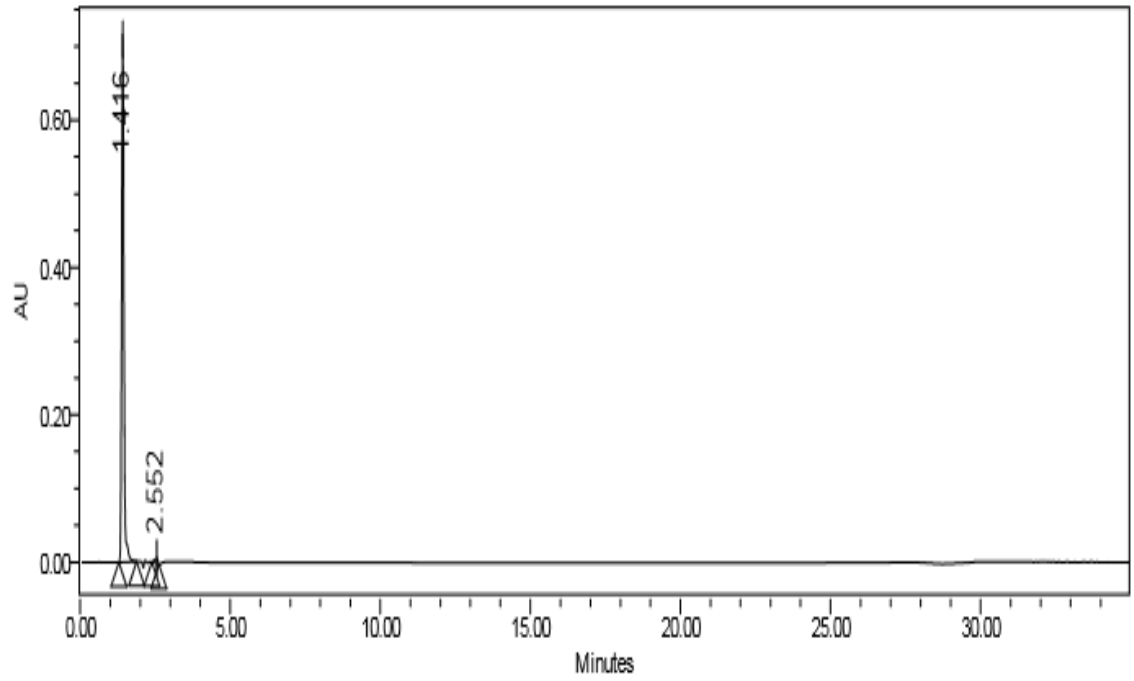


Fig No 45: LC-MS Spectrum For Title Compound IMP-DAL 7



Peak Results

	Name	RT	Area	% Area	Int Type
1		1.416	3744091	98.62	BB
2		2.552	52305	1.38	BB

Fig No 46: HPLC Spectrum For Title Compound IMP-DAL 7

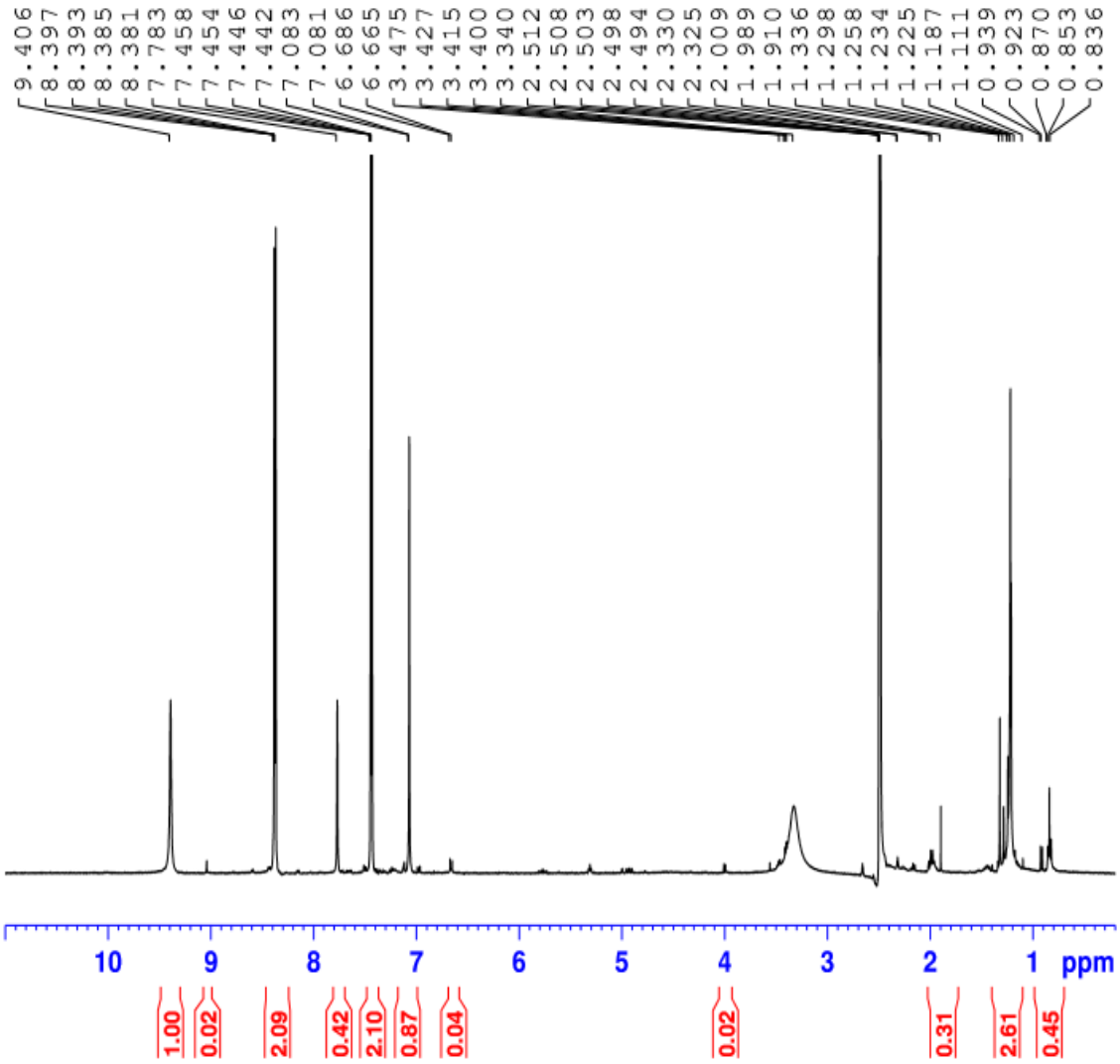


Fig No 47: ^{1}H NMR Spectrum For Title Compound IMP-DAL 7

ANTIBACTERIAL ACTIVITY RESULTS OF IMP-DAL 1:

Antibacterial Activity by Well Diffusion Method

S. No.	Concentration (µg/ml)	Bacterial Culture Zone of Inhibition in mm			
		E.coli	*P.aeruginosa*	*S. aureus*	*B.subtilis*
1	Std (Gentamicin)	26	25	25	26
