ISSN 2582 – 6050

Available Online at www.ijpba.info
International Journal of Pharmaceutical & Biological Archives 2022; 13(1):1-11

RESEARCH ARTICLE

Pharmacological Evaluation of Novel Indole Derivative for Analgesic Activity in Experimental Rats

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Received: 30 November 2021; Revised: 17 December 2021; Accepted: 01 January 2022

ABSTRACT

Pain is an unpleasant sensory and emotional experience that is subjective. The prevalence of pain increases in both developed and developing countries. In this paper, we report the synthesis of indole derivatives (M1-M4) by reacting 2-chloro-1-(1H-indol-1-yl)ethanone which react with various substituted phenol. The structure of synthesized indole derivatives was confirmed by infrared and proton nuclear magnetic resonance spectral. The subacute toxicity and acute toxicity study demonstrated that compound M3 does not significantly alter biochemicals and histopathology change in rats. The analgesic activity was evaluated *in vivo* using rats' acute pain models (Haffner's tail clip, hot plate, and acetic acid-induced writhing tests). Treatment of compound M3 significantly increased pain threshold in a tail clip and hot plate and reduced the number of acetic acid writhing in rats. Hence, compound M3 showed good analgesic activity, which is demonstrated by Haffner's tail clip, hot plate, and acetic acid-induced writhing tests for acute pain models. Overall, this study suggested that compound M3 is safe and promising analgesic activity.

Keywords: Pain, Indole derivatives, Compound M3

INTRODUCTION

Pain is a diverse, complex phenomenon that occurs as a result of the body's inflammatory reaction to tissue injury. At the site of damage, immune cells actively produce chemical mediators which lead to vasodilation, change in vascular permeability, and cellular infiltration.[1] As per the International Association for the Study of Pain (IASP), pain is a distressing sensory and emotional experience caused by acute or chronic tissue injury that is subjective in nature (https://www. iasp-pain.org/publications/iasp-news/iasp-announces-revised-definition-of-pain accessed on January 10, 2022). Pain is classified based on time of occurrence (acute and chronic) and stimulus involvement (e.g., autonomic pain, bone, joint pain, myalgia, and neuralgia).[2] Pain, on the other hand, has a protective function in nature, sending messages to the body to protect it from perceived harmful external insults. The pain involved inhibitory and excitatory pathways that influence pain's sensory and emotional components.[2] According to a comprehensive evaluation of data from 2000 to 2014, pain was reported 18 crores disability-adjusted life years globally.[3] The prevalence of pain approximately 20% of adults worldwide suffer from pain, and 10% of new cases are

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diagnosed per year.[4] According to the National Health Interview Survey, 50.2 million individuals (20.5%) in the United States experienced pain most days or every day.[5] The prevalence of pain also is increased in India. The prevalence of chronic pain was 19.23% among these individuals, with women having a greater incidence.[6] However, the cross-sectional study of Primary Health Centre reported that overall chronic pain prevalence was 19.23%. Chronic back pain was the most common (24.84%), followed by body pain (22.98%), knee (16.77%), chest (13.97%), and upper limb (13.97%) (10.87%).[7] Currently available options for pain include pharmacological treatments, for example, opioid agents (morphine and tramadol) and non-opioids agents (NSAIDs drugs) and non-pharmacological options include physical therapy and alternative medicine (e.g., chiropractic therapy, massage therapy, acupuncture, mind-body therapies, and relaxation strategies). However, existing pharmacology treatment options have various major side effects, including dependence, respiratory depression, gastrointestinal toxicity, cardiovascular toxicity, allergic reaction, sedation, and tolerance. To overall this sides effect, there is a need to discover novel pharmacological pathways and their modulators for better treatment of pain. The discovery of novel compounds in a very short period has become a focal point in the current scenario. Less toxicity and higher selectivity are two key conditions for designing a new novel molecule.[8] Keep this motivation, our laboratory synthesized indole derivatives that might have analgesic activity. The compound which is synthesized in our laboratory has an indole carbon ring. Indole is a heterocyclic molecule, which means that one or more ring carbons have been replaced by another element. Heteroatoms are non-carbon atoms found in such rings. Indoles are heterocyclic compounds composed of a pyrrole ring joined to an a-b position and a benzene ring. Indole is composed of a benzene ring and a pyrrole ring joined by a double bond. It has 10 electrons from four double bonds and one from the nitrogen atom, making it a heterocyclic structure.[9] The literature survey thus reveals that indole derivatives have good analgesic, anti-inflammatory, and anticonvulsant activity. No work has been reported on compounds that were synthesized by reaction between 2-chloro-1-(indoline-1-yl) ethanone which will be reacted with various substituted phenols. Hence, the present study was planned to evaluate the analgesic efficacy of novel indole in the rat model of acute pain.

