MICROBIAL SCREENING AND KINETICS OF METABOLISM OF CLOBAZAM

B.Thrinitha, Gufran Mohammed Abdul Rahman, Hafeeza Begum, Hafsa Fatima, Humera Begum, Kausar Fatima*

Department Of Pharmacology, Bojjam Narasimhulu College of Pharmacy, Saidabad, Vinayak Nagar Colony, Hyderabad, Telangana 500059.

ABSTRACT: ***The commenced research was proposed to produce known active/novel metabolites by microbial biotransformation easily and economically and also to develop microbial models for CYP enzymes for drug metabolism studies. This study was also aimed at the anticipation of the nature of the enzyme involved in fungal biotransformation and the affinity of the fungal enzyme towards the substrate. The present study revealed that all three fungi can be used as models for CYP3A4 enzyme to study pharmacological and toxicological properties and the biotransformation of other similar drugs. Enzyme kinetic studies revealed that metabolism mediated by enzymes present in Aspergillus ochreous possessed low Km value than enzymes present in Aspergillus fumigatus and Aspergillus niger. So the enzymes present in Aspergillus ochreous have more affinity towards Clobazam when compared with enzymes in Aspergillus fumigatus and Aspergillus niger. So maximum metabolite can be produced by Aspergillus ochreous with less quantity of Clobazam.***

Keywords: Clobazam, Aspergillus fumigatus, Aspergillus niger, Cytochrome

I. INTRODUCTION

Clobazam (CLB), is a benzodiazepine antiepileptic drug that is effective in many types of refractory epilepsy and has been in use since 1984 [**1**,**2**,**3**]. Its 1,5-benzodiazepine structure, as opposed to that of the traditional 1,4 benzodiazepines, confers its unique characteristics. It preferentially binds to the α2 subunit of GABA-A receptors over α1, rendering CLB less sedating and more suitable for chronic administration than other benzodiazepines [**1**,**2**,**3**]. Although therapeutic drug monitoring (TDM) of CLB is not routine, there is growing evidence that TDM may be of value in conditions where pharmacokinetic alterations are suspected, such as in the case of drug-drug interactions [**4**], liver disease [**5**], and extremes of age [**6**]. CLB is mainly metabolized by the liver with negligible renal elimination. N-desmethylclobazam (NDMCLB) is an active metabolite resulting from the N-demethylation of CLB (**Figure 1**). It has a longer half-life than the parent drug and is believed to contribute to the pharmacological effects of CLB [**7**]. Therefore, determination of the concentrations of both the parent drug and its metabolite is essential for TDM. The suggested reference ranges for CLB and NDMCLB are 30–300 ng/mL and 300–3000 ng/mL, respectively [**7**]. Several analytical procedures have been reported to determine CLB and NDMCLB concentrations in biological samples, utilizing various techniques including gas-liquid chromatography (GLC) [**8**,**9**,**10**], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [**11**,**12**,**13**], and high-performance liquid chromatography (HPLC) [**14**,**15**,**16**,**17**,**18**,**19**,**20**]. Herein, we present a rapid, reproducible, simple, and sensitive HPLC-UV method for quantifying CLB and NDMCLB in human plasma. The method utilizes a single liquid-liquid extraction step, small volumes of samples (0.3 mL as opposed to 1 mL in most methods) [**17**,**18**,**21**,**22**,**23**,**24**], and a simple mobile phase mixture (acetonitrile/water as opposed to using buffers) [**20**]. In addition, the method quantifies CLB and NDMCLB over the suggested reference range in patients with epilepsy and is thus practical for TDM purposes.

Herein, we present a rapid, reproducible, simple, and sensitive HPLC, MS, and H^1NMR method for quantifying CLB metabolites by microbial biotransformation. The method utilizes a single liquid-liquid extraction step, small volumes of samples (25 **µl**), and a simple mobile phase mixture (Acetonitrile: water (0.6:1)).

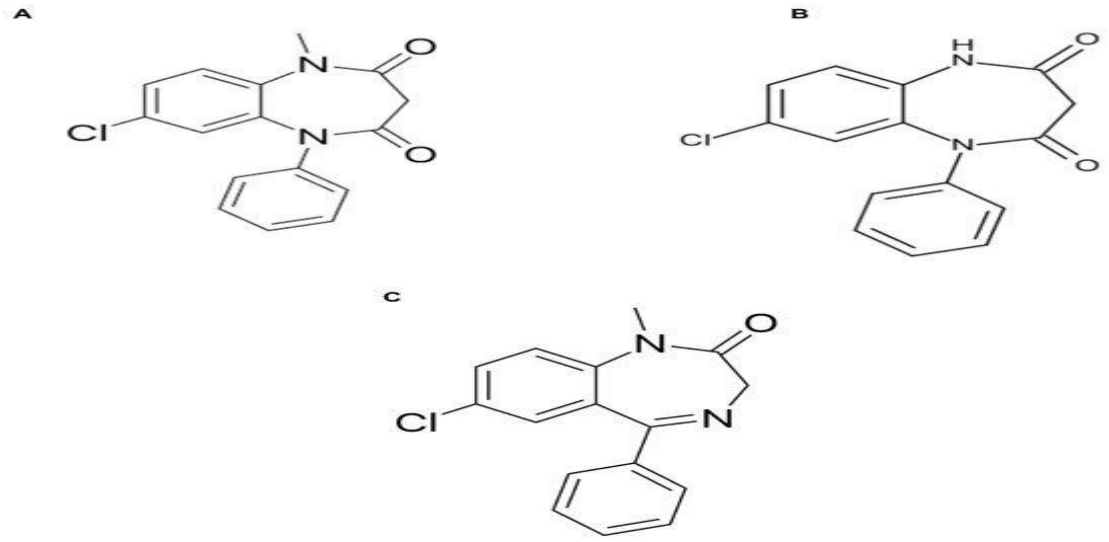


Fig. 1. Chemical structures of (A) clobazam (CLB); (B) the metabolite N-desmethylclobazam (NDMCLB); and (C) the internal standard used (diazepam).

II. MATERIALS AND METHODS

2.1 Microorganisms: *Aspergillus fumigates, Aspergillus niger, Aspergillus ochreous, Cunninghamella elegans, Cunninghamella echinate,* and *Cunnighamella blackesleeana.*

2.2 Chemicals and reagents: Dextrose, Yeast extract, and Agar-agar (SD fine chemicals Ltd, Mumbai, India); Methanol, Acetonitrile, Dichloromethane and Diethyl ether (Merck, Mumbai, India) and Clobazam (Lake chemicals, Bangalore as gift sample)

2.3 Media preparation

As the selected six cultures were fungi (*Aspergillus fumigatus, Aspergillus niger, Aspergillus ochreous, Cunnighamella echinate, Cunnighamella elegans, Cunnighamella blackesleena)*, potato dextrose broth (PDB) was used as a medium, which was suitable for fungal growth [25].

2.4 Stock culture maintenance

The viability of pure cultures was maintained by subculturing, in which pure cultures were transferred into respective fresh agar slants every six months and stored at 4°C **[**26]. For each organism, the fungal media (PDB) including 2% agar was prepared by dissolving in distilled water, and pH was adjusted to 3.5. The slants were prepared by transferring the medium into test tubes. The prepared slants were sterilized by autoclaving at 121 °C, 15 psi for 20 min. [27]. Then, the respective fungal cultures were aseptically inoculated on the slants and were incubated in an incubator for 48 -72 hrs. These stock cultures were stored in a refrigerator for further use.

2.5 Sterilization method

Sterilization of media and other materials was done in an autoclave. The containers meant for sterilization were kept in an autoclave and sterilized at 121°C, for 20 min.

2.6 Preparation of drug stock solutions

The drug stock solution was prepared by dissolving 10 mg of drug in 10 ml of methanol. Three drugs (Loratadine, Clobazam, and Chlordiazepoxide) were dissolved in methanol separately for the biotransformation study of each drug.

2.7 Fungal biotransformation-incubation protocol

Three different flasks were set for each culture for the drug as Blank I [Drug control, Blank II [Culture control]. The prepared broth medium of 50 ml was transferred to 250 ml. Erlenmeyer flasks were sterilized in an autoclave at 121°C for 15 min. and the pH of the broth was adjusted to 3.5. Then, 0.5 ml. of drug solution and respective fungal culture were aseptically added according to the protocol [28].

Blank I contained 50 ml of sterile medium to which drug solution was added and incubated without fungus. Blank II (culture control) contained 50 ml of sterile medium to which fungal culture was inoculated and incubated without substrate. Sample flasks contained a sterile medium to which both substrate and fungus were added. Blank I and Blank II were maintained to eliminate the peaks found in HPLC due to intervention between broth and drug or broth and fungus.

The drug stock solution was used as a standard in all studies to affirm the retention time of the drugs during HPLC analysis.

2.8 Fermentation

The flasks as specified in table no- 14 for all drugs and cultures were incubated using an orbital shaker incubator [CIS 24, Remi instruments, Mumbai] for 72 hrs, operated at 120 pm, at 28°C for screening studies. All the flasks Blank I, Blank II, and Sample were maintained under identical conditions to acquire the prominent growth of fungi in respective flasks. Then, the drug and metabolites were extracted and further analysed by HPLC.