2	50	15	14	15	13
3	75	17	18	17	17
4	100	22	21	20	20

Table No 10: Zone of Inhibition of IMP-DAL 1.

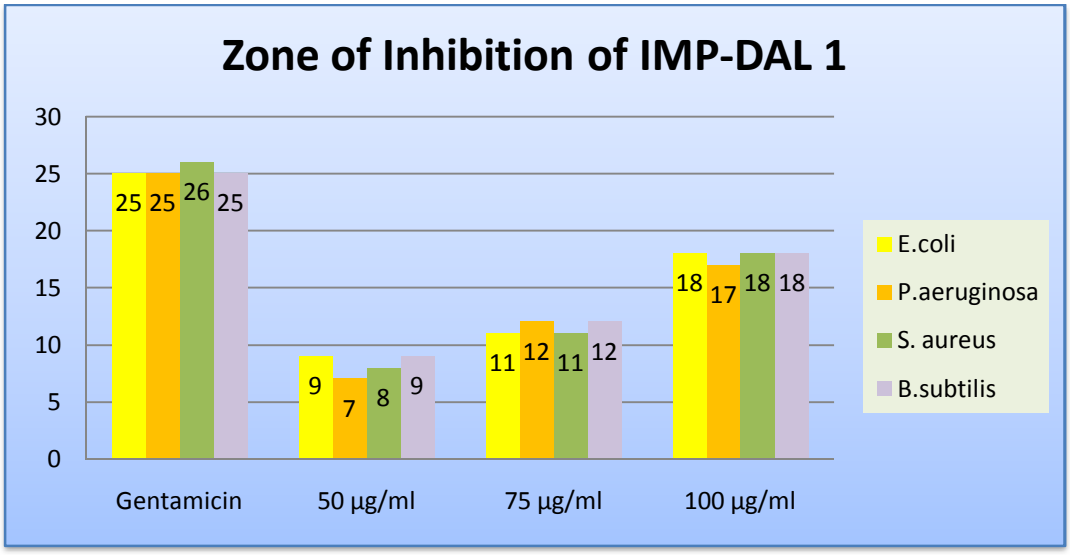


Fig No 48: Zone of Inhibition of IMP-DAL 1.

ANTIBACTERIAL ACTIVITY RESULTS OF IMP-DAL 2

S. No.	Concentration (µg/ml)	Bacterial Culture Zone of Inhibition in mm			
		E.coli	***P.aeruginosa***	***S. aureus***	***B.subtilis***
1	Std (Gentamicin)	25	25	26	26
2	50	10	08	08	09
3	75	13	13	12	12
4	100	15	15	13	14

Table No 11: Zone of Inhibition of IMP-DAL 2

.

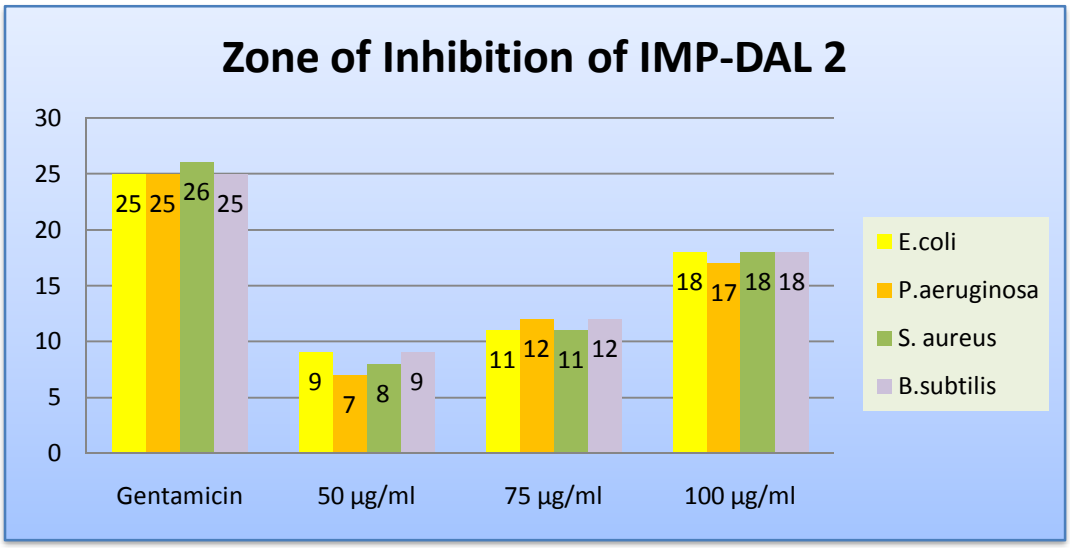


Fig No 49: Zone of Inhibition of IMP-DAL 2.

