Isolation and Phylogenetic Identification of Seed borne Mycoflora associated with Rice and Eco-friendly management by *Datura stramonium* with Phytochemical Screening : GC-MS analysis

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Abstract :

The study illustrates the efficiency of Antifungal activity of ethyl acetate extract of *Datura stramonium* against some seed borne fungi (*Aspergillus* sp, *Rhizopus* sp, *Fusarium* sp, and *Macrophomina* sp.) isolated from field crop Rice (Variety- Khas) of Indo-Gangetic area, using standard microbiological procedures and to investigate the active compound of ethyl acetate extract of *Datura stramonium* which is responsible for antifungal activity by GC-MS technique. The identification of fungi was carried out by phylogenetic tree construction and 18S rRNA sequencing. The well diffusion method was used to study the efficacy of *Datura stramonium* . The extracts were poured into the wells at different concentrations including 50 mg/ml, 100 mg/ml, 200 mg/ml and 400 mg/ml. After incubation the zone of inhibition was carried out by agar plate method was observed. The concentration of 400 mg/ml showed the maximum zone of inhibition (18.8mm±0.2) where as the concentration of 50 mg/ml showed the minimum zone of inhibition (12.7mm±0.1) for isolated *Fusarium oxysporum*. Study has also been shown the presence of Bio active compound such as N-(2-Bromobutyryl)-4-chloro-benzenesulfonamide in the leaf of *Datura stramonium* which is responsible for antifungal activity and which can be used for seed treatment against seed borne fungal disease.

Introduction :

Rice (*Oryza sativa* L.) belongs to family Poaceae with two domesticated species of genus *Oryza*. *Oryza* sativa originates from tropical and subtropical southern Asia, while the African rice, *Oryza* glaberrima, originates from West Africa [1, 2]. The crop is one of the most important food crops in the world which forms the staple diet of about 2.7 billion people and has become a commodity of strategic significance across many African countries [3]. Among the various modes of transmission of plant diseases, seeds play an important role in the transmission of pathogens and development of plant diseases. The seed-borne pathogens may be externally or internally seed-borne, extra- or intra- embryonic or associated with the seeds [4]. Seed borne fungi cause losses in terms of seed quality and quantity in all oil seed crops. These fungi also reduce the germination and storability of the oil seed. They are responsible for seed rot, seedling blight, root/shoot rot, foliar infection as well as pod blight diseases [5, 6].

Phytochemical estimation of *Datura stramonium* :

The *Datura stamonium* plant contains 0.2-0.6% alkaloids. The main alkaloids are hyoscyamine and hyoscine (scopolamine). It also contains the protein albumin and atropine. Atropine is formed from hyoscyamine by racemisation. These alkaloids are usually present in the proportion of about two parts of hyoscyamine to one part of hyoscine, but in young plants hyoscine is the predominant alkaloid. Ditiglyol esters of 3,6-dihydroxytropane and 3,6,7-trihydroxytropane have been isolated from the roots in addition to hyoscine, hyoscyamine, tropine and pseudotropine. It also contains 6- hydroxyhyoscymine, skimmianine, metelodine, acetyl derivatives of caffeic, p-coumaric and ferulic acid, stigmasterol, campesterol, with anolide I, steroidal glycosides, daturataturins A and B, flavonoids, chrysins, quercetin and their esters. With a stramonolide and coumarins (umbelliferon and scopolin) are also present in the plant. The seeds contain oil, wax, resin, extractive, gummy matter, malic acid, some salts and a peculiar alkaloid which has been named Daturia [7, 8, 9, 10].

Isolation of seed borne mycoflora :

The identification of the fungi is done based on the morphological and colony characteristics of the pathogens by direct microscopy (Figure – 1), by which the mycoflora that were associated with field crop 'Rice' (Khas) can be identified.