2.9 Induction and inhibition studies

After confirmation of metabolites formed in screening studies of fungal biotransformation, enzyme inhibition and induction studies were conducted with a fungus which has shown the formation of metabolite in HPLC analysis for each substrate by following incubation protocol. Stock solutions of substrate, inducer, and inhibitor were prepared by dissolving 10mg of each drug in 10 ml of methanol in respective volumetric flasks. 0.5 ml. of the substrate solution, inhibitor solution, inducer solution, and subcultures were aseptically added to respective sterile flasks to conduct metabolism inhibition and induction studies.

Induction and inhibition studies contained six different flasks, 5 control flasks, and one sample flask. Blank I was assigned as substrate control, composed of a sterile medium to which only substrate was added and incubated without culture. Blank II was assigned as culture control, composed of broth in which the fungi were inoculated but without substrate, inhibitor, or inducer. Blank III was inhibitor or inducer control, consisting of a sterile medium to which inhibitor or inducer was added and incubated without culture and substrate. Blank IV was substrate and inhibitor or inducer control, composed of sterile medium to which substrate and inhibitor or inducer were added and incubated without culture. Blank V was composed of a sterile medium to which culture, inhibitor, or inducer were added without substrate. The sample was composed of a sterile medium to which substrate along with inhibitor or inducer was added and incubated with culture. During inhibition or induction studies, blank flasks were prepared for each substrate to avoid the interference of extra peaks formed due to interaction between substrate and inhibitor or inducer and transformation action of microbes on inhibitor or inducer alone. The flasks as shown in Table 13 were incubated in the orbital shaker incubator [CIS 24, Remi instruments, Mumbai] for 72 hrs, operated at 120 pm, at 28°C for this study. The percentage of metabolites formed in inhibition and induction studies was calculated from the peak area of the drug and metabolite obtained in HPLC analysis.

2.10 Enzyme Kinetic Studies [29]

These studies were performed in extension to the induction and inhibition studies for all the three substrates, to examine the affinity of the respective fungal enzyme to the given substrate involved in the fungal biotransformation. The selected fungus found to form metabolite in the above studies was inoculated in labeled sample flasks containing different concentrations of the substrate like 10µg/ml, 20µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, and 60 µg/ml, and incubated under similar conditions specified in above studies. But, 10 ml of sample was aseptically collected from each flask at different incubation time intervals of 24,36,48,60, and 72 hrs to extract the metabolite and calculate the percentage of metabolite formed at each concentration of substrate and incubation time point.

2.11 Extraction Method

All incubated flaks or samples collected after a predetermined incubation period in all the above studies (biotransformation, induction, inhibition, and kinetic studies) were heated in a water bath at 50°C for 30 minutes to inactivate the fungi. Then contents of all flasks were centrifuged at 3000 rpm for 10 min. using centrifuge tubes. (Laboratory Centrifuge C-854/8, Remi instruments, Mumbai, India). The supernatant was collected from all tubes and was stored in the refrigerator. The extraction of the drug and its metabolites was conducted by withdrawing 8 ml. of supernatant collected from each of the flasks of the study and 10ml. of suitable organic solvent as shown in table no-14. Then, it was vortexed for 10 min. in cyclomixer. The organic layer was collected in screw cap bottles and kept for air drying. The dried samples were analyzed by HPLC after reconstitution with mobile phase, besides pure drug was also analyzed by HPLC to set it as a standard.

2.12 Chromatographic Conditions

Compounds were eluted by using an isocratic mixture of mobile phase (water–acetonitrile, 0.6:1, v/v) and pumped at 1 mL/min. C18 Phenomenex Luna column, All the drugs were detected at a wavelength of 230 nm. The run time was 30 min. The injection volume was 25 µl, at a temperature of 30 °C and a UV detector was used.

20 µl. of the reconstituted samples i.e., blank I [drug control], blank II [culture control], and a sample of each culture were spiked into column after spiking control (pure drug solution) to identify the retention time of the drug. Run time was fixed based on the retention time of the drugs.

The supernatant of all samples for inhibition or induction studies such as blank I [drug control], blank II [culture control], blank III [inhibitor or inducer control], blank IV [substrate + Inhibitor or inducer], blank V [inhibitor or inducer + culture] and sample [substrate + inhibitor or inducer + culture] were spiked into column after spiking

control (pure drug solution).
The reconstituted samples collected after enzyme kinetics studies were also analyzed in HPLC under the same analytical conditions.

2.13 Mass spectrometry

The metabolite identified in samples of fungi screened during HPLC analysis were isolated and further analyzed through Mass spectrometry to confirm its molar mass. The mass spectrometer (Agilent Technologies, Germany) model was API 3000MS operated in the electron spray ionization (ESI) mode. Ionization was carried out in positron mode using an ion trap detector (3.5 kV, 325°C, 210 psi).

2.14 Proton Nuclear Magnetic Resonance spectroscopy

The metabolite isolated from HPLC (as in Mass spectrometric analysis) was analyzed by 1H NMR spectroscopy using BRUKER AVANCE 400 MHz NMR spectrometer to confirm its structure. Deuterated methanol was used as a solvent to analyze drugs and their metabolites by 1H NMR analysis.

2.15 Quantification of metabolite

The peak area of metabolite acquired by HPLC from samples collected during induction, inhibition, and kinetic studies was used to quantify the metabolite formed in terms of percentage about the peak area of the drug in their respective studies.

2.16 Analysis of kinetic data

The data of percentage metabolite formed during kinetic studies were fitted in MS EXCEL by plotting a graph between time and percentage metabolite formed at different concentrations of the substrate to get the velocity of biotransformation reaction from the slope of the graph.
Then, the velocity obtained for each concentration by the above method was fitted into the Michaelis–Menten equation using nonlinear regression in GraphPad Prism 5.0 [30]. Best-fit values for *K*m and Vmax were reported to assess the possible extent of affinity of the fungal enzyme with the drug for a better understanding of fungal biotransformation of three selected drugs.

III. RESULTS

3.1 Screening studies

i. High-Performance Liquid Chromatography

Six different fungi were screened to investigate the biotransformation of Clobazam. The results of HPLC analysis of Clobazam and its metabolites in different fungal extracts are shown in figures 8-14 and data is given in table no-1. No drug peak was observed in chromatograms of the blank II (culture controls). Blank I (Drug controls) displayed the presence of a drug peak at a retention time of 6.3 min. The peak at a retention time of 1.8 min and 1.9 min. depicted solvent peaks. HPLC chromatograms of Clobazam incubated with *Aspergillus fumigatus, Aspergillus niger and Aspergillus ochreous* cultures have shown extra peaks at (CM1) 3.5 min., (CM2) 3.8 min. and (CM3) 2.6 min. respectively when compared with their controls as represented in figure 2, 3, 4, 5, 6, and 7 and table no 1. In the samples of other cultures, no extra peaks were observed when compared with their controls. Because these three cultures only metabolized Clobazam, the elutes of the extra peaks were collected from HPLC and further analyzed by both Mass and 1H NMR spectroscopy to confirm the structure of metabolites and to propose its metabolic pathway in fungi.

Table 1: The results of HPLC analysis of Loratadine and its metabolites in different culture extracts.

Name of the organism	Retention time (min.)			
	Blank I (Drug control)	**Blank II (Culture control)**	**Standard**	**Sample**
***Cunnighamella elegans* (NCIM-689)**	1.8	1.8	-	1.8
	6.3	-	6.3	6.3
***Cunnighamella echinate* (NCIM-687)**	1.8	1.8	-	1.8
	6.3	-	6.3	6.3
Cunnighamella blackesleeana (NCIM-691)	1.8	1.8	-	1.8
	6.3	-	6.3	6.3
***Aspergillus niger* (NCIM-589)**	1.9	1.9	-	1.9
				3.5*(CM_1)
	6.3	-	6.3	6.3
Aspergillus fumigatus (NCIM-902)	1.9	1.9	-	1.9
				3.8*(CM_2)

	6.3	-	6.3	6.3
Aspergillus ochreus (NCIM-1140)	1.8	1.8	-	1.8
				3.8*(CM_3)
	6.3	-	6.3	6.3

"*" – Metabolite peak

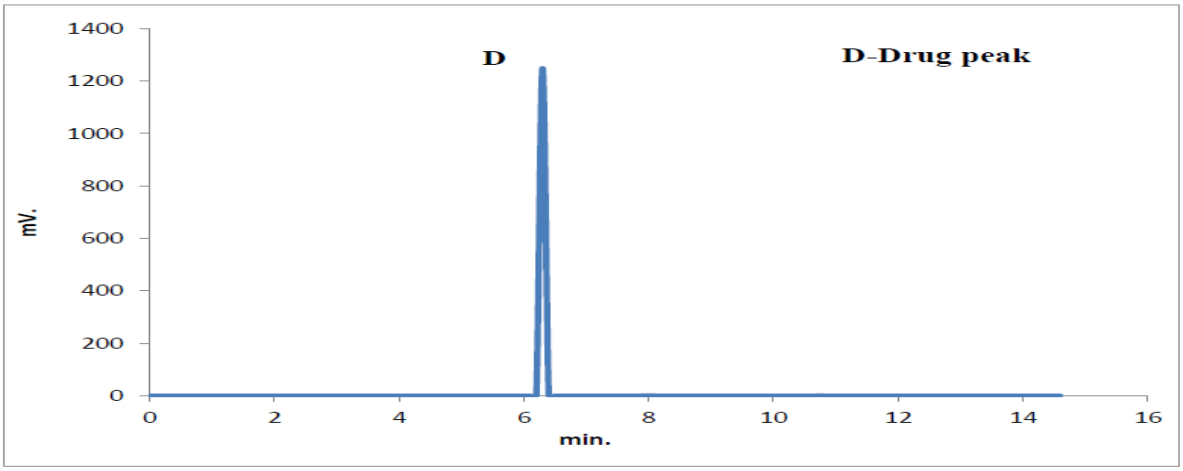


Fig.2. HPLC chromatogram of Clobazam.