ANTIBACTERIAL ACTIVITY RESULTS OF IMP-DAL 3:

S. No.	Concentration (µg/ml)	Bacterial Culture Zone of Inhibition in mm			
		E.coli	***P.aeruginosa***	***S. aureus***	***B.subtilis***
1	Std (Gentamicin)	25	26	26	26
2	50	07	06	06	04
3	75	10	10	08	09
4	100	12	11	10	11

Table No 12: Zone of Inhibition of IMP-DAL 3

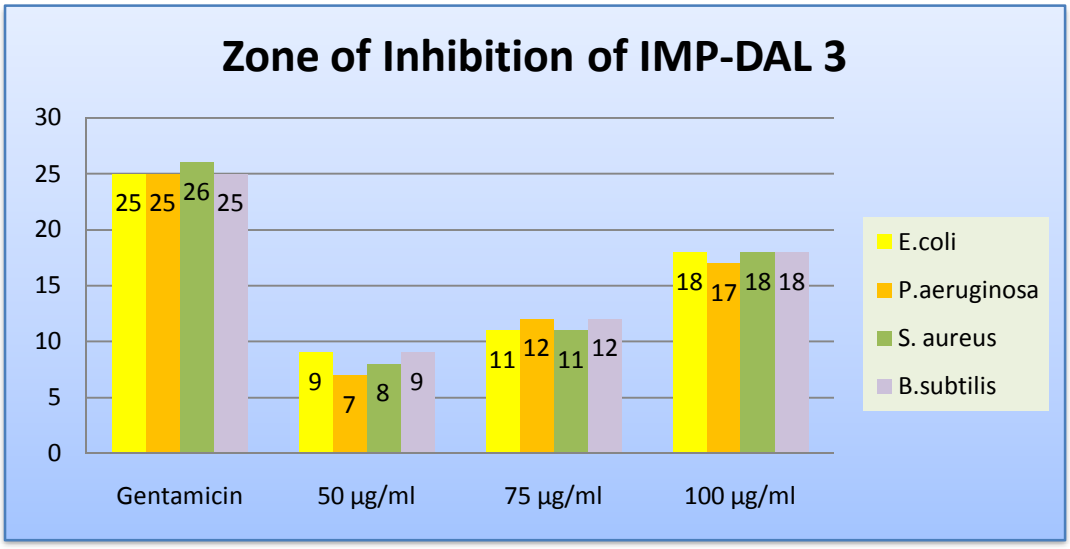


Fig No 50: Zone of Inhibition of IMP-DAL 3.

ANTIBACTERIAL ACTIVITY RESULTS OF IMP-DAL 4:

S. No.	Concentration (µg/ml)	Bacterial Culture Zone of Inhibition in mm			
		E.coli	***P.aeruginosa***	***S. aureus***	***B.subtilis***
1	Std (Gentamicin)	25	25	25	26
2	50	03	05	05	08
3	75	08	07	05	07
4	100	12	11	11	12

Table No 13: Zone of Inhibition of IMP-DAL 4

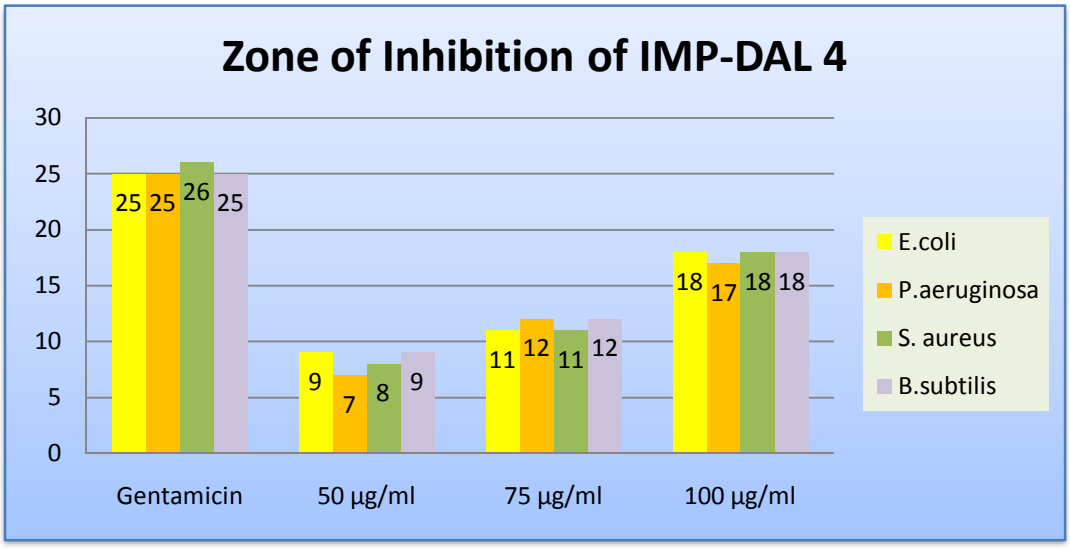


Fig No 51: Zone of Inhibition of IMP-DAL 4.