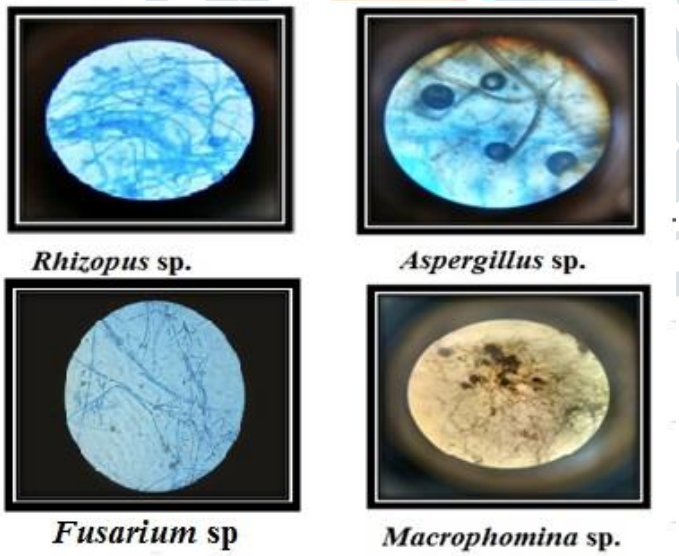


Figure no. -1

Percentage of Incidence of seed borne mycoflora :

Seeds which were collected from different locations were tested by two different methods methods – the Standard blotter method and the Agar plate method.Those were Standard blotter method and Agar plate method. The data obtained in standard blotter method (Table 1 ,Figure 2) and in Agar Plate method (Table 2,

Figure 1) indicated the association of Three fungal species viz., *Aspergillus* sp., , *Fusarium* sp., *Macrophomina* sp, Out of these Both of the Standard Blotter and Agar Plate method *Fusarium* sp. was found predominant(49.00

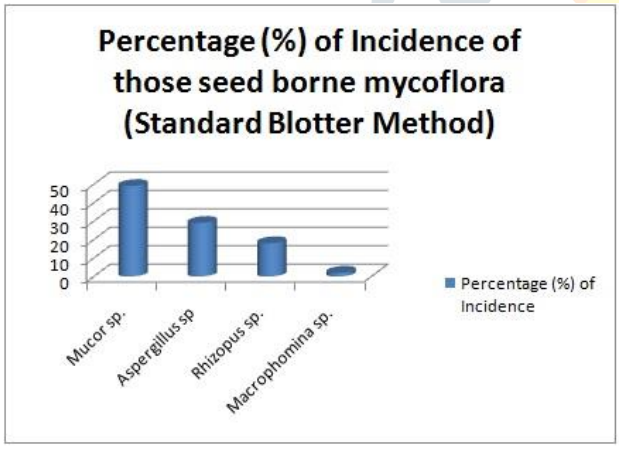
percent in Standard Blotter method and 38.00 percent in agar plate method) and total infection percentage varied from approx 97.0-99.00%.

Name of Seed borne mycoflora/ Fungi	Percentage of Incidence
***Mucor* sp.**	49.00
***Aspergillus* sp.**	29.00
***Rhizopus* sp.**	18.00
***Macrophomina* sp.**	02.00
TOTAL PERCENTAGE	98.00%

Table-1 : Percentage of Incidence of those seed borne mycoflora (Standard Blotter Method)

Name of Seed borne mycoflora/ Fungi	Percentage of Incidence
***Fusarium* sp.**	39.00
***Aspergillus* sp.**	27.00
***Rhizopus* sp.**	22.00
***Macrophomina* sp.**	9.00
TOTAL PERCENTAGE	97.00%

Table-2 : Percentage of Incidence of those seed borne mycoflora (Agar Plate Method)