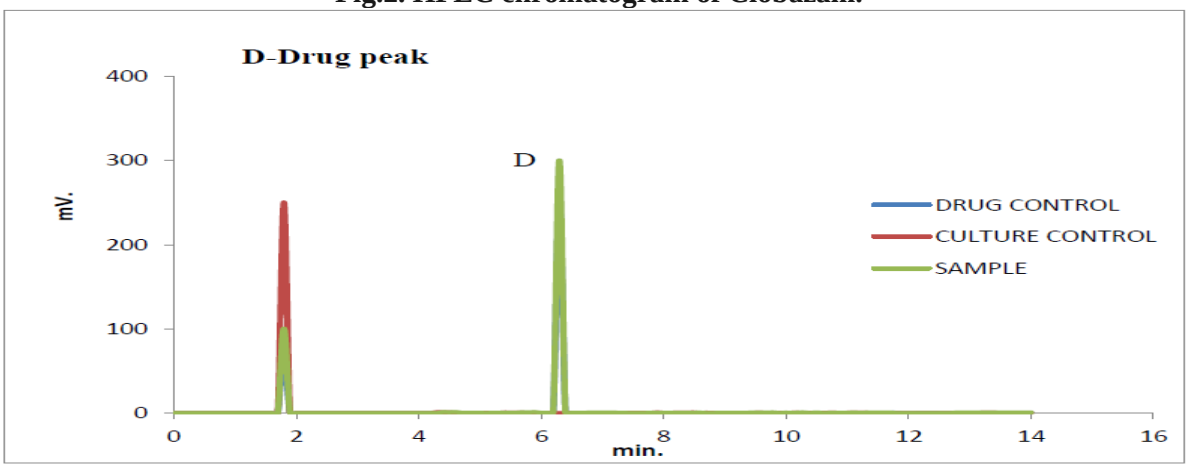


Fig.3. HPLC chromatogram of Clobazam from culture extracts of *Cunnighamella elegans*

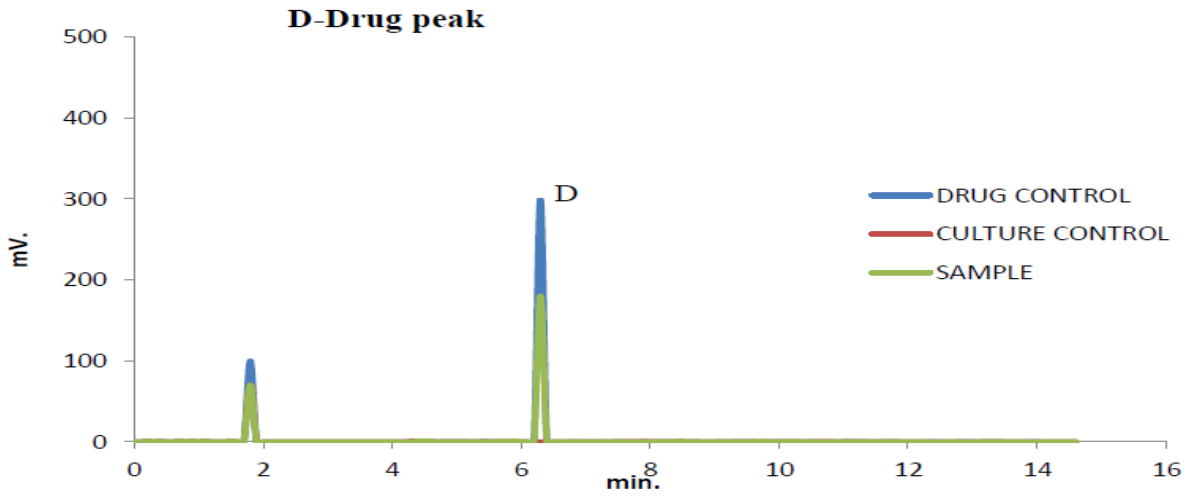


Fig.4. HPLC chromatogram of Clobazam from culture extracts of *Cunnighamella echinate.*

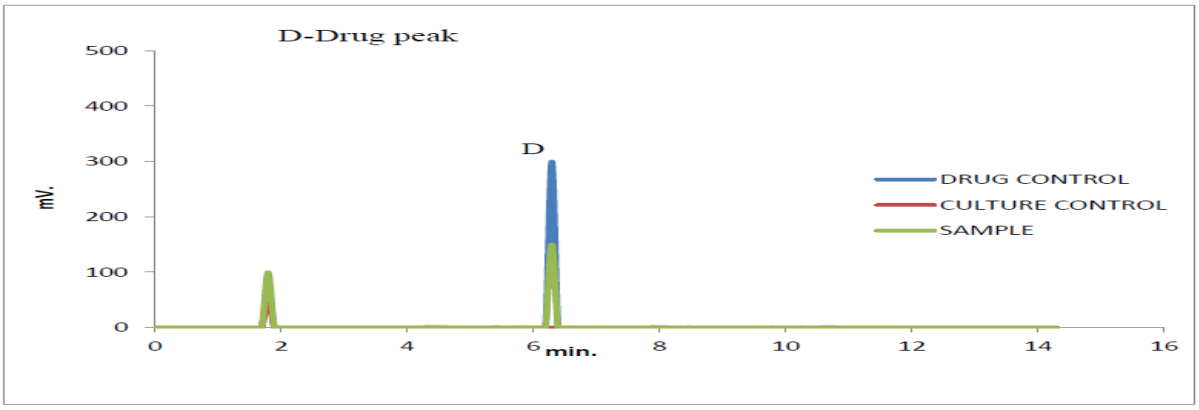


Fig.5. HPLC chromatogram of Clobazam from culture extracts of *Cunnighamella blackesleena.*

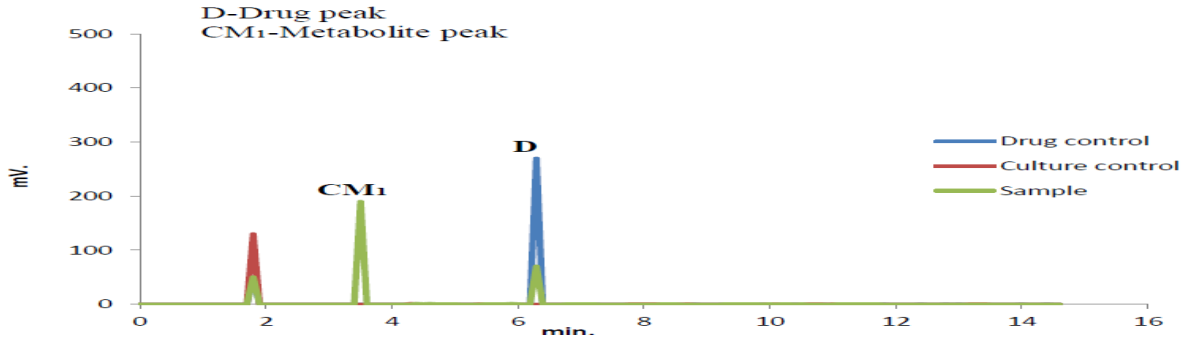


Fig.6. HPLC chromatogram of Clobazam from culture extracts of *Aspergillus fumigatus.*

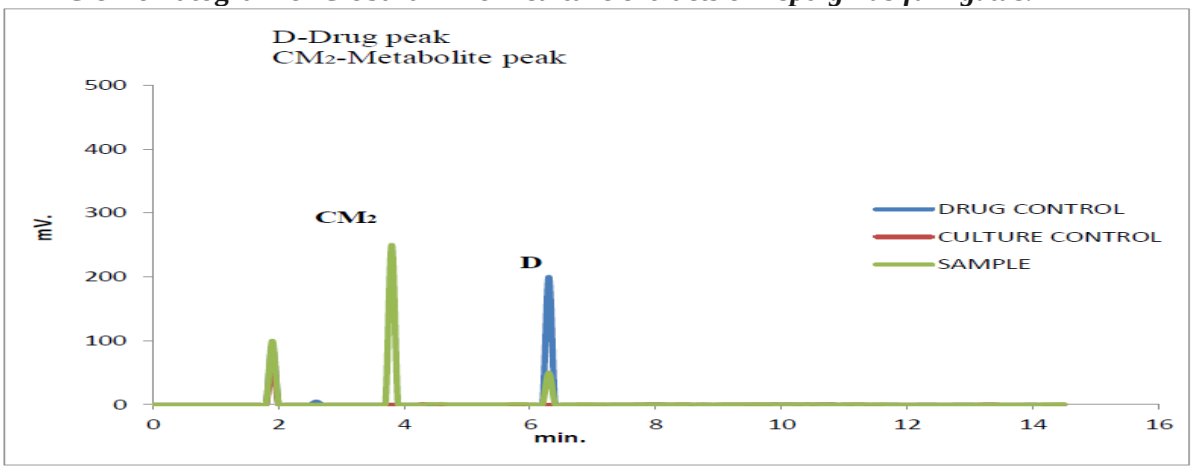


Fig.7. HPLC chromatogram of Clobazam from culture extracts of *Aspergillus niger.*