MATERIALS AND METHODS

Chemical and Solvents

All laboratory and analytical graded chemicals and solvents were purchased. The melting point was determined by the capillary method. Synthesized compound was characterized by proton nuclear magnetic resonance (^{1}H-NMR) (AV-300 BROKE JES at 300 MHz spectrophotometer, IIT, Delhi), infrared (IR) in KBr, and TLC analysis.

Synthesis Schemes for Indole Derivatives

Indole derivatives were synthesis using two steps reaction. In the first step, indole moiety was allowed to react with chloroacetyl chloride in toluene which forms 2-chloro-1-(indoline-1-yl) ethanone. In second step, 2-chloro-1-(indoline-1-yl) allows to react acetone in the presence of potassium carbonate with various substituted phenols to final indole derivatives (2-(2-hydroxyphenyl)-1-(1H-indol-1-yl) ethanone (M-2), 2-(2-nitrophenoxy)-1-(1H-indol-1-yl) ethanone (M-1), 2-(2-amino phenoxy)-1-(1H-indol-1-yl) ethanone (M-3), and 1-(1H-indol-1-yl)-2-phenoxyethanone (M4). Then, final synthesized molecules structure was confirmed by ^{1}H-NMR and IR.

Animals

Albino rat's male or female (between 150 and 200 g) was used in the experiment after ethics approval (IAEC/2019/837ac/M. Pharm/07) of IFTM University. Animals were fed a standard diet and tap water *ad libitum*. All animals were kept at standard controlled temperature (31 ± 1°C), humidity (60 ± 0.2%), and a 12 h light and 12 h dark cycle. All experiments were performed by IAEC guidelines.

Acute Toxicity Studies

An acute oral toxicity study was carried out by OECD-423 criteria (acute toxic class method). In this study, albino rats of either sex were chosen at random using random sampling procedures. The animal was fasted overnight and merely given water. The produced chemical M3 was then delivered orally by intragastric tube at doses of 5, 50, 300, and 2000 mg/kg for 2 weeks to assess behavioral changes and mortality.[10]

Subacute Toxicity Studies

An acute oral toxicity study was performed as per OECD-407 guidelines (acute toxic class method).[11] In brief, albino rats of either sex selected by random sampling techniques were employed in this study. The animal was kept fasting overnight, providing only water. The single dose of M3 compound was administered orally at 1000 mg/kg by intragastric tube, and animals were observed for 2 weeks for morality and behavioral changes. Finally, animals were sacrificed for his to pathology analysis, and blood was isolated for biochemical analysis. Based on LD_{50}, three different doses were selected, that is, low, medium, and high.

Experimental Design and Treatment Schedule

Compound M2 was selected for further research based on the acute toxicity study. Compound M3 was dissolved in normal saline and orally administrated to rats. Animals were divided into the followings groups:

Group I – Normal group (normal saline, p.o.)
Group II – Standard group diclofenac (20 mg/kg, i.p.)
Group III – Treated group low dose (100 mg/kg, p.o.)
Group IV – Treated group medium dose (200 mg/kg, p.o.)
Group V – Treated group high dose (400 mg/kg, p.o.).

Induction of Acute Pain Models

Thermal-induced pain (hot plate method)

Hot plate methods were performed based on the previously reported methods.[12] Thermal pain was induced in animals by a hot plate. In this procedure, animals were kept on a hot plate with a temperature of 55°C. Time is taken to show jumping, licking behaviors were recoded, and cutoff time was kept at about 15 s to avoid unnecessary pain and damage.

Mechanically induced pain (Haffner's tail clip method)

Haffner's tail clip method was performed based on the reported method.[13] Briefly, the tail is tightly clipped with the object that generates pain in the tail. Thus, the rat will start biting that portion of the tail. If given drugs have analgesic potential, the rat will not bite its tail so frequently. The rats that did not show any response within 15 s will reject the experiments.