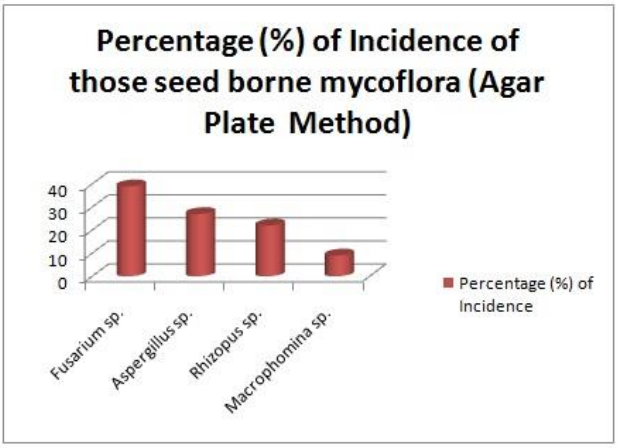


Figure no. - 2

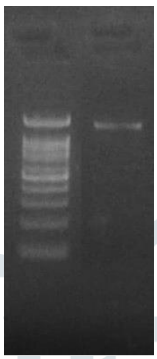
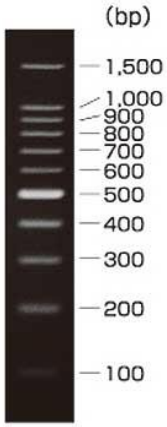
Identification by 18S rRNA sequencing methods :

As the percentage of incidence of *Fusarium* sp. is predominantly high in both the Standard Blotter and the Agar Plate method, we identified this fungus upto species level by 18S rRNA sequencing and Phylogenetic tree analysis technique.The results of screening given below-

1. Data Result

Sample *Fusarium* sp. which was labelled as Test_Seq, showed high similarity with ***Fusarium oxysporum strain EMT.*** based on nucleotide homology and phylogenetic analysis

2. gDNA and ITS Amplicon QC Data

gDNA	ITS PCR Amplicon	Ladder Specification
		

3. Sanger Sequencing Chromatogram Sequences

>Forward Seq data

CATTATATAAGTTATCGGTTATTTGATAGTACCTTAGTACTTGGATAACCGTGGGAATTCTATAGCTAATACATGCTAAAA
ATCCCGACTTCGGAAGGGAGGTATTTATTATATTAAAAACCATTGCCCTTCCGGGCTCACTGGTGATTCATGATAACTCCT
CGAATCCCATGGCCTTGTGCCGGCGATGGTTCATTCAAATTTCTTCCCTATCAACTTTCGATGTTAGGGTATTCGCCAATC
ATGCTTGCAACGGGTAACAGAGGGTTAGGGCTCGACCCCGAACAAGGAACCTGGAAAACGGCTACTACGTCCGAGGAAGGC
AGCCGGCTGCGCCAAATTCCCCACTCCGCGAAACTGGGGAGGTATGTAGACAACTAATATCCTGGATCCATGGGCTCTCTT
GGGTCTTGTATATTGGAAATAGAGTAACATATTCTAAATCCACTTAACAGATTGAAAAGGTGAGAGCGTGCAAGTCTGGGA
TGCCGCGCGAGCCGTCGGTAATTCCAGACTCGCAATATGCGAAATATGTAAAGTTTGTAT

4. Sequence Homology: Top Blast Hits

Table 3: Top 10 Blast Hits

Description	Max Score	Total Score	Query Coverage	E Value	Identity	Accession
Fusarium sp. MF395 18S ribosomal RNA gene, partial sequence	576	576	99%	6e-163	87.43%	KM096248.1
Fusarium oxysporum strain EMT 18S ribosomal RNA gene, partial sequence	572	572	99%	7e-162	87.43%	KT182633.1
Fusarium oxysporum strain FII-FF4 internal transcribed spacer 1, partial sequence	569	569	99%	9e-161	87.06%	JX967527.1
Fusarium oxysporum isolate M7SA48 18S ribosomal RNA gene, partial sequence	568	568	99%	3e-160	87.25%	MH051075.1
Fusarium oxysporum isolate M6SB2 18S ribosomal RNA gene, partial sequence	568	568	99%	3e-160	87.25%	MH051040.1
Fusarium oxysporum isolate C7SA7 18S	568	568	99%	3e-160	87.25%	MH050993.1