ANTIBACTERIAL ACTIVITY RESULTS OF IMP-DAL 5:

S. No.	Concentration (µg/ml)	Bacterial Culture Zone of Inhibition in mm			
		E.coli	***P.aeruginosa***	***S. aureus***	***B.subtilis***
1	Std (Gentamicin)	26	26	25	25
2	50	05	04	04	05
3	75	07	06	06	06
4	100	09	10	10	08

Table No 14: Zone of Inhibition of IMP-DAL 5

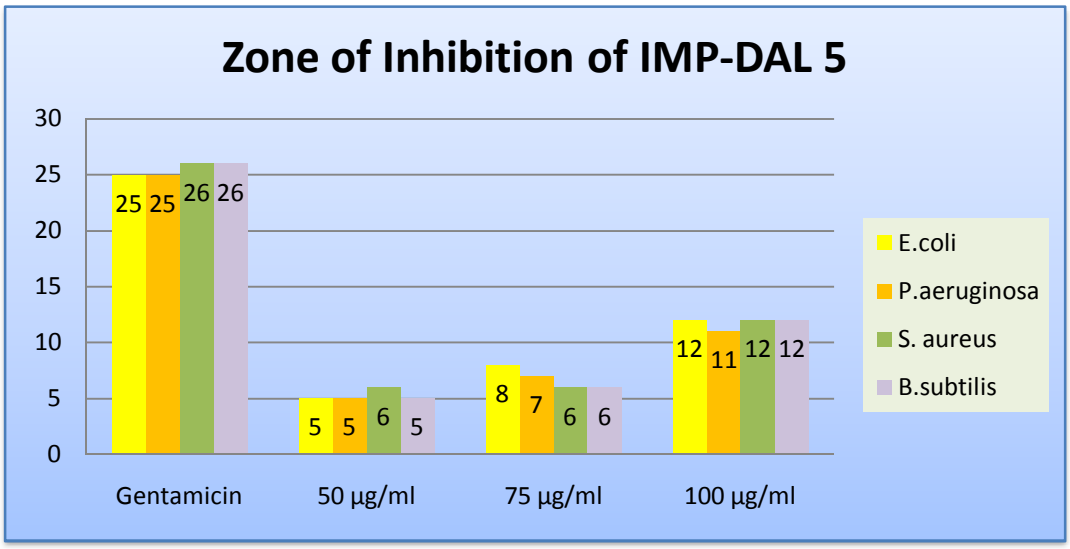


Fig No 52: Zone of Inhibition of IMP-DAL 5.

ANTIBACTERIAL ACTIVITY RESULTS OF IMP-DAL 6:

S. No.	Concentration (µg/ml)	Bacterial Culture Zone of Inhibition in mm			
		E.coli	***P.aeruginosa***	***S. aureus***	***B.subtilis***
1	Std (Gentamicin)	25	25	26	26
2	50	05	05	06	05
3	75	08	07	06	06
4	100	12	11	12	12

Table No 15: Zone of Inhibition of IMP-DAL 6

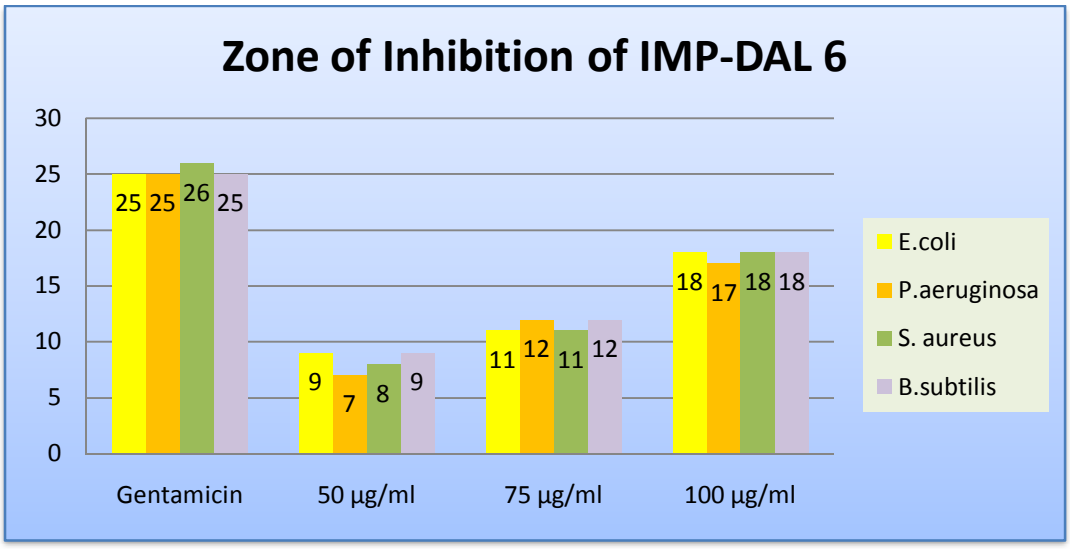


Fig No 53: Zone of Inhibition of IMP-DAL 6.

ANTIBACTERIAL ACTIVITY RESULTS OF IMP-DAL 7:

S. No.	Concentration (µg/ml)	Bacterial Culture Zone of Inhibition in mm			
		E.coli	***P.aeruginosa***	***S. aureus***	***B.subtilis***
1	Std (Gentamicin)	25	25	26	25
2	50	09	07	08	09
3	75	11	12	11	12
4	100	18	17	18	18

