

# Isolation of $\beta$ -Asarone from *Acorus calamus* Linn. and Evaluation of its Anticonvulsant Activity using MES and PTZ Models in Mice

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## ABSTRACT

**Introduction:** *Acorus calamus* is being used in Ayurveda as Medhya Rasayana to enhance mental ability and used in treatment of various brain related disorders. **Objective:** Extraction of volatile oil from *Acorus calamus* rhizomes and Isolation of  $\beta$ -Asarone from it and anticonvulsant activity of volatile oil in mice using MES and PTZ models. **Method:** The volatile (calamus) oil was extracted using hydro steam distillation. Calamus oil was subjected to column chromatography using Silica gel in glass column for isolation of  $\beta$ -Asarone. Calamus oil was evaluated for anticonvulsant activity at 30, 100 and 300mg/kg dose using MES, scPTZ, minimum clonic seizure (at 6 Hz.) model and Rotarod test was used to determine the neurotoxicity of volatile oil. **Results:**  $\beta$ -Asarone was isolated and confirmed by structure elucidation using <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, IR, UVS and Mass spectroscopy. Calamus oil was found to be inactive in preliminary screening at all dose levels but has shown neurotoxicity at 300 mg/kg. Although, volatile oil was found to be active in minimal clonic seizure test at 6 Hz. At 100 mg/kg, it displayed protection even up to 4.0hr indicating longer duration of action.

ED<sub>50</sub> (48.13 mg/kg) and TD<sub>50</sub> (224.13 mg/kg) of volatile oil at 6 Hz in mice also determined at 95% C.I. Protective index(PI) value of volatile oil was found to be 4.65. The experimental results were compared with the standard drug Phenytoin (30 mg/kg). **Conclusion:** The calamus oil has shown a protective index that can be compared with Phenytoin and can be used as an alternate of synthetic antiepileptic drugs.

**Key words:** Anticonvulsant, *Acorus calamus*,  $\beta$ -Asarone, Calamus Oil.

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## INTRODUCTION

Epilepsy is a neurological disorder of varied etiology, characterized by paroxysmal, excessive and hyper synchronous discharge from large number of neurons. Despite the optimal use of available antiepileptic drugs (AEDs), many patients fail to experience seizure control and others do so only at the expense of significant toxic side effects that range in severity from minimal brain impairment to death from aplastic anemia or hepatic failure.<sup>1</sup> It is estimated that available medication controls seizures in only 50 % of patients or decrease incidence in only 75 % of patients. *Acorus calamus* Linn. (Araceae) commonly known as sweet flag or sweet root. The reported uses associated with *Acorus calamus* are anticonvulsant,<sup>2</sup> antispasmodic,<sup>3</sup> neuroprotective, antiulcerogenic,<sup>4</sup> anti-tumor,<sup>5</sup> antimicrobial,<sup>6,7</sup> anti-inflammatory,<sup>8,9</sup> antioxidant, anticholinesterase<sup>10</sup> and hepatoprotective.<sup>11</sup> The rhizomes possessing sweet aromatic odour is reported to have strong anticonvulsant effects. The volatile oil from rhizomes of *Acorus calamus* was reported to contain the active anticonvulsant compound  $\beta$ -Asarone in significant amounts.<sup>7,9</sup>  $\beta$ -Asarone easily passes through the blood-brain-barrier (BBB) and shows significant pharmacological effects on the cardiovascular and central nervous systems.<sup>12</sup> Methanolic extract of *Acorus calamus* was also reported to possess significant anti-seizure effect in subcutaneous pentylenetetrazol (scPTZ) induced convulsion model when administered orally in the doses of 100 and 200 mg/kg. It was also found to increase the latency period in mice significantly in scPTZ model.<sup>2</sup> Asarones were found to alter fighting behaviours along with conditioned responses and neuroprotection in convulsions.<sup>13</sup> Bramhi Ghrita, a polyherbal formulation containing *Bacopa monneri*, *Evolvulus alsinoids*, *Acorus calamus*, *Saussurea lappa* and cow's ghee was also reported to possess anti-seizure effects