ribosomal RNA gene, partial sequence						
Fusarium oxysporum isolate M5SA98 18S ribosomal RNA gene, partial sequence	568	568	99%	3e-160	87.25%	MH050977.1
Fusarium solani strain FsP small subunit ribosomal RNA gene, partial sequence	568	568	99%	3e-160	87.25%	MK027283.1
Fusarium solani strain FsQ small subunit ribosomal RNA gene, partial sequence	568	568	99%	3e-160	87.25%	MK027282.1
Fusarium solani strain FsF small subunit ribosomal RNA gene, partial sequence	568	568	99%	3e-160	87.25%	MK027273.1

5. Phylogenetic Analysis

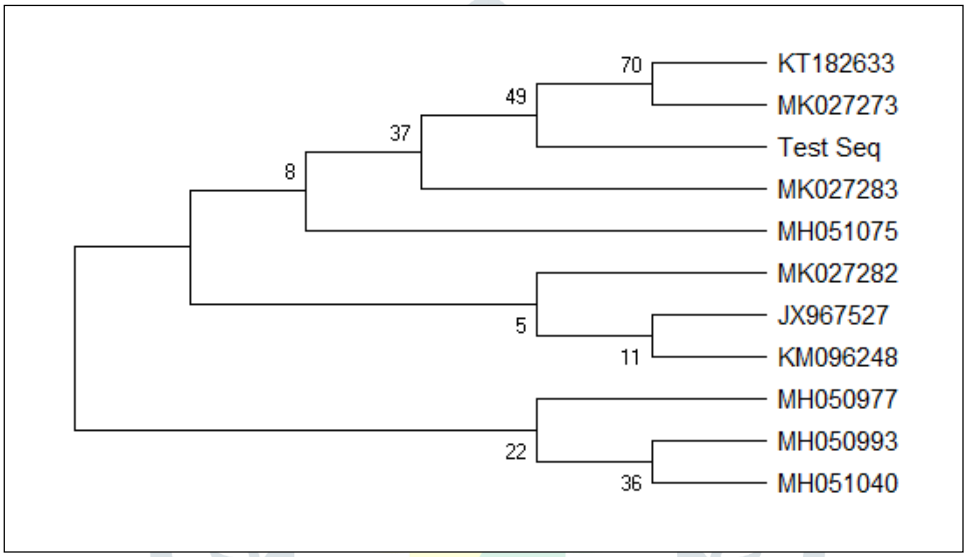


Figure 3: Phylogenetic Tree

6. Distance Matrix Analysis

Test_Seq		0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
JX967527	0.114		0.001	0.002	0.007	0.001	0.005	0.002	0.003	0.003	0.002
KM096248	0.114	0.003		0.004	0.008	0.002	0.005	0.003	0.004	0.004	0.003
KT182633	0.107	0.003	0.009		0.001	0.001	0.001	0.004	0.003	0.003	0.004
MK027273	0.114	0.073	0.086	0.001		0.006	0.006	0.004	0.004	0.004	0.004
MK027282	0.114	0.001	0.005	0.001	0.071		0.004	0.002	0.003	0.003	0.003
MK027283	0.114	0.033	0.041	0.001	0.063	0.033		0.003	0.004	0.004	0.003
MH050977	0.114	0.004	0.009	0.010	0.016	0.006	0.011		0.003	0.003	0.003
MH050993	0.114	0.008	0.013	0.008	0.017	0.010	0.012	0.007		0.002	0.003
MH051040	0.114	0.009	0.014	0.007	0.021	0.011	0.015	0.010	0.007		0.004
MH051075	0.114	0.005	0.009	0.013	0.014	0.006	0.008	0.012	0.012	0.013	

The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Kimura 2-parameter model [11]. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1771 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [12].