Table No 16: Zone of Inhibition of IMP-DAL 7

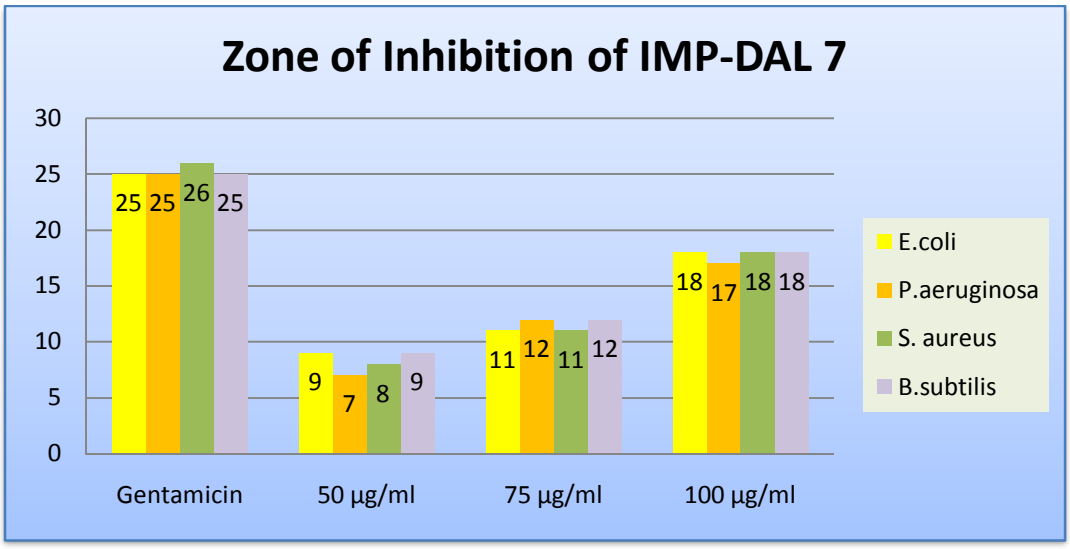


Fig No 54: Zone of Inhibition of IMP-DAL 7.

PERCENTAGE ZONE OF INHIBITION OF SYNTHESIZED DALFAMPRIDINE GENOTOXIC IMPURITIES

Compound code	*E.coli*		*P.aeruginosa*		*S. aureus*		*B.subtilis*	
	Inhibition zone in mm	% of inhibition	Inhibition zone in mm	% of inhibition	Inhibition zone in mm	% of inhibition	Inhibition zone in mm	% of inhibition
STANDARD	25	100.00	26	100.00	26	100.00	25	100.00
IMP-DAL 1	22	88.00	21	80.76	20	76.92	20	80.00
IMP-DAL 2	15	60.00	15	57.69	13	50.00	14	56.00
IMP-DAL 3	12	48.00	11	42.30	10	38.46	11	44.00
IMP-DAL 4	12	48.00	11	42.30	11	42.30	12	48.00
IMP-DAL 5	09	36.00	10	38.46	10	38.46	08	32.00
IMP-DAL 6	12	48.00	11	42.30	12	46.15	12	48.00
IMP-DAL 7	18	72.00	17	65.38	18	69.23	18	72.00

Table No 17 : Percentage Zone of Inhibition for standard and synthesized compounds

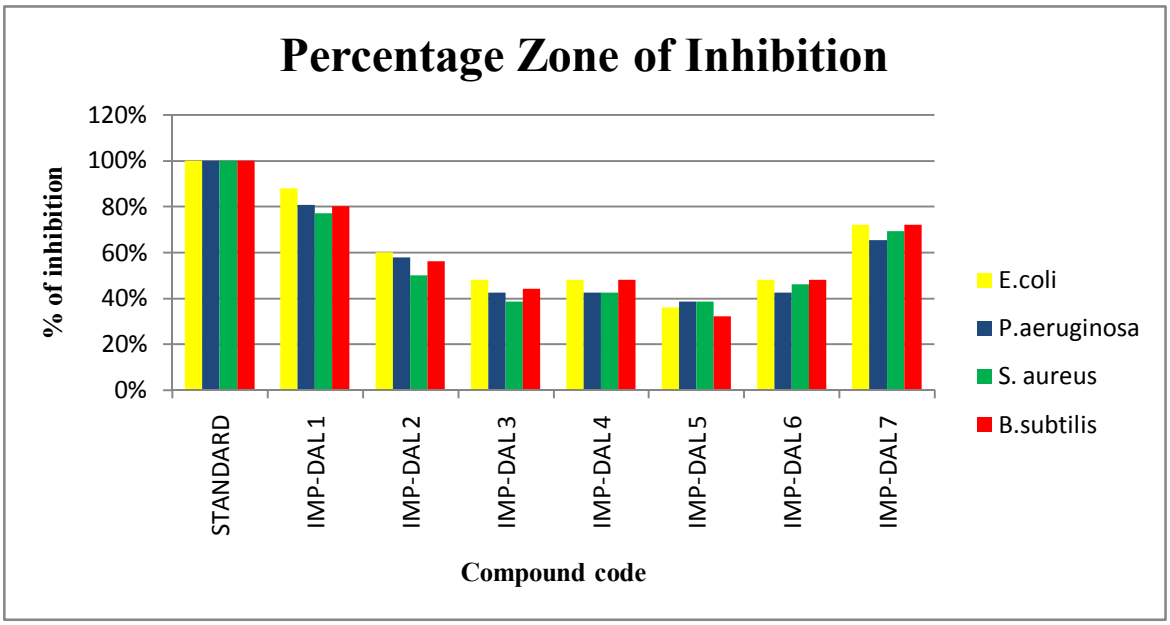


Fig No 55: Percentage Zone of Inhibition for standard and synthesized compounds

7.2 DISCUSSION

Dalfampridine is an organic compound with the chemical formula $C_5H_4N\text{-}NH_2$ and molecular weight 94.04 gm/mole. It is chemically pyridine-4-amine, used primarily as a research tool for subtypes of potassium channels. It is use to manage some of the symptoms of multiple sclerosis and is indicated for symptomatic improvement of walking in adults with several variations of the disease. It is very effective avicide and bird repellent and is highly toxic to human; it strongly excites central nervous system.

Fampridine is also marketed as ampyra in the United States by Acorda Therapeutics and as Fampyra in Europe. In Canada, the medication has been approved for use by Health Canada since February 10, 2012. Dalfampridine is a non-selective K+ channel blocker, which can block a wide variety of K+ channels with different state dependences. Therefore, in search for clues for the structural determinants of K+ channels that are important for the state dependences of drug-channel interactions, 4-AP serves as a useful tool. Potassium channel blocker used to help multiple sclerosis patients walk. This is the first drug that was specifically approved to help with mobility in these patients. Fampridine has been shown to improve visual function and motor skills and relieve fatigue in patients with multiple sclerosis (MS).