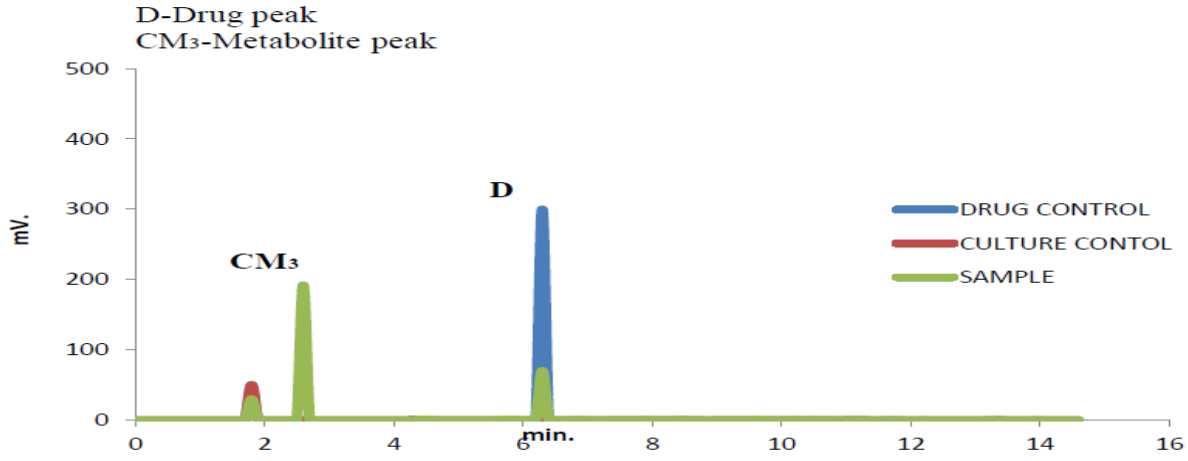


Fig.8. HPLC chromatogram of Clobazam from culture extracts of *Aspergillus ochreous.*

ii. Mass spectrometry

Mass spectrum of Clobazam and metabolites and fragmentation patterns are shown in figures 8-14. The mass spectrum of Clobazam was compared with mass spectra of metabolites (CM1, CM2, and CM3). The mass spectrum of the drug has shown a molecular ion peak at m/z 301(M+1) which is equal to the molecular weight of Clobazam as in figure 8.

Metabolite-CM1

The mass spectrum of CM1, a metabolite produced by *Aspergillus fumigatus* has shown a molecular ion peak at m/z 303.75[M+1] that is equal to the molecular weight of 4-hydroxy or clobazam as shown in figure 9. The structure of metabolite, CM1 is also supported by its mass fragments at m/z 268, 275, and 285 as per the mass fragmentation pattern shown in figure 10.

Metabolite- CM2

The mass spectrum of CM2, a metabolite produced by *Aspergillus niger*, has shown a molecular ion peak at m/z 319.74 [M+1], which is equal to the molecular weight of 3,4-dihydroxy or clobazam as shown in figure 11, and fragments at m/z 283, 284, 291 and 301 were also supported the structure of Clobazam metabolite, CM2 as shown in figure 12.

Metabolite-CM3

The mass spectrum of CM3, a metabolite produced by *Aspergillus ochreous*, has shown a molecular ion peak at m/z 333.75 [M+1] which is equal to the molar mass of 3-methoxy 4-hydroxy or clobazam in the mass spectrum shown in figure 13. The structure of metabolite, CM3 is supported by mass fragments at m/z 283, 298, 301, and 315 as per the mass fragmentation pattern shown in figure 14.

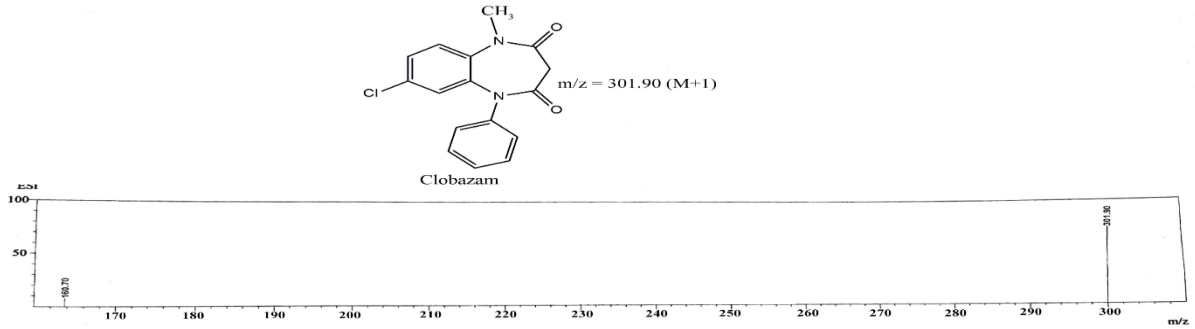


Fig.9. Mass spectrum of Clobazam.