Evaluation of Antifungal activity of *Datura stramonium* :

As the percentage of incidence of *Fusarium oxysporum* is high among other mycoflora, Various concentrations (mg/ml) of plant extracts of *Datura stramonium* were used for seed treatment of seeds infected by *Fusarium oxysporum* by the soaking methods (Table 4, Figure 4). Extract (in ethyl acetate, chloroform and benzene) of botanicals were applied at four concentrations, 50 mg/ml, 100mg/ml, 200 mg/ml and 400 mg/ml.

Table-4 : Antifungal activity of different solvent extracts of leaf of *Datura stramonium*

Extracts	Concentrations (mg/ml)	Zone of Inhibition (mm) observed in *Fusarium oxysporum*
Ethyl Acetate	400	18.8 ± 0.2
	200	17.2 ± 0.1
	100	16.6± 0.2
	50	12.7 ± 0.1
Chloroform	400	17.8 ± 0.1
	200	15.4 ± 0.1
	100	13.7± 0.2
	50	12.5 ± 0.1
Benzene	400	15.0± 0.2
	200	13.6 ± 0.1
	100	11.3± 0.2
	50	10.7 ± 0.1

(Means with standard deviation)

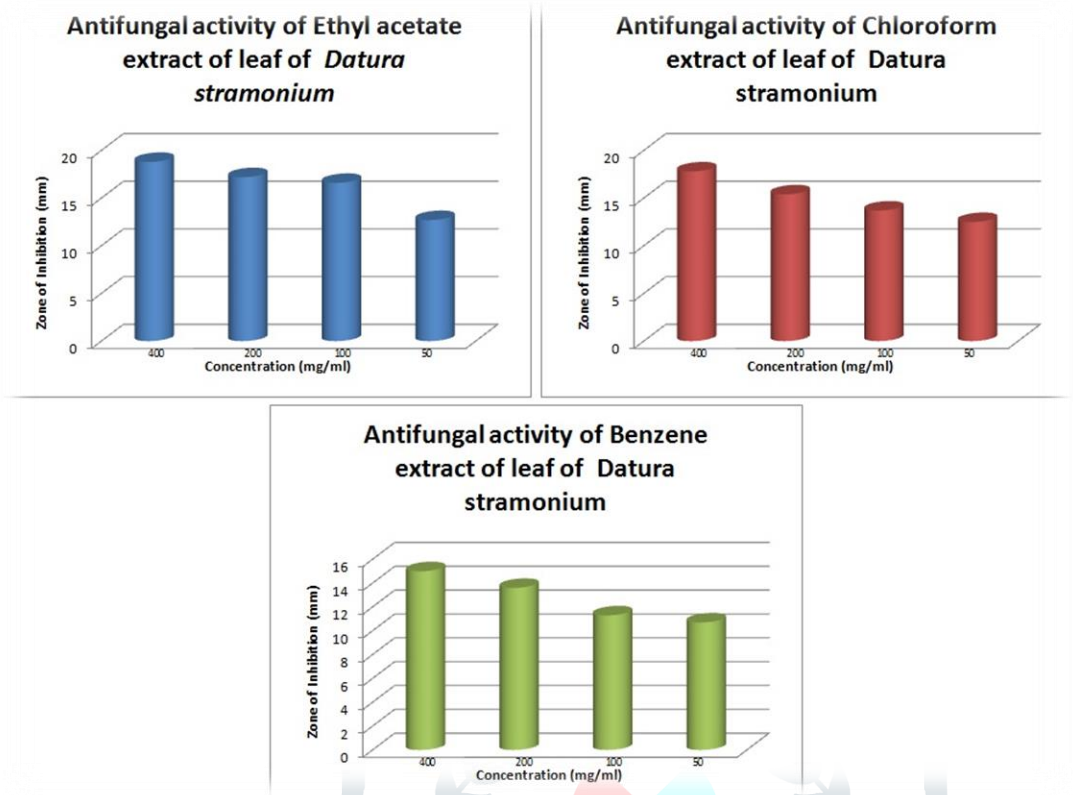


Figure no. 4

Estimation of Bio-active compounds suspected for antifungal activity of plant extract by Gas Chromatography- Mass spectrometry :