Dalfampridine impurities are synthesized, characterized and evaluated such as 1,2-Di(pyridine-4-yl)hydrazine, 4-Aminopyridine-N-oxide, 2-Aminopyridine, 3-Aminopyridine, Pyridine-4-carboxamide, Pyridine-4-carboxylic acid, (1, 3-Di (pyridin-4-yl) urea. Name of that synthesized recations and detailed descriptions, difficulties during synthesis and evaluation were discussed below:

N-oxidation: Pyridine N- oxidation was tried with mCPBA but the result was not achieved since oxidation of nitrogen is very poor. Alternatively hydrogen peroxide was used in presence of acetic acid gives us oxidized product.

Azo formation : Chemistry involved in formation of diazo group by using nitro compounds is very new and coupling reagents were tried with titanium chloride in presence stannous chloride. And reaction was achieved by using stannous chloride in presence of aqueous base.

N- deoxygenation: Selective N- deoxidation in presence of diazo group was very challenging since possibility of aza bond breakage observed by using palladium carbon metallic reagent in presence of hydrogen. Alternatively selection of metallic reagents was done with repetitive trials with available literature source.

To achieve selective deoxigenation 1 eqivalent of raney nickel was used in presence of hydrogen 5 mbar pressure so controlled removal of two unit of oxygens in pyridine was achieved successfully.

Selective diazo Reduction : Collected literature says reduction was performed by metallic reagents in presence of hydrogen and the same chemistry was applied to get the desired product but achievement was not done. So extensive literature was made, hydrazine hydrate was the selective reagent for the diazo reduction of pyridine containing nucleus and it was achieved to get targeted molecule successfully.

Nitro to amine reduction of pyridine containing N- oxide: Pyridine N oxide group was very sensitive towards palladium carbon / hydrogen exposure and very unstable. By understanding the chemistry of pyridine N- oxide nucleus, nitro reduction was achieved with controlled hydrogen pressure with minimal usage of metallic reagent like palladium carbon.

Nitro to amine reduction : Zinc ammonium formate was used in presence of methanol as a suitable solvent to form zinc amalgam complex, formed complex helped to reduce nitro group of pyridine nucleus with higher rate of reaction helps us to get the good yield of targeted molecule.

Diamide formation : Pyridine amines was successfully coverted to amide linkage by the usage of 1,1' Carbonyl Di imidazole. Carbonyl group from the CDI drawn the amine proton to form diamides pyridine nucleus.

Antibacterial activity:

The synthesized dalfampridine genotoxic impurities were evaluated against gram +ve bacteria like Staphylococcus-aureus, Bacillus-subtilis and gram -ve bacteria like Pseudomonas-aeruginosa, Escherichia-coli using standard drug Gentamycin. Zone of inhibition in mm was determined. The results were listed in table No. . The explanations revealed that, IMP-DAL 1, IMP-DAL 2, IMP-DAL 7 exhibits substantial antibacterial activity against and its direction Escherichia-coli > Pseudomonas-aeruginosa > Bacillus-subtilis > Staphylococcus-aureus. IMP-DAL 3, IMP-DAL 4, IMP-DAL 5, IMP-DAL 6 also have antibacterial activity but its showed mild antibacterial activity contrary to gram +ve bacteria and gram -ve bacteria test organisms. Concentration of test solutions were 100 µg/ml, 75 µg/ml and 50 µg/ml used for antibacterial study but 100 µg/ml is showed more activity compared to other concentrations.

8.1 SUMMARY

Main aim and focus of research work has been designed to prepared dalfampridine genotoxic impurities with different types of reaction mechanisams like, selective nitration, nitro to amine conversion, N- oxidation, diazotitation, azo coupling, reduction of N=N by hydrazine hydrate, N-deoxygenation, acid to acid chloride formation, mono and di amide formation.

Section 1, gives an introduction to the development, biological importance endowed by compounds containing dalfampridine impurities which has different substitutions. The chemistry of Dalfampridine and synthetic methods of Dalfampridine has also been illustrated briefly.

Section 2, focuses on the aim and scope of the work, entire research of this dissertation by explaining the need to develop newer compounds and to make the synthetic routes more appropriate by different reaction applications.

Section 3, an elaborated review of literature of various substituted Dalfampridine and its impurities with which their biological activity and genotoxicity of fampridine impurity has been described.

Section 4, deals with the details regarding the chemicals, reagents, culture media, test organisams and methodology used in the entire research work. This section also provides the synthetic schemes used to synthesize the intermediate, final compounds and preliminary screening of antibacterial activity of synthesized compounds . The physical constants, TLC, recrystallisation solvents of the synthesized compounds were also incorporated in this section.

Section 5, all the synthesised molecules were characterized by subjecting to analytical studies like ^{1}H-NMR, LCMS and HPLC spectroscopy. All the final molecules assessed for *in-vitro* antibacterial action on gram + ve and gram - ve

bacteria using standard drug Gentamycin. This section deals with the details of pharmacological activity results of anti-bacterial property of the synthesized compounds.

Section 6, deals with the details of synthesise of individual molecule, problem faced during the synthesis, the spectral analyses with reasoning and discusses on the results of antibacterial activity.

Section 7, deals with summarise the every step of Dalfampridine impurities synthesis, identification, characterization and its biological activity of this dissertation work. summarizes all the theoretical and experimental activities and topics that led to the formation of this dissertation.

Section 8, a conclusion of the work in relation to objectives and the results obtained has been depicted.

Section 9, enlists all the references that led to the formation of this dissertation.