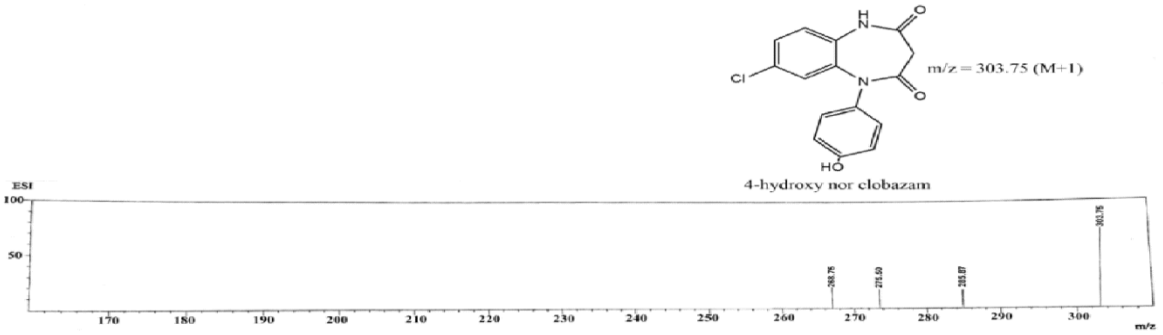


Fig.10. Mass spectrum of Clobazam metabolite, CM1

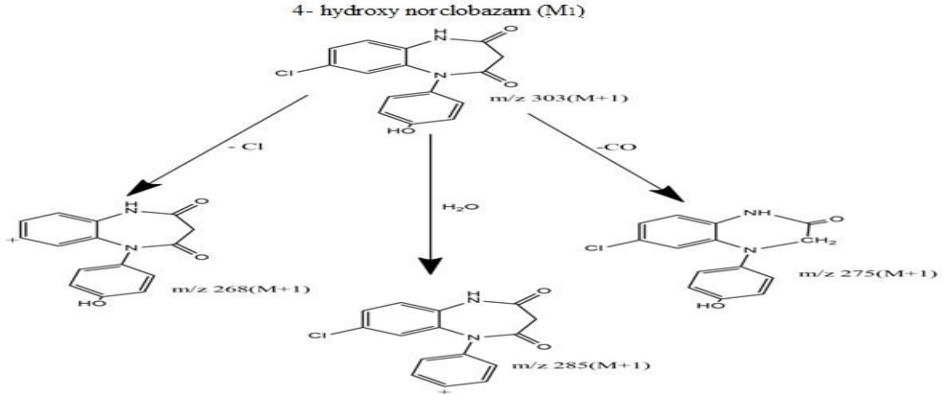


Fig.11. Mass fragmentation pattern of Clobazam metabolite, CM1

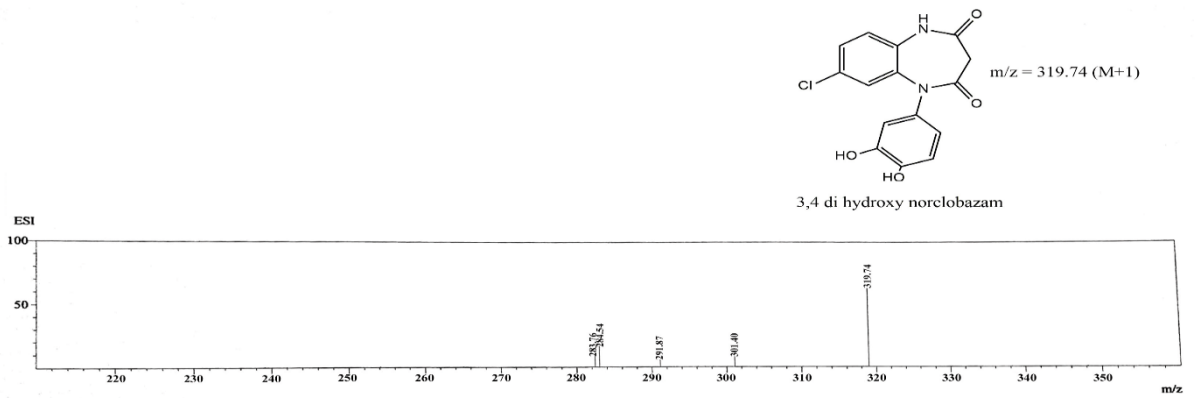


Fig.12. Mass spectrum of Clobazam metabolite, CM2

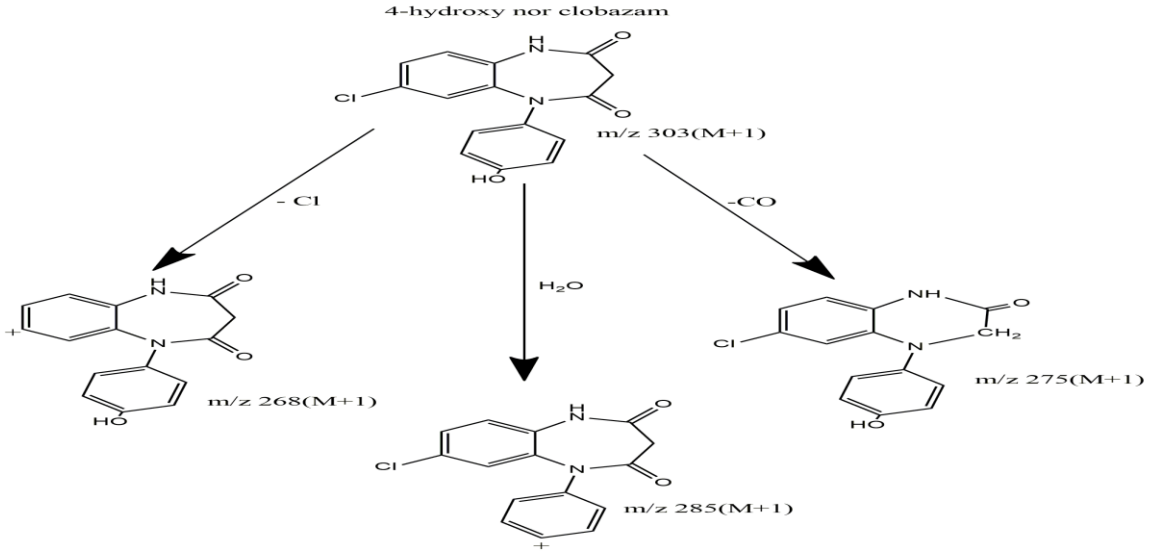


Fig.13. Mass fragmentation pattern of Clobazam metabolite, CM2.

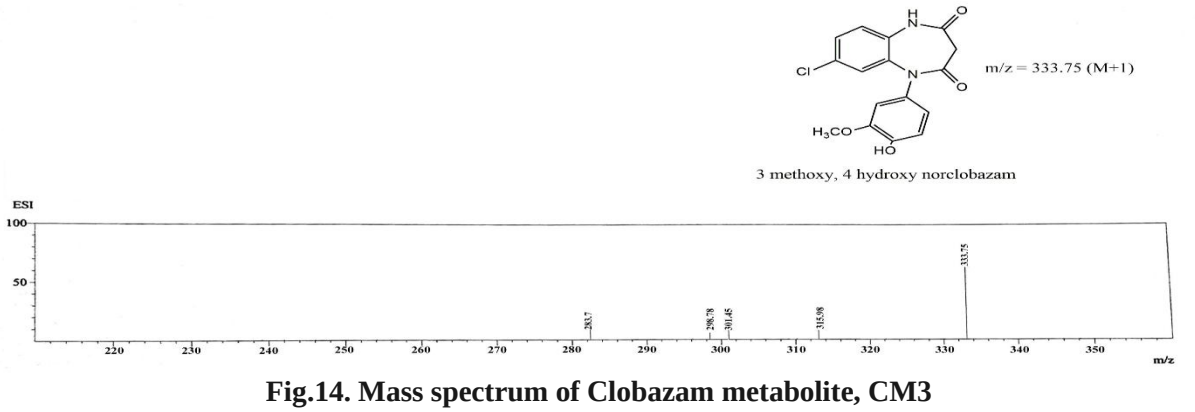


Fig.14. Mass spectrum of Clobazam metabolite, CM3

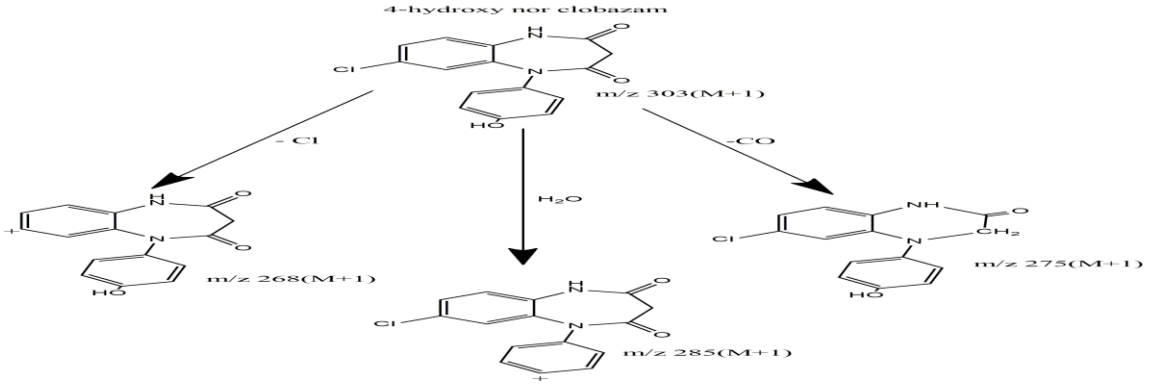


Fig.15. Mass fragmentation pattern of Clobazam metabolite, CM3.

iii. ^{1}HNMR Spectroscopy

The structures of CM1, CM2, and CM3 were further confirmed by 1H NMR spectroscopy. ^{1}H NMR spectrum of Clobazam shown in figure 15 was used to compare with ^{1}H NMR spectra of metabolites.

Metabolite-CM1

4-hydroxy or clobazam, the structure of CM1 was confirmed by the appearance of a peak at δ 4.91 in its 1HNMR spectrum (figure 16) indicated the addition of hydroxyl group. And absence of a peak at δ 3.52 indicated the removal of a methyl group from the structure of the drug, which represented the formation of or clobazam, an active metabolite of Clobazam.

Metabolite-CM2

Structural confirmation of 3,4-dihydroxy or clobazam, CM2 was done by observation of a doublet at δ 4.91 in 1HNMR spectrum of CM2 dictated the presence of two hydroxy groups. The absence of peak at δ 3.52 indicated the removal of methyl group from the structure of the drug, that represented the formation of norclobazam an active metabolite of Clobazam. 1H NMR spectrum of CM2 is shown in figure 17.

Metabolite-CM3

3 methoxy 4-hydroxy norclobazam, the structure of CM3 was confirmed by the presence of a triplet at δ 3.67 in 1HNMR stated the incidence of one methoxy group , appearance of a peak at δ 4.91 indicated the addition of hydoxy group. And absence of peak at δ 3.52 represented the removal of methyl group from the structure of the drug to form norclobazam an active metabolite of Clobazam as shown in figure 18.

1H NMR proton assignment of Clobazam and its three metabolites (CM1,CM2 and CM3) is shown in table 2.