Free radicals play a crucial role in the development of tissue damage in pathological events. The extraction method presented is simple, rapid and inexpensive, with reduced solvent consumption. GC-MS method used for the analysis of the obtained extracts can be an interesting tool for testing the amount of some active principles in herbs used in cosmetic, drugs, pharmaceutical or food industry. Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.The Gas chromatogram (GC) of Ethyl acetate extract of *Datura stramonium* showing the GC-MS profile of the compounds identified is given in the Figure 5. The peaks in the chromatogram were integrated and were compared with the database of spectrum of known components stored in the GC-MS library. The detailed tabulations of GC-MS analysis of the extracts are given in Table 5 separately. The peak is found at RT 11.253 min with a peak area of 100%. It shows typically N-(2-Bromobutyryl)-4-chloro-benzenesulfonamide, along with suspected 2-bromo-4-fluorophenyl-4-Nitrobenzoic acid, 5-fluoro-2-nitrophenyl-3-Bromobenzoic acid, O-Ethyl-O-(1,1-difluoro-2-bromoethyl)-N-(1-methylpropyl)-

phosphorothioamidate. This compound is a Alkaloid with a molecular formula is $C_{10}H_{11}BrClNO_3S$ and molecular weight is 338.9 (Figure- 5). Phytochemical analysis by GCMS analysis of the plant extract revealed the presence of different bio-active compound .

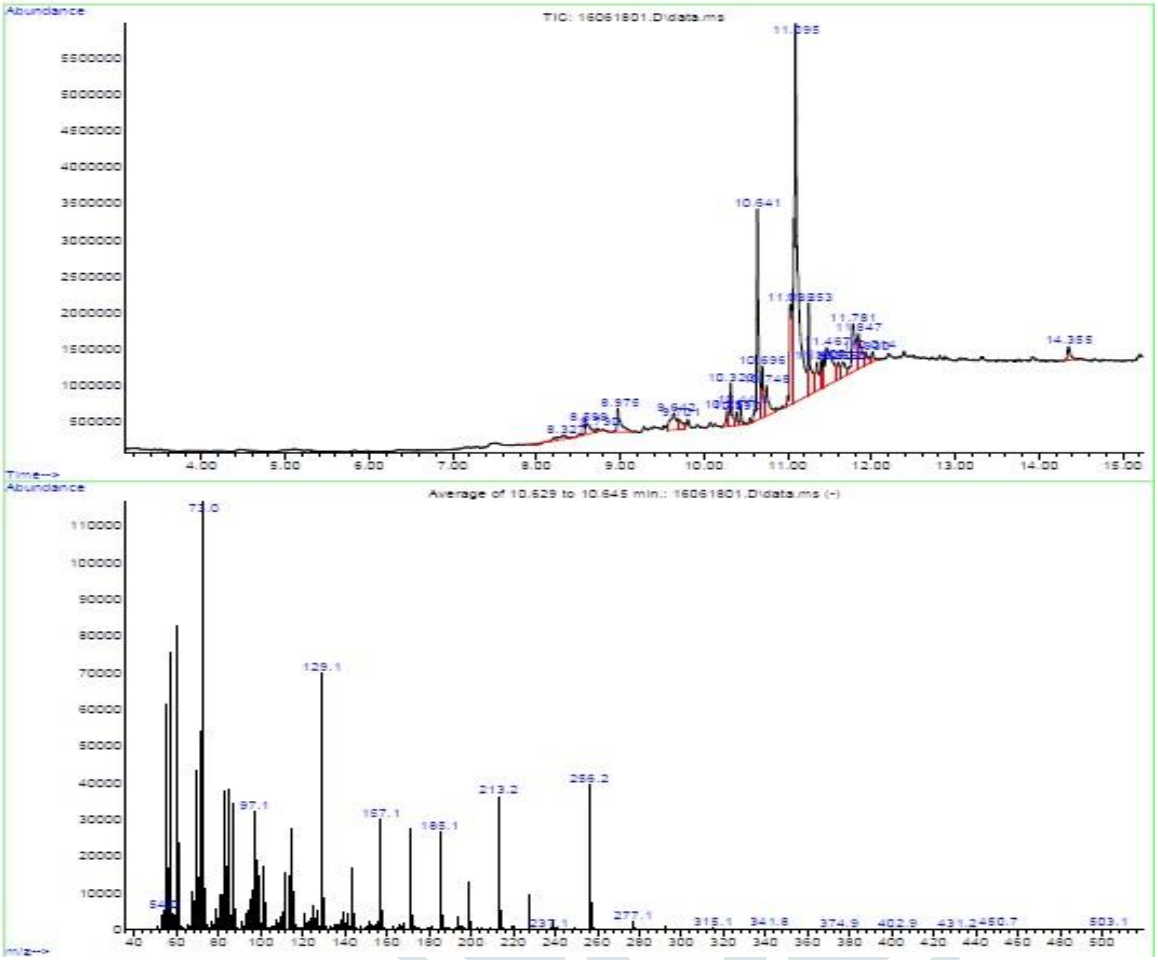
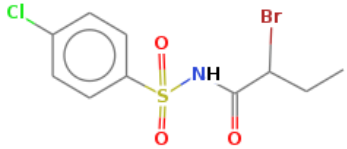