Chemical-induced pain (acetic acid-induced method)

Acetic acid-induced method was performed based on the previously reported method.[14] For generating pain, acetic acid was induced into the peritoneal cavity of rats, then when chemicals writhing behavior was recorded in animals.

Statistical Analysis

Data were analyzed by GraphPad Prism (version 5.0). The result was expressed as mean ± SD. Statistical difference between control and experimental values was analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test level $P < 0.05$, which was considered statistically significant.

RESULTS

Characterization of Indole Derivatives Compounds

Four different indole derivatives (M1, M2, M2, and M4) were synthesized. The synthesized indole derivatives' physical characterization was done by melting point, % of yield, and Rf value. The % yield and melting point in M3 indole derivative were higher (68%, 172°C) as compared to M1 (62%, 46°C), M2 (59%, 48°C), and M4 (65%, 40°C). However, the M1 derivative showed a higher Rf

value than M2, M3, and M4 indole derivative [Table 1].

Indole Derivative Structure Confirmation by IR and ^{1}H-NMR

Synthesized indole derivative structure was confirmed by IR spectroscopy and 1H-NMR. IR and 1H-NMR spectra of synthesized indole derivative present in Supplement file 1. Due to the high % of the yield of M3, indole derivative was selected for further experimentations.

Acute Toxicity Studies

Orally administered compound M3 at the dose of 5, 50, 300, and 2000 mg/kg by the intragastric route, and we do not observe any gross behavioral changes and mortality until 2 weeks. They represent that compound M3 was practically nontoxic, and it can be used further to evaluate the subacute toxicity and analgesic activity.

Subacute Toxicity Studies

In subacute toxicity studies, the single dose of M3 compound was administrated; then, animals were observed for 2 weeks. Animals were sacrificed for hematological and biochemical parameters analyzed along with histological examination. Compound M3 administration found non-significant changes in hematology parameters of major organs (such as heart, liver, and kidney) [Figure 1], hematological parameters (hemoglobin, red blood cells [RBCs], white blood cells [WBCs], deferential neutrophils, lymphocytes, monocytes, eosinophil, basophils, packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin [MCH], and MCH concentration [MCHC] except platelets) [Table 2]. While biochemical parameters include Na^+, K^+, Ca^{2+}, albumin, SGOT, SGPT, and total bilirubin also increase non-significant [Table 3]. Therefore, the subacute studies suggested that synthesized compound M3 was safe with no significant toxicity effect on the major organs, biochemicals, and hematological parameters.

Effect of M3 Compound on Thermal-induced Pain

Thermal pain in rats was induced using Eddy's hot plate. Treatment of compound M3 showed a significantly increased maximum reaction time in dose-dependent manners as compared to control ($P < 0.001$). However, the standard also showed a significant increase in mean reaction time ($P < 0.001$). However, compound M3 and standard treatment effect were shown to be persistent till 180 min significantly [Table 4].

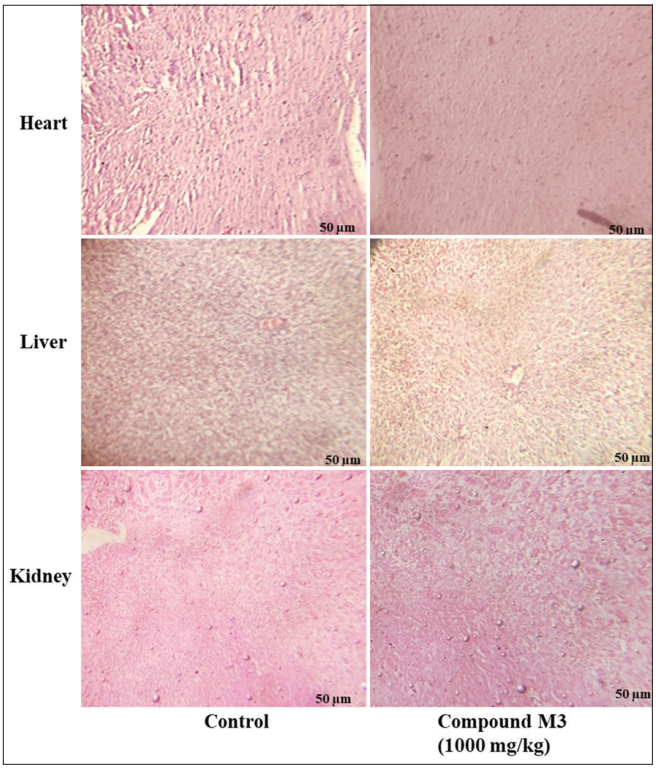

Figure 1: Hematoxylin and eosin staining of major organs (heart, liver, and kidney)