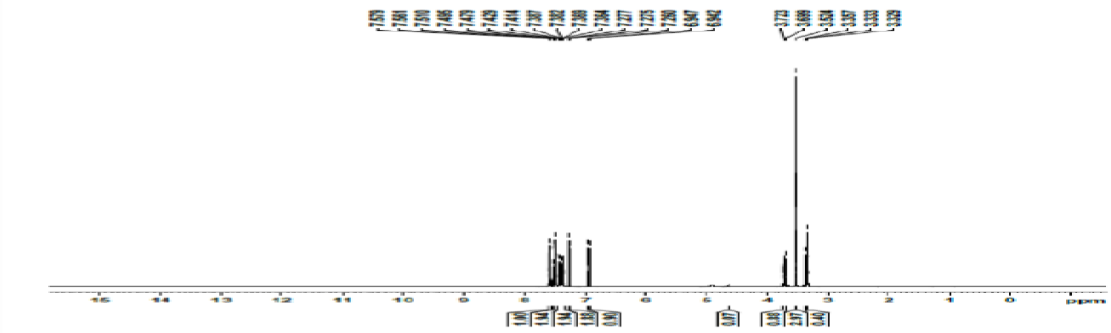


Fig.16. 1HNMR spectrum of Clobazam

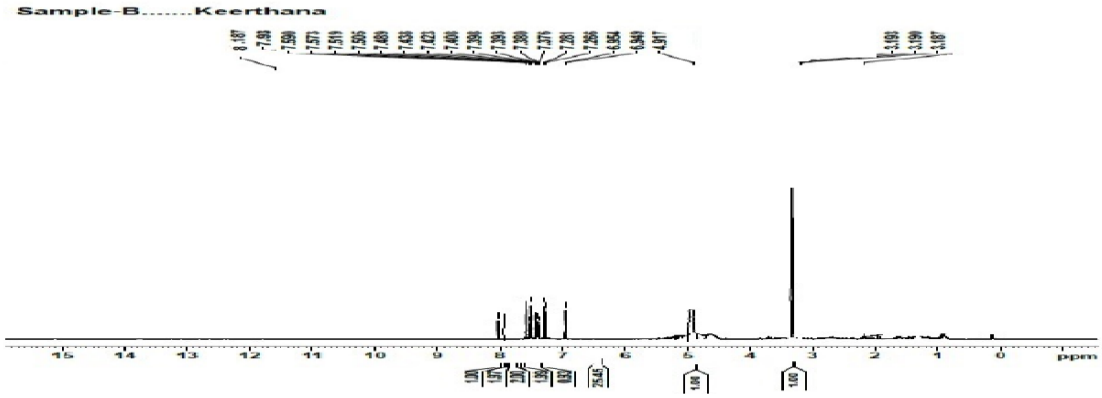


Fig.17. 1HNMR spectrum of Clobazam metabolite, CM1

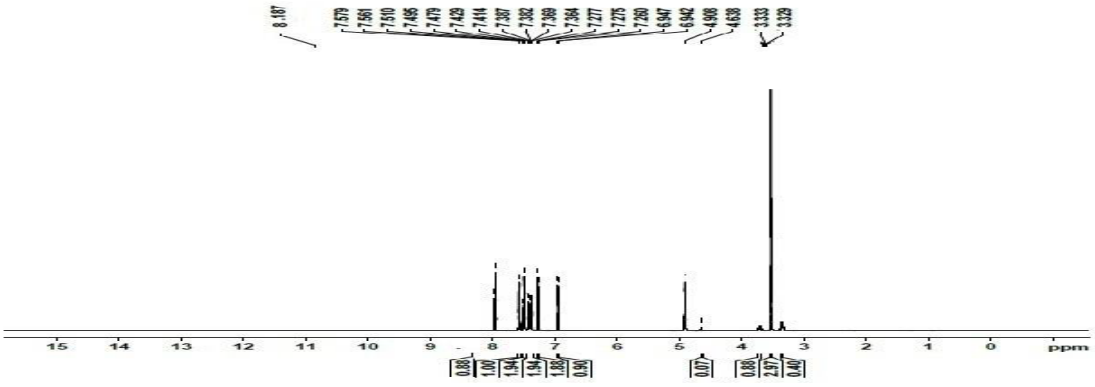


Fig.18. 1HNMR spectrum of Clobazam metabolite, CM2

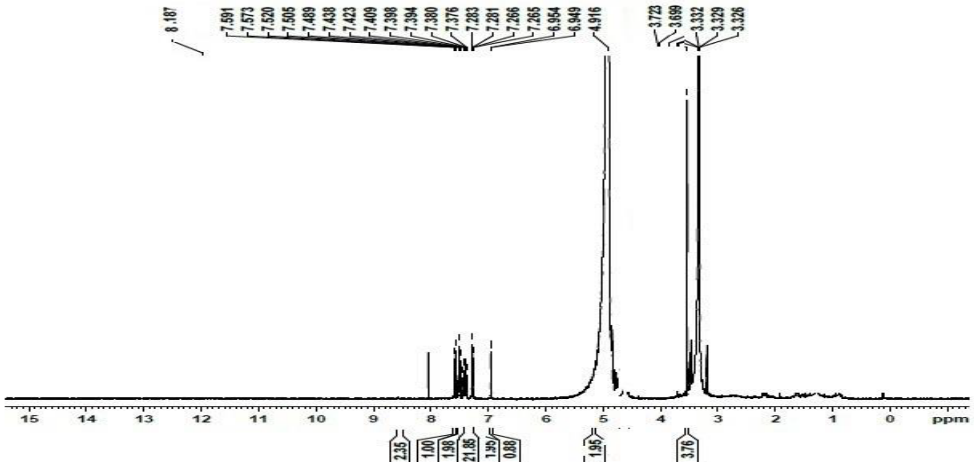


Fig.19. 1HNMR spectrum of Clobazam metabolite, CM3

Table 2: 1HNMR Proton assignment of Loratadine and its metabolites.

Compound name	1H NMR proton assignment	Figure no
Clobazam	3.32-3.35(t,2H,CH2), **3.52 (s,3H,CH3),**6.94(s, H, CH),7.26 (s, H,CH) , 7.27 (s, H,CH), 7.41-7.42(s,2H,CH) , 7.47 (m,3H,CH).	24
CM1	3.18-3.19 (d, 2H,CH2), **4.91 (s,1H,OH)**, 6.94-6.95 (d,2H, CH), 7.26-7.59 (m,4H,CH), 7.93 (s, H,CH), **8.18 (s, H, NH).**	25
CM2	3.32-3.33(d, 2H, CH2),4.64 - **4.91 (d,2H,OH)**, 6.94 (s, 2H, CH), 7.26-7.59 (m,4H,CH), **8.18 (s, H, NH).**	26
CM3	3.23-3.33 (d, 2H,CH2), **3.69-3.72(t,3H CH3), 4.91 (s,1H,OH)**, 6.94-6.95 (d,2H, CH), 7.26-7.59 (m,4H,CH), **8.18 (s, H, NH).**	27

***** Bold face indicates the additional peaks observed in the respective 1H NMR spectra.

3.2. Enzyme Kinetic Studies

Enzyme kinetic studies of Clobazam metabolism were conducted with three organisms *Aspergillus fumigatus, Aspergillus niger and Aspergillus ochreus* for CM1, CM2 and CM3. For each culture, six different substrate concentrations (10µg/ml to 60µg/ml) were used. Samples were collected during incubation after regular intervals of 24,36,48,60 and 72 hrs. The results of HPLC analysis obtained in the form of peak area were used to calculate percentage metabolite formed at different time points at each concentration. For each concentration of substrate, a graph was plotted between percentage of metabolite vs incubation time. Six different plots were obtained for six concentrations of substrate for each metabolite. Slope values obtained from each plot were considered as velocity of metabolic reaction by respective fungi.

3.2.1 Effect of incubation period on percentage metabolite formation

During enzyme kinetic studies, samples were analyzed by HPLC after collecting at regular incubation time intervals of 24,36,48,64 and 72 hrs. The graphs were plotted between percentage of metabolite vs incubation time to find out the effect of incubation time on metabolite formation.

Metabolite-CM1

As the incubation time was increased the percentage metabolite (CM1) formation was also increased up to 72 hrs of incubation with all six concentrations of substrate as shown in figure 19.

Metabolite (CM2)

In case of formation of metabolite (CM2) by *Aspergillus niger*, as the incubation time was increased, the percentage of metabolite formation was also increased with six concentrations of substrate. Graphs were plotted between percentage of metabolite, CM2 formation vs incubation time are represented in figure 20.

Metabolite (CM3)

As the incubation time was increased the percentage of metabolite (CM3) formation was also increased with six concentrations of substrate. Graphs were plotted between percentage of metabolite, CM3 formation vs incubation time are represented in figure 21.

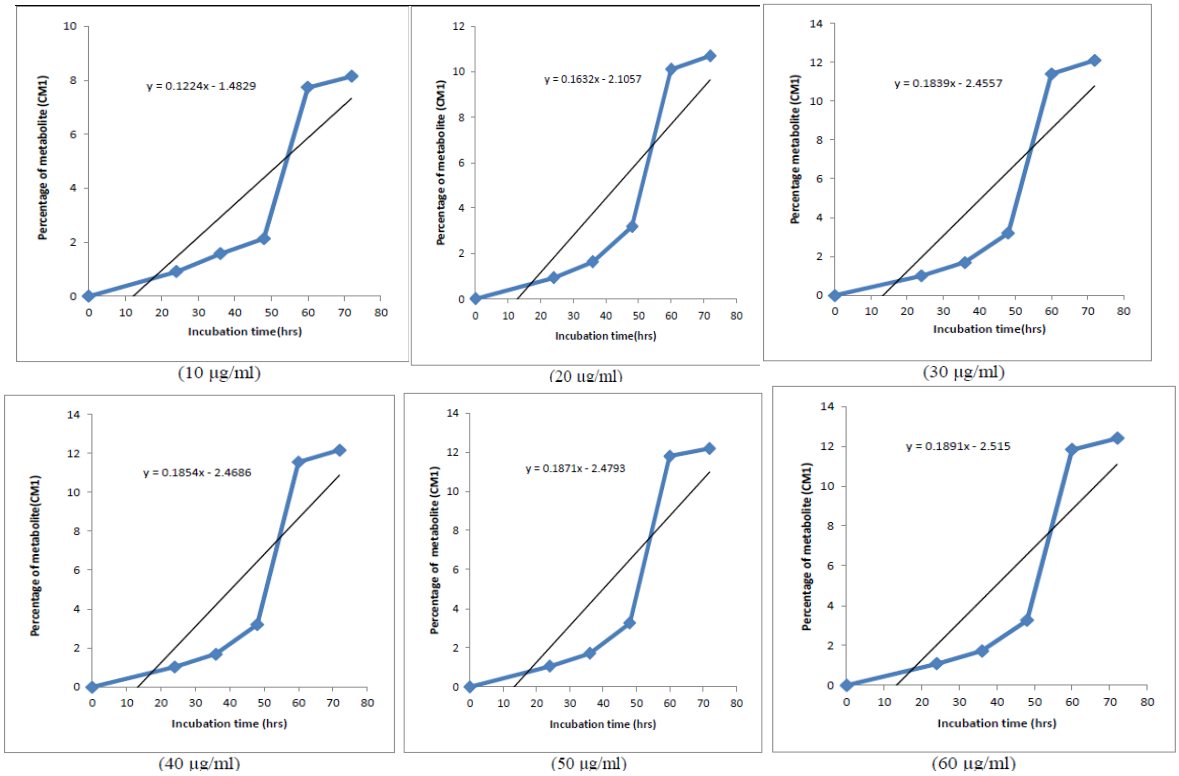


Fig.20. Percentage of metabolite, CM1 formed at different incubation time points with different concentrations of Clobazam.