in MES and scPTZ models. Additionally, it has also reduced alertness, spontaneous locomotor activity and reactivity. It was also reported to potentiate Phenobarbital induced sleep and increases the pain threshold while antagonized the behavioral effects of d-amphetamine.<sup>14</sup> Men tat syrup, a product from Himalaya Herbal Healthcare, has also shown a pivotal role in the prognosis of children with febrile convulsions. It mainly contains herbs such as *Bacopa monnieri*, *Nardostachys jatamansi*, *Centella asiatica*, *Acorus calamus* and *Prunus amygdalus*. It has exhibited a significant improvement in children with febrile seizures.<sup>15</sup> *A. calamus* has also shown potent synergistic effects on anticonvulsant properties of prototype drugs Phenytoin and Phenobarbital. It has reduced the ED<sub>50</sub> of Phenytoin and Phenobarbital from 13.5 mg/kg to 9.25 mg/kg and 8 mg/kg to 5 mg/kg respectively, at a dose of 185 mg/kg. It has also displayed the significant increase in the antiepileptic activity of Phenytoin and Phenobarbital in the sub-effective dosage of 10 mg/kg and 2 mg/kg respectively.<sup>16</sup> It is also reported to prevent ferric chloride induced epileptogenic, in the dose of 200 mg/kg. It has also shown a significant decrease in the activity of superoxide dismutase and catalase enzymes with a decrease in the lipid peroxidation in cerebral cortex, thus indicating a potent anticonvulsant profile.<sup>17</sup>

In the present study,  $\beta$ -Asarone was isolated from volatile oil of *Acorus calamus* using column chromatography. Calamus oil was extracted using hydro steam distillation and evaluated for its anticonvulsant properties using MES, scPTZ and minimal clonic seizure (at 6 Hz) models. Rotarod test model was used to determine the neurotoxicity of Calamus oil.

## MATERIALS AND METHODS

### Plant material

The plant material (rhizomes) of *Acorus calamus* was purchased from NC Herbs suppliers Barraut, Uttar Pradesh. Identification and taxonomical authentication was done by Dr. H.B. Singh, Head, Raw Materials Herbarium and Museum (RHMD) at National Institute of Science Communication and Information Resources, New Delhi (NISCAIR). The voucher specimen no. of authenticated plant material NISCAIR/RHMD/Consult/-2010-11/1587/185. Rhizomes are also preserved in the departmental museum of Ram-Eesh Institute of Vocational and Technical Education, Greater Noida.

### Extraction of volatile oil and isolation of fraction containing Beta-Asarone

The air dried rhizomes of *A. calamus* were size reduced to 1-3 cms and extraction of the volatile oil was carried out in Clevenger assembly by hydro-distillation method. The oil yield was 5-10 ml/kg. The extracted oil was dehydrated over anhydrous Sodium sulphate and stored at 4<sup>o</sup> C. The oil was subjected to column chromatography using glass column. The silica gel (60-120 mesh, Loba Chemie, India) was first activated in oven at 110<sup>o</sup> C for 30 min and then used for column packing using Wet-packing technique. The column was eluted with solvents of increasing polarity from Petroleum ether to Methanol (procured from Rankem Laboratory, India) and simultaneously, TLC was performed for identification of  $\beta$ -Asarone. Silica Gel G (CDH Laboratory Reagents, India) was used for preparing TLC. The chloroform: petroleum ether fraction showing single spot of maximum intensity was collected and subjected to spectral analysis (IR, <sup>1</sup>H NMR, <sup>13</sup>C NMR and mass spectroscopy) for identification of  $\beta$ -Asarone (BA).

### Preparation of test drugs for Anticonvulsant screening

The volatile oil of *Acorus calamus* (liquid) was prepared as suspension in methyl cellulose.

### Drugs and Chemicals

Phenytoin Sodium (Epsolin, Cadila Healthcare Limited) dissolved in normal saline was prepared on the day of the experimentation. Phenytoin (30 mg/kg) was administered orally.