8.2 CONCLUSION

From the planned reaction mechanism and chemistry of pyridine nucleus was understood made an attempt to synthesize different pyridine derivatives, that is fampridine genotoxic impurities from waste application of chemical reactions like N-oxidation, Azo formation, N- deoxygenation, selective deoxigenation, Selective diazo Reduction, Nitro to amine reduction of pyridine containing N- oxide, Nitro to amine reduction, Diamide formation.

All the targeted molecules synthesized with good yield and high purity. To achieve high purity attention was drawn forwards automated combiflash and it was performed by developing different analytical methods for separation of synthesized genotoxic impurities.

The synthesized genotoxic impurities were characterized using hyponated analytical instruments like NMR, LCMS-MS, HPLC Combiflash.

The HPLC purity of genotoxic impurities listed below

S.NO	NAME OF THE IMPURITY	% PURITY
1.	IMP-DAL 3	99.93%
2.	IMP-DAL 6	99.70%
3.	IMP-DAL 4	99.05%
4.	IMP-DAL 5	99.05%
5.	IMP-DAL 7	96.62%
6.	IMP-DAL 2	94.42%
7.	IMP-DAL 1	92.15%

Table No 18: HPLC purity of synthesized dalfampridine genotoxic impurities

By the above achievement and all the characterization data's of genotoxic impurities can be used as reference standards in pharmaceutical industry to evaluate the drug substance.

Novel produced compounds, assessed for its *in-vitro* anti-bacterial activity compared to both gram-positive and gram-negative bacteria using the standard drug Gentamycin. It was concluded as IMP-DAL 1, IMP-DAL 2, IMP-DAL 7 exhibited substantial antibacterial activity against its direction *Escherichia-coli* > *Pseudomonas-aeruginosa* > *Bacillus-subtilis* > *Staphylococcus-aureus*. IMP-DAL 3, IMP-DAL 4, IMP-DAL 5, IMP-DAL 6 showed mild antibacterial activity contrary to gram +ve bacteria and gram -ve bacteria test organisms. 100 µg/ml of synthesized molecules showed more activity compared to other concentrations.

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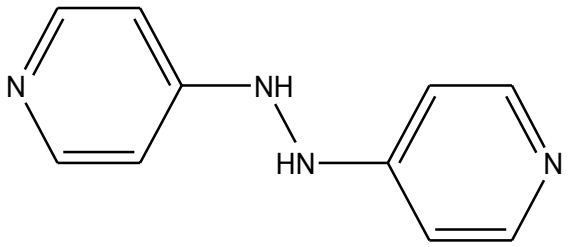
Certificate of Analysis

DALFAMPRIDINE DIMER IMPURITY

(REFERENCE SUBSTANCE)



1,2-Bis(4-pyridyl)hydrazine

PURITY (HPLC): 92.1%

CATALOG #: 100287 **Lot #:** PADM/IMP/295

PACK SIZE: 85 mg **CAS #:** 19808-51-6

DATE OF COA: 03 January 2018

EXPIRATION DATE: 02 January 2019

RECEIPT DATE:_____

Note: This space is provided for convenience only and its use is not required.

STORAGE: Store at 2˚C to 8˚C.

CHEMICAL FORMULA: $C_{10}H_{10}N_4$ **Molecular weight:** 186.21 g/mol

PHYSICAL DESCRIPTION: Brown solid.

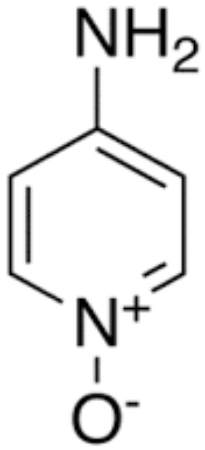
HAZARDS: Read Safety data sheet before using. All chemical reference materials should be considered potentially hazardous and should be used only by qualified laboratory personnel.

INSTRUCTIONS FOR USE: Do not dry, use as is. The internal pressure of container may be slightly different from the atmospheric pressure at the user's location. Open slowly and carefully to avoid dispersion of the material. This material is intended for Analytical use only. Not for drug, human consumption household or other uses.

Certificate of Analysis

DALFAMPRIDINE N-OXIDE IMPURITY

(REFERENCE SUBSTANCE)



4-Pyridinamine 1-Oxide

PURITY (HPLC): 94.4%

CATALOG #: 100289 **Lot #:** PADM/IMP/297

PACK SIZE: 125 mg **CAS #:** 3535-75-9

DATE OF COA: 27 December 2017

EXPIRATION DATE: 26 December 2018

RECEIPT DATE:_____

Note: This space is provided for convenience only and its use is not required.

STORAGE: Store at 2˚C to 8˚C.

CHEMICAL FORMULA: $C_4H_6N_2O$ **Molecular weight:** 110.11 g/mol

PHYSICAL DESCRIPTION: Brown solid.

HAZARDS: Read Safety data sheet before using. All chemical reference materials should be considered potentially hazardous and should be used only by qualified laboratory personnel.

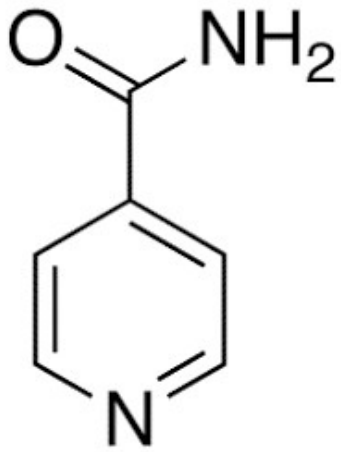
INSTRUCTIONS FOR USE: Do not dry, use as is. The internal pressure of container may be slightly different from the atmospheric pressure at the user's location. Open slowly and carefully to avoid dispersion of the material. This material is intended for Analytical use only. Not for drug, human consumption household or other uses.