Table 1: Percentage yield, melting point, and Rf value of the synthetic compound

S. No.	Compound code	Compound chemical name	Melting point °C	% yield	R_f value
1.	M_1	2-(2-nitrophenoxy)-1-(1H-indol-1 yl) ethanone	46	62	4.7
2.	M_2	1-(1H-indol-1-yl)-2-phenoxyethanone	48	59	3.9
3.	M_3	2-(2-amino phenoxy)-1-(1H-indol-1-yl) ethanone	172	68	4.3
4.	M_4	2-(2-hydroxyphenoxy)-1-(1H-indol-1-yl) ethanone	40	65	4.6

Table 2: Hematological parameters of treated rats in subacute toxicity

S. No.	Parameters	Unit	Control	Compound M3 (1000 mg/kg)
1.	Hemoglobin	g/L	149.53±0.53	148.44±0.27
2.	Total RBCs	10^{12}/L	7.50±0.25	7.31±0.33
3.	Total WBC_s	10^9/L	7.49±0.22	7.66±0.5
4.	Deferential neutrophils	%	13.5±1.52	13.55±1.48
5.	Lymphocytes	%	84.19±2.39	86.44±1.34
6.	Monocytes	%	3.33±0.05	3.1±0.09
7.	Eosinophil'	%	00±00	00±00
8.	Basophils	%	00±00	00±00
9.	Platelets	10^9/L	820.21±33.33	825.70±41.11
10.	Packed cell volume	L/L	0.39±0.01	0.41±0.07
11.	Mean corpuscular volume	fL	53.41±0.75	54.88±0.11
12.	MCH	Pg	18.55±0.17	19.01±0.25
13.	MHC	g/L	300.12±1.11	303.15±2.11

Data expressed as Mean±SD (*n*=10) and statistical significance was analyzed using one-way ANOVA followed by Tukey's multiple comparison test

Table 3: Biochemical parameter of treated rats in subacute toxicity

S. No.	Parameter	Unit	Control	Compound M3 (1000 mg/kg)
1.	Sodium	mmol/L	124.3±0.56	128.00±0.94
2.	Potassium	mmol/L	5.33±0.01	5.98±0.04
3.	Chloride	mmol/L	97.8±0.03	99.89±0.04
4.	Calcium	mmol/L	9.55±0.04	9.78±0.05
5.	Albumin	g/L	29.83±0.03	32.31±0.01
6.	SGPT (AST)	U/L	53.67±1.23	56.33±2.42
7.	SGOT (ALP)	U/L	75.41±5.43	76.19±4.91
8.	Total bilirubin	mg/dl	0.45±00	0.47±0.02

Data expressed as Mean±SD (*n*=10) and statistical significance was analyzed using one-way ANOVA followed by Tukey's multiple comparison test

Table 4: Eddy's hot plate observation

Group	Treatment	Dose mg/kg	Reaction time (s) Mean±(SD)				
			0 min	30 min	60 min	120 min	180 min
I	Control	Normal saline	8.4±1.21	8.3±1.20	7.1±1.03	8.5±1.14	7.1±1.61
II	Standard (Diclofenac sodium)	20 mg/kg	10.03±1.01**	14.1±1.00**	14.09±1.23**	13.33±1.11**	13.00±0.99**
III	A low dose of M3	100 mg/kg	9.01±1.21**	11.11±1.31**	13.00±1.02**	13.11±1.00**	10.01±1.05**
IV	Medium dose of M3	200 mg/kg	9.03±1.00**	12.01±1.00**	13.05±1.19**	14.05±1.15**	12.69±1.31**
V	High dose of M3	400 mg/kg	8.99±1.21**	11.55±2.11**	13.99±1.31**	13.60±1.01**	12.89±2.05**

Data expressed as Mean±SD (*n*=6) and statistical significance was analyzed using one-way ANOVA followed by Tukey's multiple comparison test. ***P*<0.01 represents significance compared to control

Effect of M3 Compound on Mechanical-Induced Pain

Mechanical pain in rats was induced using Haffner's tail clip method which was presented. Treatment of compound M3 was non-significantly increased reaction times at low and high doses ($P > 0.05$) while medium dose showed and standard (diclofenac) significantly increase increased reaction times ($P < 0.001$) at 0 min. However, after 15 min of treatment of standard (diclofenac sodium) and compound M3 showed a significant increase in mean reaction time at low dose, medium dose as well as high dose ($P < 0.001$). The result indicated that the compound M3 has analgesic potential and M3 showed an increase in pain threshold throughout the entire observation for 60 min [Table 5].