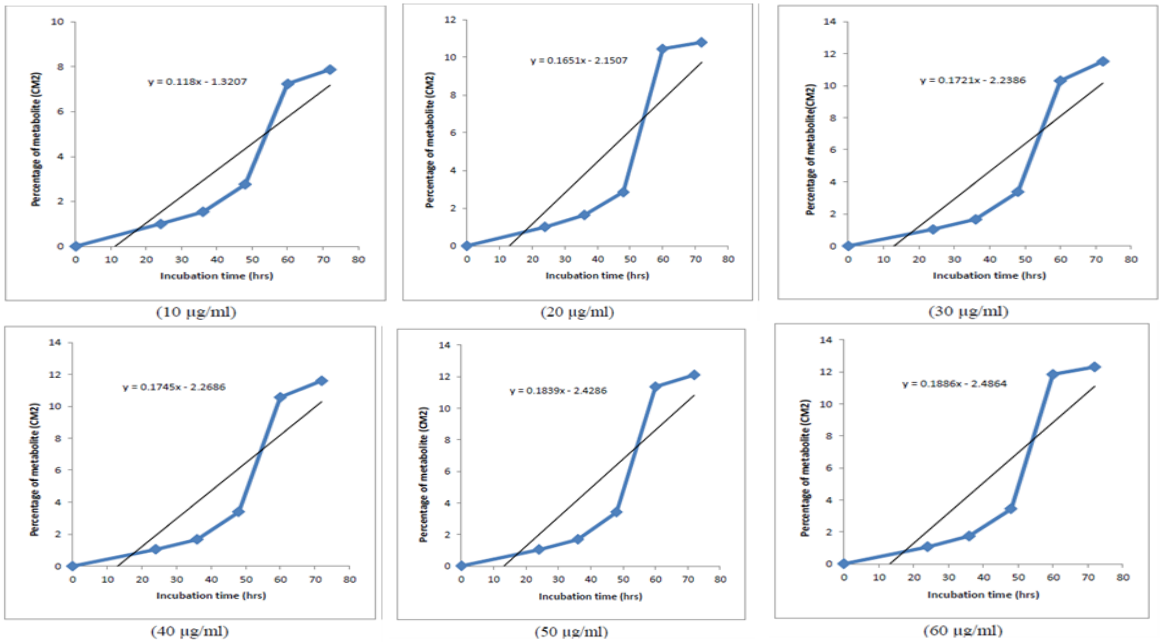


Fig.21. Percentage of metabolite, CM2 formed at different incubation time with different concentrations of Clobazam.

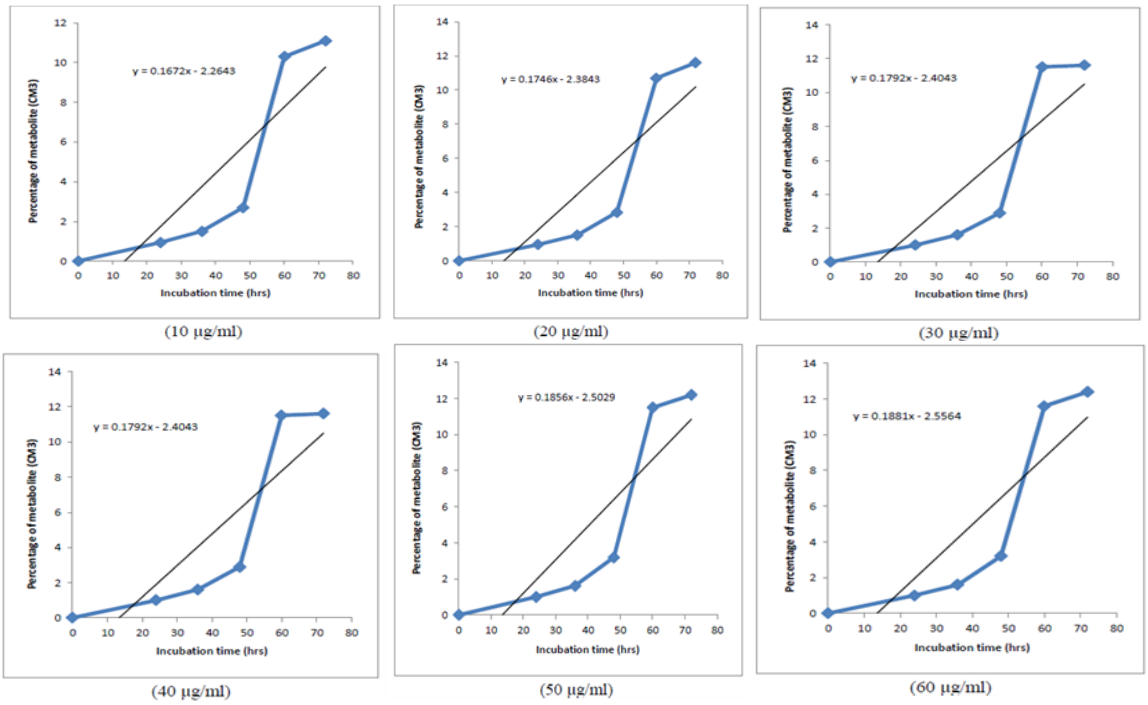


Fig.22. Percentage of metabolite, CM3 formed at different incubation time points with different concentrations of Clobazam.

Table 3: Percentage of Clobazam metabolites formed during kinetic studies

Con. µg/ml	Incubation time (hrs.)														
	24			36			48			60			72		
	CM1 (%)	CM2 (%)	CM3 (%)	CM1 (%)	CM2 (%)	CM3 (%)	CM1 (%)	CM2 (%)	CM3 (%)	CM1 (%)	CM2 (%)	CM3 (%)	CM1 (%)	CM2 (%)	CM3 (%)
10	0.9	1.0	0.94	1.57	1.53	1.50	2.13	2.76	2.75	7.73	7.23	10.3	8.14	7.8	11.1
20	0.93	1.0	0.95	1.63	1.63	1.50	3.18	2.85	2.84	10.1	10.4	10.7	10.7	10.8	11.6
30	1.01	1.03	0.99	1.69	1.66	1.60	3.20	3.37	2.89	11.4	10.3	11.5	12.1	11.5	11.6
40	1.04	1.04	0.99	1.70	1.67	1.61	3.21	3.39	3.20	11.5	10.5	11.5	12.1	11.6	11.7
50	1.06	1.04	1.0	1.71	1.68	1.62	3.26	3.40	3.20	11.8	11.3	11.5	12.2	12.1	12.2
60	1.08	1.06	1.0	1.72	1.73	1.6	3.26	3.43	3.20	11.8	11.8	11.6	12.4	12.3	12.4

Slopes values were obtained from above plots for three metabolites for six different concentrations, which were considered as velocity of reactions by respective organisms as represented in table 4.

Table 4: Slope values (velocity) of Clobazam metabolites for six different concentrations.

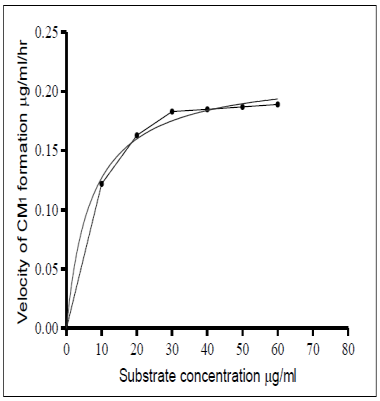
Substrate concentration (µg/ml)	Slope values or velocity (µg/ml/hr)		
	CM1	CM2	CM3
10	0.122	0.118	0.167
20	0.163	0.165	0.174
30	0.183	0.172	0.179
40	0.185	0.174	0.179
50	0.187	0.183	0.185
60	0.189	0.188	0.188

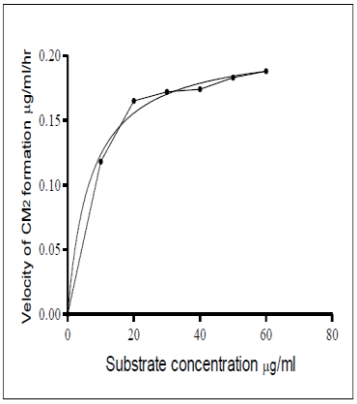
3.2.1.1. Effect of substrate concentration on percentage of metabolite formation

Six different substrate concentrations (10 µg/ml - 60µg/ml) were used to study the influence of substrate concentration on percentage of metabolite formation after 72 hrs of incubation (CM1, CM2 and CM3). After 72 hrs of incubation, percentage of formation of CM1, CM2 and CM3 was increased with increase in substrate concentration from 10 to 60 µg/ml.