### Anticonvulsant activity screening

The anticonvulsant screening and ED<sub>50</sub> and TD<sub>50</sub> evaluation were carried out under Anticonvulsant Screening Program at National Institute of Neurological Disorders and Stroke (NINDS), National Institute of Health (NIH), using their reported procedures<sup>18</sup> by MES, scPTZ, minimum clonic seizure (at 6 Hz.) models and Rotarod test was used to determine the toxicity of volatile oil. In quantitative screening, groups of eight mice were given a range of intraperitoneal doses (per oral for rats) of the test drug until at least three points were established in the range of 10–90% seizure protection or minimal-observed neurotoxicity. From the plots of these data, the respective ED<sub>50</sub> and TD<sub>50</sub> values, 95% confidence intervals, slope of the regression line, and the standard error of the slopes were calculated by means of a computer program written by the NINDS.

### Maximal Electroshock Model (MES)

Commonly this test is conducted in adult male albino mice. For mice, current (60 Hz) is delivered via corneal electrodes primed with an electrolyte solution containing an anesthetic agent. The animals were administered with the test drug solution in methyl cellulose, prior to electric shock.

The characteristics of electroshock seizures are a tonic limb flexion of 1-2 sec followed by a tonic limb extension of roughly 10-12 sec and finally generalized clonic movements for 12 sec. The total duration of seizure is approximately 25 sec. Only abolition of the limb tonic extensor spasm

is recorded as the measure of anticonvulsant potency. The component is considered abolished if the hind leg extension does not exceed a 90<sup>o</sup> angle with the plane of the body.

### The Subcutaneous Pentylentetrazole (Metrazole) seizure Tests (scPTZ)

This is a model that primarily identifies compounds that raises seizure threshold. The behavioral seizure produced is not typical of absence epilepsy but clonic in nature like other rodent models of absence seizures, PTZ induced seizures are potentiated by GABA agonist. With some minor exceptions, the pharmacological profile of the scPTZ seizure model is consistent with the human condition.<sup>14,15</sup> The Car worth Farms No. 1 mice received pentylentetrazol at a dose of 85 mg/kg. It produces clonic seizures lasting for a period of at least 5 sec in 97% (CD<sub>97</sub>) of animals tested. At the anticipated time of testing the convulsant is administered subcutaneously. The test compound is administered intraperitoneally in mice. Animals were observed over a 30-min period. Absence of clonic spasms in the observed time indicates a compound's ability to abolish the effect of pentylentetrazol on seizure threshold.<sup>19,20</sup>

### Rotarod test

At 30 min. after the administration of the test compound, the animals were tested on a 1-in diameter knurled plastic rod rotating at 6 rpm for 1 min. Neurotoxicity was indicated by the inability of an animal to maintain equilibrium in each of three trials.

### Minimum clonic seizure test

The animal clonic seizure is used to assess a compound's efficacy against electrically induced seizures but uses low frequency (6 Hz) and longer duration of stimulation (3 sec). Test compounds are pre-administered to mice which are then challenged with sufficient current delivered through corneal electrodes to elicit a psychomotor seizure in 97% of animals (32 mA for 3 s). Protection was defined as the absence of minimal clonic phase with stereotyped, automatist behaviors. This behavior is described as like aura of human patients with partial seizures.

## RESULTS

The volatile oil was obtained from rhizomes of *A. calamus* and subjected to column chromatography and the fraction containing  $\beta$ -Asarone was eluted with the chloroform: petroleum ether (b.p. - 60<sup>o</sup>-80<sup>o</sup>C) in the ratio of 4:1. The fraction has shown a dense spot in the TLC with Rf value-0.549 (solvent system- chloroform: benzene, 4:1). The eluted fraction was further subjected to spectral characterization and the spectral reports are given as IR spectra and Mass spectra is given in appendix A, <sup>1</sup>H NMR spectra is given in appendix B and <sup>13</sup>C NMR spectra is given in appendix C.