Table 5: Observation table of Haffner's tail clip model

Group	Treatment	Dose mg/kg	Reaction time (s)			
			0 min	15 min	30 min	60 min
I	Control	Normal saline	4.09±0.75	4.20±0.63	4.81±0.79	4.97±0.55
II	Standard (diclofenac sodium)	20 mg/kg	5.79±0.13***	6.93±0.11***	9.84±0.19***	9.66±0.21***
III	Low dose of M3	100 mg/kg	5.03±1.19	5.93±0.89***	6.87±0.91***	7.00±0.92***
IV	Medium dose of M3	200 mg/kg	5.89±0.82***	6.01±1.00***	6.89±0.83***	7.22±0.91***
V	High dose of M3	400 mg/kg	5.13±0.67	6.01±0.79***	6.99±0.72***	8.12±0.83***

Data expressed as Mean±SD (n=6) and statistical significance was analyzed using one-way ANOVA followed by Tukey's multiple comparison test. ***P<0.001 represents significance compared to control

Effect of M3 Compound on Chemical-Induced Pain

Chemical-induced pain was caused by acetic acid-induced writhing. The administration of the compound M3 at 100, 200, and 400 mg/kg showed a significantly reduced number of writhes (acetic acid-induced abdominal constriction) in experimental rats compared with control (P < 0.001). The standard drug (diclofenac) also considerably reduced the number of writhes as compared to control [Table 6].

Table 6: Observation table of acetic acid-induced model

Group	Treatment	Dose mg/kg	No. of writhing
I	Control	1% acetic acid	83.3±6.5
II	Standard (diclofenac sodium)	20 mg/kg	33.55±4.4***
III	Low dose of M3	100 mg/kg	61.33±3.33***
IV	Medium dose of M3	200 mg/kg	43.09±3.24***
V	High dose of M3	400 mg/kg	40.11±2.25***

Values are expressed in Mean±SD (n=6) and statistical significance one-way ANOVA followed by Tukey's multiple comparison test. ***P<0.001 represents significance compared to control

DISCUSSION

Literature review reported that indole derivatives possess different biological activities, including anti-inflammatory, antibacterial, antifungal, analgesic, and anticonvulsant activity.[15] Given these observations, we have synthesized some novel indole derivatives and evaluated them for their analgesic activity. Thus, four molecules (M1–M4) were synthesized by mixing indole with chloroacetyl chloride in toluene to form 2-chloro-1-(1H-indol-1-yl) ethanone, which interacts with various substituted phenol in acetone in the presence of potassium carbonate to yield final indole derivatives. The compounds were obtained in solid form, with yields ranging from 58% to 69%. The purity and homogeneity of all compounds were shown by TLC and their melting points. The structures of these compounds were confirmed using IR and ^{1}H-NMR. Four molecules (M1, M2, M3, and M4) were synthesized and analyzed by IR and ^{1}H-NMR spectra, however, due to the low percent yield, only one molecule with a high percent yield (M3) was chosen for future investigation. This chemical was investigated and tested for *in vivo* analgesic efficacy. Assessing toxicity is a critical preparatory step before conducting effectiveness studies when screening new compounds for pharmacological activity. The calculation of LD50 is a basic step in the study of toxicity.[16] The acute oral toxicity study may provide preliminary information about agents' toxic action, which could help decide the dose of the novel compound *in vivo* studies.