Figure no. 5 : GC-MS Chromatogram

RETENTION TIME	MOLECULAR WEIGHT	COMPOUND	COMPOUND NAME	STRUCTURE
AVERAGE OF 11.241 To 11.258 min 16061801.D/data.ms(-)	338.9	• N-(2-Bromobutyryl)-4-chloro-benzenesulfonamide	N/A	

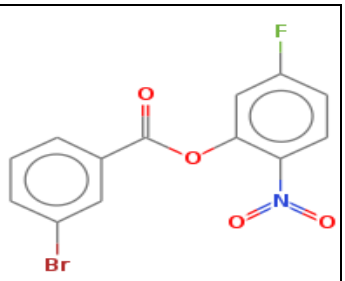
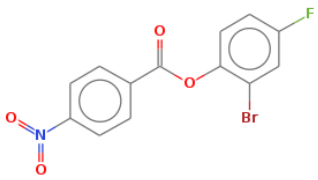
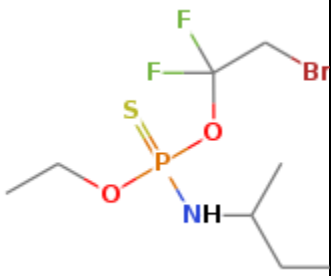
AND PEAK AREA (%)=11.253		• 3-Bromobenzoic acid, 5-fluoro-2-nitrophenyl ester	N/A	
		• 4-Nitrobenzoic acid, 2-bromo-4-fluorophenyl ester		
		• O-Ethyl-O-(1,1-difluoro-2-bromoethyl)-N-(1-methylpropyl)-phosphorothioamidate	O-Ethyl-O-(2-bromo-1,1-difluoroethyl)-N-(1-methylpropyl)phosphonothioamidate	

Table 5 : GCMS analysis of Compound by its molecular weight of Ethyl acetate extract of *Datura stramonium*

Conclusion:

Based on the findings, it may be concluded that antifungal activity is both solvent and pathogen dependent. The ethyl acetate extract showed dose dependent antifungal activity. The results of this study are very encouraging, however, further study on chemical constituents and their mechanisms in exhibiting certain biological activities are needed to understand the complex pharmacological effects of the plant species. The plant is active against *Fusarium oxysporum* , a seed borne mycoflora. The present GC-MS results of *Datura stramonium* indicated the presence of some bio-molecules which have important medicinal activities which correspond well with the reports of the medicinal activities of this plant. Further work is required to confirm the efficacy of this plant. Therefore, it could be used as an alternate source for the treatment of seed borne diseases caused by those pathogens.

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