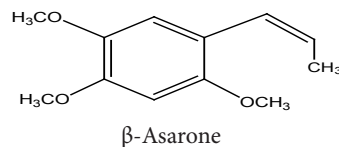
### Spectral Observations

<sup>1</sup>H NMR Data: 7.156-6.992 (d,1H, ArH), 6.811 (s,1H, ArH), 6.673-6.632 (d,2H, ArH,J=12.3), 6.571-6.519 (d,1H,ethylenic-H, J=15.6), 6.412-6.374 (d,1H,ethylenic-H, J=11.4), 6.155-6.080 (m,1H,ethylenic-H), 5.703-5.618 (m,1H,ethylenic-H), 3.791-3.692 (m,18H,9\*2 -OCH<sub>3</sub>), 1.827-1.768 (m,6H,3\*2 -CH<sub>3</sub>).

<sup>13</sup>C NMR Data: 151 (s, C-5), 150 (s, C-7), 142.7 (s, C-8), 124.96-124.67 (t, C-3), 123.28 (s, C-2), 114.3 (s, C-9), 110.04 (s, C-4), 98.69-97.28 (m, C-6), 56.51-55.72 (m, C10, C11, C12), 14.53 (s, C-1).

Mass (ESI) Data: 416.2, 209.11

The spectral data analysis supported the reported structure characteristics of  $\beta$ -Asarone.



**Table 1: Quantitative anticonvulsant data in mice at 6 Hz (test drug administered i.p)**

Test	Time (Hrs.)	ED <sub>50</sub> (mg/kg)	95 % Confidence interval	Std. Error
6 Hz	1	48.13	37.73-67.34	1.73
TOX	1	224.13	199.76-243.71	5.61

Note:

a. 6 Hz = Minimum clonic seizure test at 6 Hz.

b. TOX= Rotarod toxicity test.

**Table 2: Preliminary anticonvulsant data of volatile oil of *A. calamus***

Test	Dose (mg/kg)	Time (H)	
		0.5	4.0
MES	30	N/F	N/F
	100	0/1	0/1
	300	0/3	0/3
scPTZ	30	0/1	0/1
	100	0/1	0/1
	300	0/1	0/1
TOX	30	0/4	0/2
	100	0/8	0/4
	300	4/4	2/2

Note:

a- N/F = Number of animals active or toxic / Number of animals tested.

b- MES= Maximal Electro Shock test.

c- scPTZ=Subcutaneous Pentylenetetrazole (Metrazole) seizure test.

d- TOX= Rotarod toxicity test.

### MES, scPTZ, minimal clonic seizure and rotarod neurotoxicity screening

Calamus oil was subjected to preliminary anticonvulsant screening at 30, 100 and 300mg/kg dose using MES, scPTZ, minimal clonic seizure test and neurotoxicity parameters in mice. Phenytoin (30mg/kg) was used as standard. The results of MES and scPTZ are given in Table 2 while the observations for response comments are given in Table 3. The results of minimum clonic seizure test are given in Table 4. Observations for rotarod neurotoxicity screening were given in Table 2. Results of ED<sub>50</sub> and TD<sub>50</sub> evaluation were specified in Table 1. The protective index (PI) value for calamus oil in 6Hz studies can be calculated as 4.65. Tremors and diarrhea are the common biological responses observed in animals during toxicity screening.

## DISCUSSION

The rhizomes of *Acorus calamus* Linn contains appreciable amount of calamus oil. Calamus oil was extracted using hydro steam distillation employing heavy oil Clevenger assembly. During extraction of volatile oil, the rhizomes have shown tremendous swelling. The yield of isolated volatile oil was 10-20 ml/kg. The isolated oil was collected and dried over anhydrous Sodium sulphate and stored in refrigerator. The isolation of  $\beta$ -Asarone was performed using glass column and silica gel for column chromatography as adsorbent. The column was packed using wet packing method. Elute from the column at Chloroform: Ethyl acetate mixture in ratio of 4:1 given intense dark spot on TLC. Elute was sent for spectral analysis like UV, IR, <sup>1</sup>H-NMR, <sup>13</sup>C-NMR and Mass spectroscopy. The spectral reports when characterized revealed the identity of the eluted fraction as  $\beta$ -Asarone. The method of isolation is easy and the yield of  $\beta$ -Asarone from calamus oil is appreciably high.