Furthermore, if the animals survive at a high dosage (e.g., 2000 mg/kg), no additional acute testing will be performed.[10] In this investigation, the compound M3 at a level of 2000 mg/kg had no negative effects on the treated rats after 14 days of observation. As a result of this investigation, M2 did not induce acute toxicity effects at the level evaluated, and the LD50 value might be >2000 mg/kg. Because no harmful effects were discovered during the critical toxicity research, a follow-up study was done to assess M3's subacute toxicity for up to 28 days. Subacute studies give information on dosing regimens, target organ toxicity, and identify visible adverse effects that may impact the average life span of experimental animals. After 28 days of M3 compound therapy, no significant changes in histopathology of major organs, biochemical analysis, or hematological

parameters were identified. The hematological findings revealed a non-significant change in RBC indices, WBC counts, neutrophils, lymphocytes, and monocytes levels. Serum biochemistry was examined to discover potential changes in renal and hepatic activities influenced by total compound protein, with total bilirubin potentially influencing hepatocellular and secretory processes of the liver.[17,18] The lack of significant changes in SGOT, SGPT, ALP, and creatinine levels, which are strong indices of liver and kidney function, indicates that the compound M3 did not affect rat hepatocytes and kidneys after a 28-day subacute administration. Haffner's tail clip, hot plate, and acetic acid-induced writhing tests were used to assess the compound's analgesic efficacy. These are common pharmacological paradigms for assessing synthetic chemical analgesia.[19] For centrally active analgesics, both Haffner's tail clip and the hot plate procedures are commonly utilized.[20] While these tests are not applicable to peripherally acting medications, they are amenable to the acetic acid-induced writhing test.[21] The present study found that the chemical generated significant antinociceptive actions when tested utilizing various pain models. The new findings further show that phenol derivatives operate both centrally and peripherally. The pain models utilized in this study were chosen to measure both centrally and peripherally mediated effects. The overall study suggested that indole derivatives (M3) were safe and possessed good analgesic activity.

ACKNOWLEDGMENTS

The authors acknowledge the financial assistance for IFTM University for this work.

CONFLICTS OF INTEREST

The author declares that they have no conflicts of interest.

INFORMED CONSENT

Not required.

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SUPPLEMENT FILES

IR SPECTRA OF INDOLE DERIVATES (M1-M4)