Certificate of Analysis

ISONICOTINAMIDE

(REFERENCE SUBSTANCE)

4-Pyridinecarboxamide



PURITY (HPLC): 99.1%

CATALOG #: 100320 **Lot #:** PADM/IMP/328

PACK SIZE: 100 mg **CAS #:** 1453-82-3

DATE OF COA: 10 November 2017

EXPIRATION DATE: 09 November 2018

RECEIPT DATE:_____

Note: This space is provided for convenience only and its use is not required.

STORAGE: Store at 2˚C to 8˚C.

CHEMICAL FORMULA: $C_5H_6N_2O$ **Molecular weight:** 122.12 g/mol

PHYSICAL DESCRIPTION: White solid.

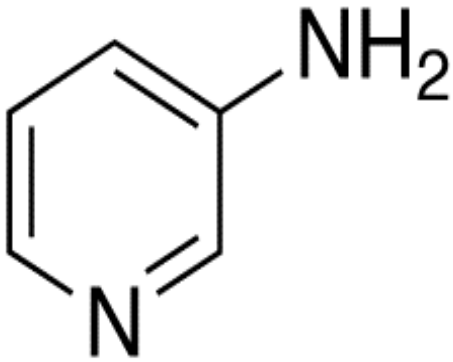
HAZARDS: Read Safety data sheet before using. All chemical reference materials should be considered potentially hazardous and should be used only by qualified laboratory personnel.

INSTRUCTIONS FOR USE: Do not dry, use as is. The internal pressure of container may be slightly different from the atmospheric pressure at the user's location. Open slowly and carefully to avoid dispersion of the material. This material is intended for Analytical use only. Not for drug, human consumption household or other uses.

Certificate of Analysis

3-AMINOPYRIDINE

(REFERENCE SUBSTANCE)



3-Pyridinamine

PURITY (HPLC): 99.1%

CATALOG #:	100321	**Lot #:**	PADM/IMP/329
PACK SIZE:	45 mg	**CAS #:**	462-08-8
DATE OF COA:	10 November 2017		

EXPIRATION DATE: 09 November 2018

RECEIPT DATE:_____

Note: This space is provided for convenience only and its use is not required.

STORAGE: Store at 2˚C to 8˚C.

CHEMICAL FORMULA:	$C_5H_6N_2$	**Molecular weight:**	94.11 g/mol
PHYSICAL DESCRIPTION:	Brown solid.		

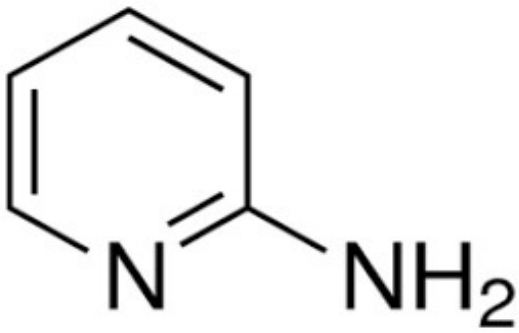
HAZARDS: Read Safety data sheet before using. All chemical reference materials should be considered potentially hazardous and should be used only by qualified laboratory personnel.

INSTRUCTIONS FOR USE: Do not dry, use as is. The internal pressure of container may be slightly different from the atmospheric pressure at the user's location. Open slowly and carefully to avoid dispersion of the material. This material is intended for Analytical use only. Not for drug, human consumption household or other uses.

Certificate of Analysis

2-AMINOPYRIDINE

(REFERENCE SUBSTANCE)



2-Pyridinamine

PURITY (HPLC): 99.9%

CATALOG #: 100322 **Lot #:** PADM/IMP/330

PACK SIZE: 190 mg **CAS #:** 504-29-0

DATE OF COA: 10 November 2017

EXPIRATION DATE: 09 November 2018

RECEIPT DATE:_____

Note: This space is provided for convenience only and its use is not required.

STORAGE: Store at 2˚C to 8˚C.

CHEMICAL FORMULA: $C_5H_6N_2$ **Molecular weight:** 94.11 g/mol

PHYSICAL DESCRIPTION: White solid.

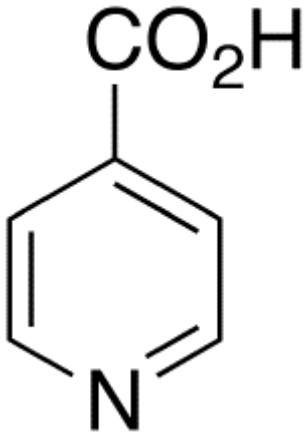
HAZARDS: Read Safety data sheet before using. All chemical reference materials should be considered potentially hazardous and should be used only by qualified laboratory personnel.

INSTRUCTIONS FOR USE: Do not dry, use as is. The internal pressure of container may be slightly different from the atmospheric pressure at the user's location. Open slowly and carefully to avoid dispersion of the material. This material is intended for Analytical use only. Not for drug, human consumption household or other uses.

Certificate of Analysis

ISONICOTINIC ACID

(REFERENCE SUBSTANCE)



4-Pyridinecarboxylic Acid

PURITY (HPLC): 99.7%

CATALOG #: 100323 **Lot #:** PADM/IMP/331

PACK SIZE: 170 mg **CAS #:** 55-22-1

DATE OF COA: 10 November 2017

EXPIRATION DATE: 09 November 2018

RECEIPT DATE:_____

Note: This space is provided for convenience only and its use is not required.

STORAGE: Store at 2˚C to 8˚C.

CHEMICAL FORMULA: $C_6H_5NO_2$ **Molecular weight:** 123.11 g/mol

PHYSICAL DESCRIPTION: White solid.

HAZARDS: Read Safety data sheet before using. All chemical reference materials should be considered potentially hazardous and should be used only by qualified laboratory personnel.

INSTRUCTIONS FOR USE: Do not dry, use as is. The internal pressure of container may be slightly different from the atmospheric pressure at the user's location. Open slowly and carefully to avoid dispersion of the material. This material is intended for Analytical use only. Not for drug, human consumption household or other uses.