**Table 3: Response comments for volatile oil of *A. calamus***

Test	Dose (mg/kg)	Time (H)	Comments
scMET	300	0.5	Died during test without seizure
		4	Died during test without seizure
TOX	300	0.5	Tremors
		2	Unable to grasp rotarod
		4	Tremors

Note:

a- scPTZ=Subcutaneous Pentylenetetrazole (Metrazole) seizure test.

b- TOX= Rotarod toxicity test.

**Table 4: Anticonvulsant activity of volatile oil of *A. calamus* at 6 Hz. MES**

Test	Dose (mg/kg)	Time (Hrs.)				
		0.25	0.5	1.0	2.0	4.0
6 Hz	100	N/F	N/F	N/F	N/F	N/F
6 Hz	100	0/4	2/4	4/4	¾	2/4
TOX	100	0/4	0/4	0/4	0/4	0/4

Note:

6 Hz = Minimum clonic seizure test at 6 Hz.

The volatile oil which contains about 80 percent of  $\beta$ -Asarone was screened for anticonvulsant activity as it was reported to possess the anti-convulsant potential. Brahmi ghrita and Mentet syrup containing *Acorus calamus* are already being prescribed for increasing mental ability and treatment of brain disorders. The active fraction containing volatile oil was screened for its anticonvulsant potential using MES, scPTZ, minimal clonic seizure test and neurotoxicity parameters in mice and results are compared with the standard Phenytoin. Calamus oil was found to be inactive in preliminary screening at all dose levels but has shown neurotoxicity at 300mg/kg. Although, volatile oil was found to be active in minimal clonic seizure test at 6 Hz. At 100mg/kg, it has displayed protection even up to 4.0hr indicating longer duration of action. Further evaluations of ED<sub>50</sub> and TD<sub>50</sub> observations have shown the values of 48.13 and 224.13mg/kg respectively. The protective index (PI) of calamus oil was calculated as 4.65 which proves that calamus oil can be successfully employed for the long-term management of epilepsy with least side effects as compared to synthetic analogues. The calamus oil can also be used to reduce the effective dose of synthetic antiepileptic drugs if used as an adjuvant with them. This can not only minimize the associated toxicities with synthetic antiepileptic drugs but can also help in increasing patient compliance.

## CONCLUSION

The present study showed various positive approaches for the treatment, long term management and prevention of epileptic seizures. Volatile oil from the rhizomes of *Acorus calamus* showed a better protective index of 4.65 as compared to standard drugs. The magical drug need to be explored more to increase its therapeutic potential minimizing the associated side effects.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

None.

## ABBREVIATIONS USED

**CPCSEA:** Committee for Control and Supervision of Experiments on Animals; **NMR:** Nuclear Magnetic Resonance; **IRS:** Infrared Spectroscopy; **UVS:** Ultraviolet Spectroscopy; **BBB:** Blood Brain Barrier; **NIS-CAIR:** National Institute of Science Communication and Information Resources; **ED:** Effective dose; **TD:** Toxic Dose; **scPTZ:** Subcutaneously Pentylentetrazol; **scMET:** Subcutaneously Metrazole; **MES:** Maximal Electroshock; **AFI:** Ayurvedic Pharmacopoeia of India; **AED:** Anti Epileptic Drugs. **RHMD:** Raw Materials and Herbarium Department; **PI:** Protective Index.

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## PICTORIAL ABSTRACT



## SUMMARY

- $\beta$ -Asarone was isolated from rhizomes of *Acorus calamus* using Column chromatography.
- Identity of the column elute was established as  $\beta$ -Asarone by characterization of the UV, IR,  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$  and Mass Spectras.
- Anticonvulsant activity of the volatile oil containing  $\beta$ -Asarone was screened in MES, scPTZ, Minimal clonic seizure model screening at 6Hz and toxicity was screened by Rotarod model.
- *Acorus calamus* possesses significant anticonvulsant activity with Protective index of 4.65 minimal clonic seizure model and can be a better option for long term management of epilepsy.