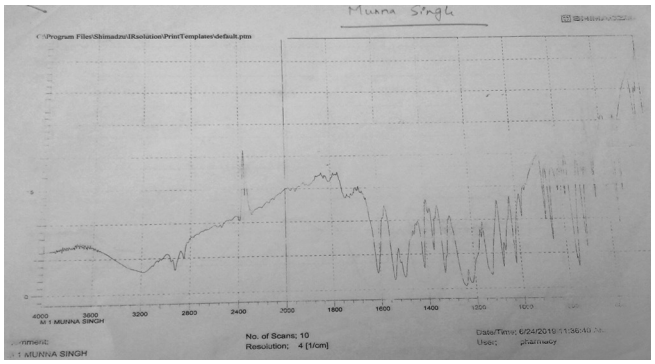


Figure 1: IR spectra of M1 compound

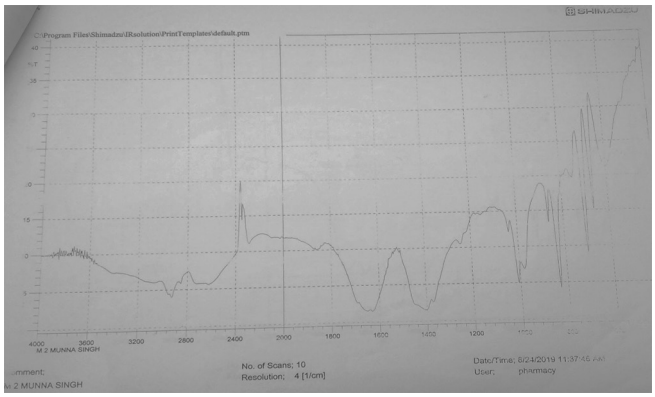


Figure 2: IR spectra of M2 compound

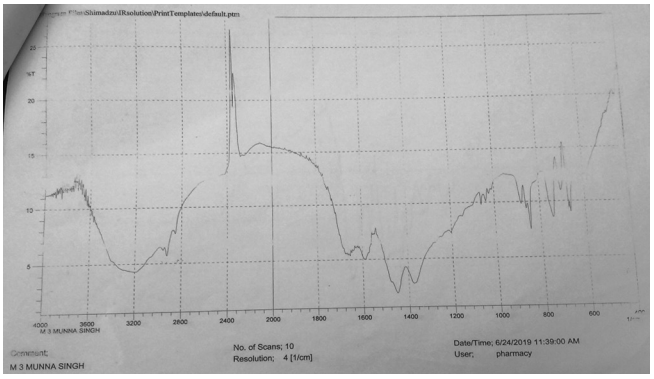


Figure 3: IR spectra of M3 compound

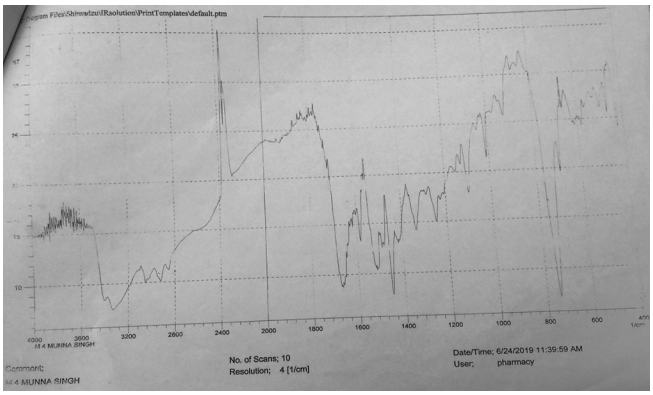


Figure 4: IR spectra of M4 compound

Table 1: IR interpretation of M1, M2, M3 and M4 compounds

S. No.	Observed peak (cm^{-1})	Functional group assigned	Characteristic peak
Compound M1	3190	C-H str (aromatic)	2800-3200
	1240	C-C str (aromatic)	1000-1250
	850	C-H bend (aromatic)	700-900
	2900	C-H str (Aliphatic)	2900-2700
	1590	C=O str	1600-1700
	1190	C-O str	1225-1200
	1380	C-N str	1400-1600
Compound M2	2920	C-H str (aromatic)	2800-3200
	1010	C-C str (aromatic)	1000-1250
	840	C-H bend (aromatic)	700-900
	2838	C-H str (Aliphatic)	2900-2700
	1630	C=O str	1600-1700
	1260	C-O str	1225-1200
	1400	C-N str	1400-1600
Compound M3	3190	C-H str (aromatic)	2800-3200
	1240	C-C str (aromatic)	1000-1250
	850	C-H bend (aromatic)	700-900
	2900	C-H str (Aliphatic)	2900-2700
	1590	C=O str	1600-1700
	1190	C-O str	1225-1200
	1380	C-N str	1400-1600
Compound M4	3040	C-H str (aromatic)	2800-3200
	1250	C-C str (aromatic)	1000-1250
	710	C-H bend (aromatic)	700-900
	2840	C-H str (Aliphatic)	2900-2700
	1660	C=O str	1600-1700
	1400	C-O str	1225-1200
	1450	C-N str	1400-1600

Table 2: NMR interpretation of M1 compound

Compound Name	Chemical shift (δ)(ppm)	No. of protons	Inferences
M1	6.50-7.83	6	Indole
	4.2	2	CH_2
	6.99-7.34	5	Phenol
M3	6.50-8.11	6	Indole
	4.8-5.00	2	CH_2
	6.52-6.8	5	Phenol
M4	6.50-7.10	6	Indole
	4.1-4.30	2	CH_2

1^H NMR SPECTRA OF INDOLE DERIVATES (M1-M4)