## ABOUT AUTHOR



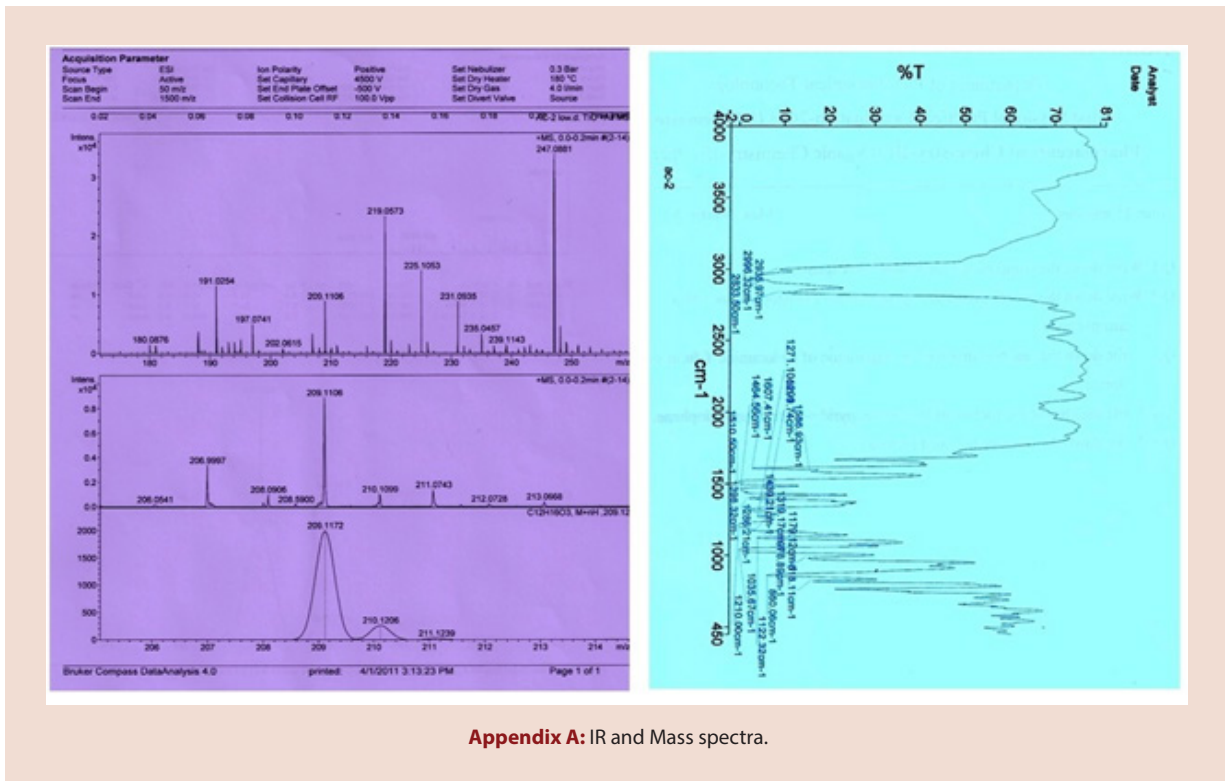
**Rahul Kaushik** did his B.Pharm and M.Pharm (Pharmacognosy) from Dr. APJ Abdul Kalam Technical University, Lucknow. He is working in the Ram-Eesh Institute of Vocational & Technical Education, Greater Noida as Assistant Professor. He is also pursuing PhD from Dr. APJ Abdul Kalam Technical University, Lucknow. With over 6 Years of Academic and Industrial experience he has authored more than 10 research and review articles in International and National journals and attended more than 15 National conferences and seminars. His area of interest is Standardization of Herbal drugs, Chromatography, Phytochemistry and Herbal Anticonvulsants.



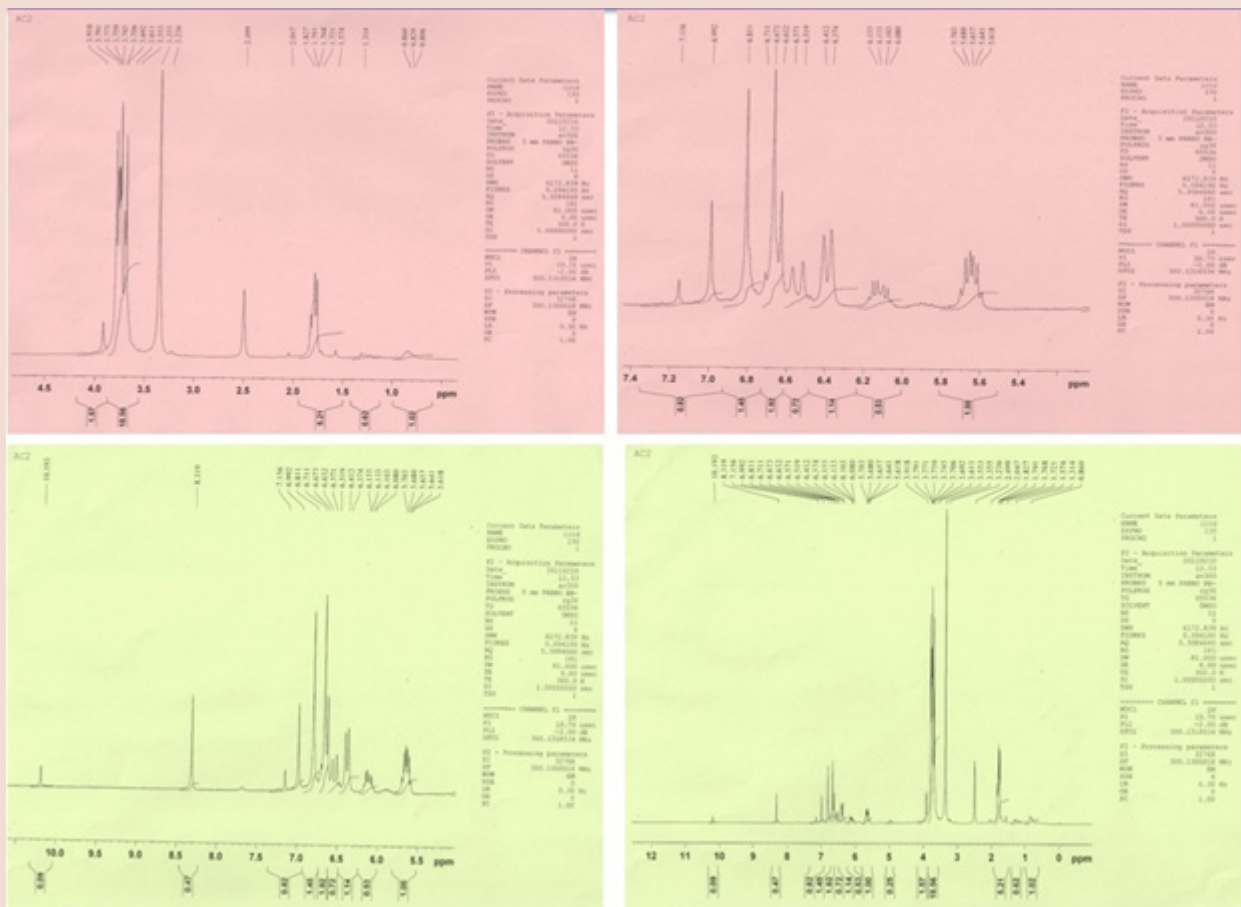
**Prof. (Dr.) Jainendra Jain** did M.Pharm from Banaras Hindu University Varanasi and Ph.D from Uttar Pradesh Technical University Lucknow. Currently he is associated with Ram-Eesh Institute of Vocational and Technical, Greater Noida as Principal. He has to his credit 37 research papers in National and International journals of high repute and also presented 29 poster presentations in various National and International conferences. He has guided more than 20 UG, 12 PG and 7 PhD scholars. He is recipient of many grants from All India Council of Technical Education (AICTE) Govt. of India, Department of Science and Technology, Dr. APJ Abdul Kalam Technical University and so on. He has successfully organized many National level workshops and seminars. He has keen interest in designing and development of both herbal and synthetic Anticonvulsants.