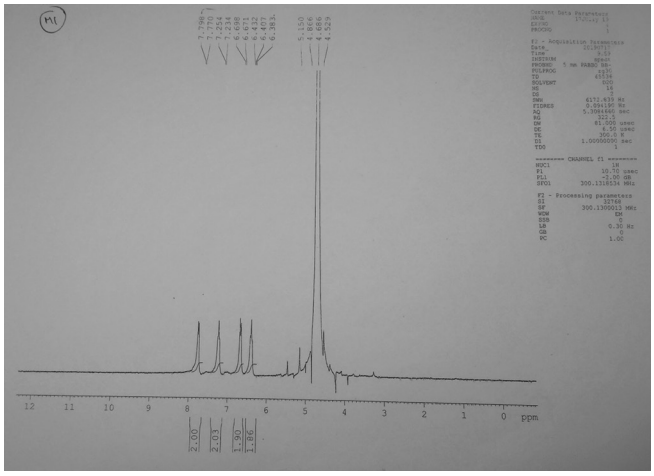


Figure 5: NMR spectra of M1 compound

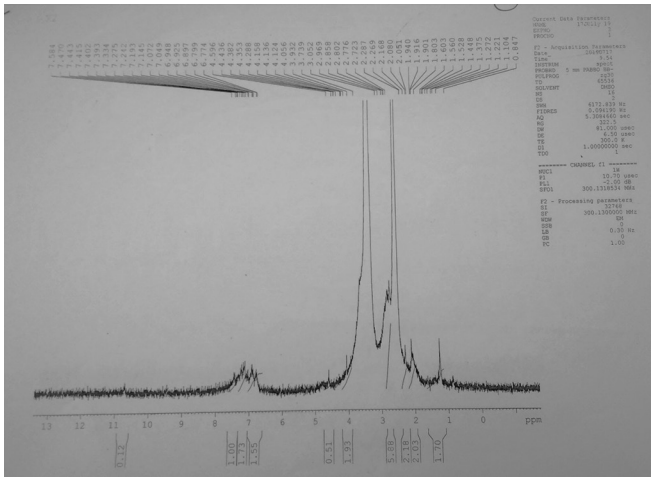


Figure 6: NMR spectra of M2 compound

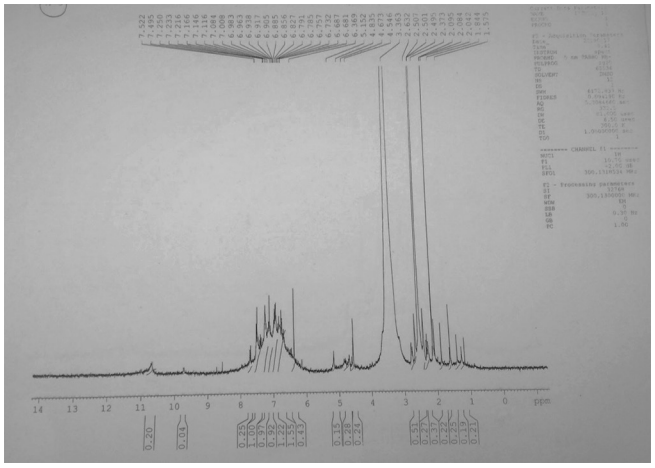


Figure 7: NMR spectra of M3 compound

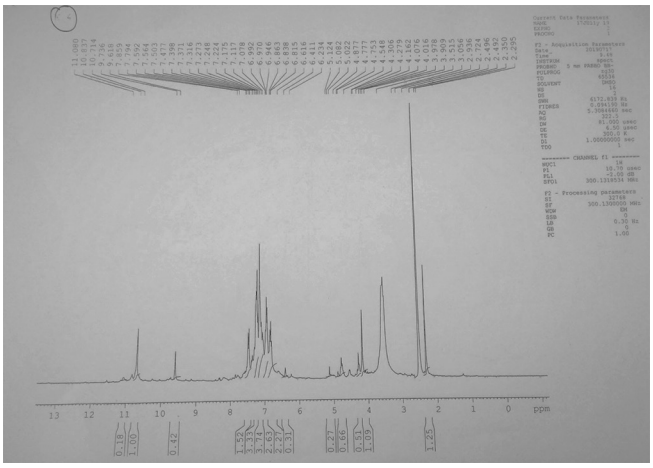
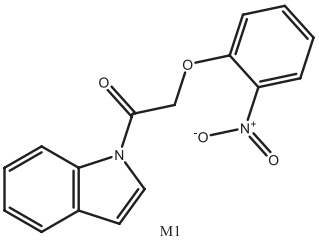
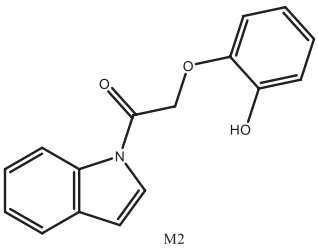


Figure 8: NMR spectra of M4 compound

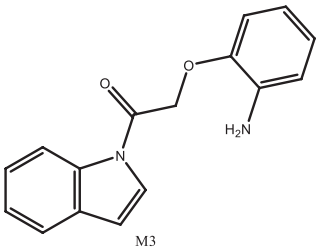
FINAL STRUCTURE OF INDOLE DERIVATES (M1-M4)



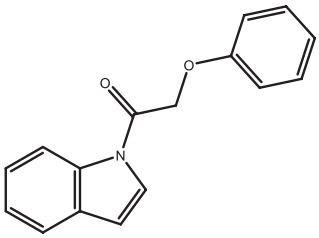
M1



M2



M3



M4