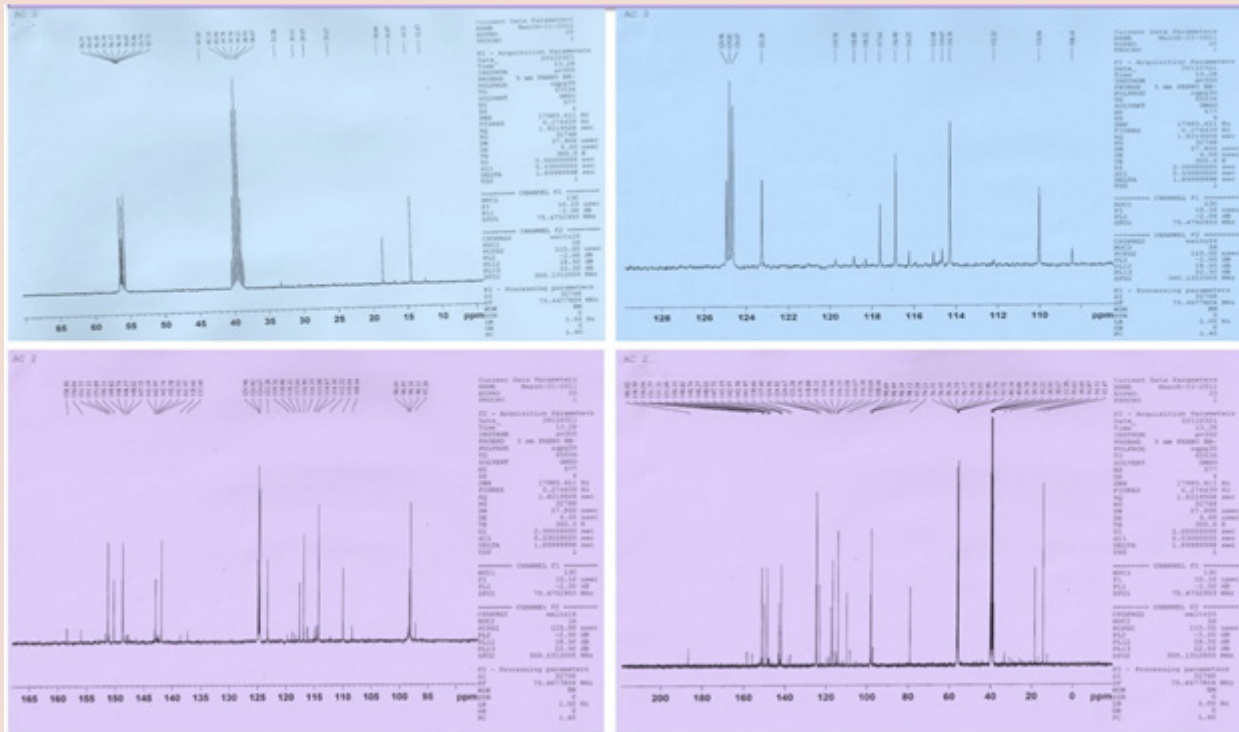
**Dr. Lubhan Singh** secured Gold medal in his M.Pharm (Pharmacology) from BPUT Rourkela, Orissa and Ph.D. from Uttarakhand Technical University, Dehradun. He is currently associated with Ram-Eesh Institute of Vocational and Technical, Greater Noida as Associate Professor and Head of Pharmacology Department. He has to his credit more than 35 research and review articles in National and International journals and has presented more than 50 posters in various National and International conferences and seminars. He has filed 01 patent and guided more than 100 UG and 10 PG students. He has coordinated 04 National conferences and seminars. He has received seminar grant from SERB. He has interest in CNS acting molecules.



Appendix A: IR and Mass spectra.



Appendix B: <sup>1</sup>H-NMR spectra of eluted fraction.



**Appendix C:** <sup>13</sup>C-NMR spectra of eluted fraction.