Then, the velocity of reaction was plotted against substrate concentration (figure 22-24) by applying Michaelis-Menten kinetics to get kinetic parameters Km and Vmax.

Velocity of metabolic reactions for formation of three metabolites, CM1, CM2 and CM3 formed by *Aspergillus fumigatus*, *Apergillus niger and Aspergillus ochreus* respectively, increased with increase in substrate concentration. So, Michaelis-Menten plots were plotted between velocity of metabolites (CM1, CM2 and CM3) formation vs substrate concentration as shown in figure 22-24.





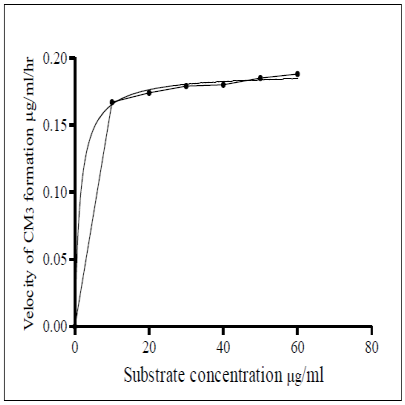


Fig.23. Michaelis-Menten plot of CM1 Fig.24. Michaelis-Menten plot of CM2 Fig.25. Michaelis-Menten plot of CM3

Table 5: Kinetic parameters of Loratadine metabolism by three organisms

Kinetic Parameters	CM1 (by *Cunnighamella elegans)*	CM2 (by *Cunnighamella echinulata)*	CM3 (by *Aspergillus niger*)
Km (µg/ml)	7.064	6.997	1.433
Vmax (µg/ml/hr)	0.2165	0.2100	0.1890

IV. DISCUSSION

4.1 Screening Studies

The ability of fungi for their capacity to biotransform the drug Clobazam, a CYP3A4 substrate [31] to its active metabolite was investigaed using six fungal cultures. The similar enzymatic systems of mammals like CYP 450 enzymes important for biotransformation of Clobazam may be present in fungi, hence, any one of these may have capacity to biotransform Clobazam to its active metabolite, nor clobazam. The conversion of Clobazam to its active metabolite was examined in the present study with the help of fermentation method and HPLC analysis. Six fungi were incubated on rotary shaker for 72 hrs as protocol and analysed their extracts by HPLC. Among six, the samples of *Aspergillus fumigatus*, *Aspergillus niger* and *Aspergillus ochreus* have shown extra peaks in HPLC compared to their respective controls. Hence, these were contemplated as the fungi able to biotranform Clobazam to its metabolites CM1,CM2 and CM3 respectively. When samples of other organisms were correlated with thier controls, identical peaks were found in samples and controls, this indicated no biotransformation and metabolite formation by other organisms.

Metabolite- CM1

The sample of *Aspergillus fumigatus* has shown an extra peak at retention time of 3.5min. when compared with its controls. The extra peak indicated the formation of Clobazam metabolite (CM1). The structure of metabolite, CM1 was elucidated by mass spectrometry and 1H NMR spectroscopic studies. Mass spectrum of Clobazam metabolite (CM1) formed by *Aspergillus fumigatus* has shown 2Da higher molecular weight than that of Clobazam, (m/z 303.75) which suggested the loss of methyl group from parent drug structure (as norclobazam structure) and addition of one hydroxy group to parent drug. The structure of metabolite CM1 was also supported by the mass fragments at m/z 268, 275 and 285. 1H NMR spectrum of CM1 has shown a peak at δ 8.18 represented the presence of NH group (possible on demethylation) and absence of peak at δ 3.52 indicated removal of methyl group from the structure of the drug and appearance of a singlet at δ 4.91 indicated the addition of one hydroxy group to parent drug structure. So, the metabolite CM1 structure was confirmed as 4-hydroxy nor clobazam.

Metabolite- CM2

The sample of *Aspergillus niger* has shown an extra peak at retention time of 3.8min.when compared with its controls. The extra peak denoted the formation of Clobazam metabolite (CM2) by these fungi. The structure of metabolite, CM2 was assessed by mass spectrometry and 1H NMR spectroscopic studies. Mass spectrum of Clobazam metabolite (CM2) formed by *Aspergillus niger* has shown 18 Da higher molecular weight than that of Clobazam, (m/z 319.74) which suggested the loss of methyl group from parent drug structure and addition of two hydroxy groups to the parent drug structure. The structure of metabolite, CM2 was also supported by the mass fragments at m/z 283,284, 291 and 301. ^{1}H NMR spectrum of CM2 has shown a peak at δ 8.18 represented the presence of NH group (possible on demethylation) and absence of peak at δ 3.52 indicated removal of methyl

group from the structure of the drug and appearance of a doublet at δ 4.91 indicated the addition of two hydroxy groups. So, the metabolite CM2 was confirmed as 3,4- dihydroxy norclobazam.

Metabolite- CM3

The sample of *Aspergillus ochreus* has shown an extra peak at retention time of 2.6min when compared with its controls. The extra peak indicated the formation of Clobazam metabolite, CM3 by *Aspergillus ochreus*. Mass spectrum of Clobazam metabolite (CM3) formed by *Aspergillus ochreus* has shown 32 Da higher molecular weight than that of Clobazam which suggested the loss of methyl group from parent drug structure and addition of one hydroxy group and methoxy group to the parent drug. The structure of metabolite, CM3 was supported by the mass fragments at m/z 283, 298, 301 and 315. ^{1}H NMR spectrum of CM3 has shown peak at δ 8.18 represented the presence of NH group (possible on demethylation) and absence of peak at δ3.52 indicated removal of methyl group from the structure of the drug and appearance of a singlet at δ 4.91 indicated the addition of one hydroxy group and a triplet at δ 3.69 indicated the addition of methoxy group to the parent drug structure. So, the metabolite CM3 was confirmed as 3-methoxy, 4-hydroxy norclobazam.

^{1}H NMR proton assignments of Clobazam and metabolites (CM1, CM2 and CM3) was also supported the structures of individual meatbolites. The metabolites CM1 CM2 and CM3 are monohydroxy, di hydoxy and mono hydroxy, methoxy derivatives of norclobazam. Hence, the formation of norclobazam as an intermediate product by *Aspergillus fimugatus, Aspergillus niger* and *Aspergillus ochreus* was noticed from above results.

Clobazam is extensively metabolised to an active metabolite 'norclobazam' in human, by *in-vitro* liver microsomes [31] and other mammals similar to metabolites acquired by *Aspergillus fumigatus, Aspergillus niger* and *Aspergillus ochreus* in the present study.

4. 2. Enzyme Kinetic Studies

The enzyme kinetic studies are vital to find out the affinity of fungal enzyme towards the substrate by estimating kinetic parameters, Km and Vmax. Kinetics of microbial metabolism of Clobazam by three fungi was studied using substrate concentrations ranging from 10 µg/ml to 60 µg/ml and estimating the percentage of metabolite formed at different incubation periods for each concentration.

Metabolite- CM1

On summarising the results of kinetics of CM1 formation, it was noticed that, as the incubation time was increased, the percentage metabolite (CM1) formation was also increased along with increase in the substrate concentration in the metabolism of Clobazam by *Aspergillus fumigatus*. The percentage of metabolite, CM1 formation was maximum at 72 hrs of incubation when compared with 24,36,48 and 60 hrs of incubation. The study of effect of substrate concentration on percentage of metabolite, CM1 formation revealed that, the maximum percentage of metabolite (CM1) was observed at 60 µg/ml substrate concentration at 72 hrs of incubation. Velocity of the metabolism reaction was obtained from slope values of lines found in graphs plotted between incubation time and percentage of metabolite (CM1) for each concentation of substrate. Then, Michaelis-Meneten kinetics was applied to the data of velocity of metabolism reaction vs substrate concentration in order to estimate Km and Vmax. Km and Vmax values of metabolism reactions mediated by enzymes in *Aspergillus fumigatus* to form CM1 were 7.06 µg/mland 0.21µg/ml/hr respectively. These values stated that metabolism reaction which formed CM1proceeded with the maximum velocity (Vmax) of0.21µg/ml/hr, and half maximum velocity of reaction was found at the concentration (Km)of 7.06µg/ml.

Metabolite- CM2

The enzyme kinetic studies of fungal metabolism mediated by enzymes in *Aspergillus niger* to form CM2 was found that, as the incubation time was increased, the percentage of metabolite formation was also increased along with increase in the substrate concentration in the metabolism of Clobazam. The percentage of metabolite, CM2 formation was maximum at 72 hrs of incubation when compared with 24,36,48 and 60 hrs of incubation. Velocity of metabolic reactions for six concentrations were obtained from slope values of lines found in graphs plotted between incubation time and percentage of metabolite (CM2). The study of effect of substrate concentration on percentage of metabolite, CM2 formation revealed that, the maximum percentage of metabolite (CM2) was observed at 60 µg/ml substrate concentration at 72 hrs of incubation. The Michaelis–Menten kinetics was applied to biotransformation kinetics of CM2 formation because, the velocity of reaction was increased with increase in the substrate concentrations from 10 µg/ml to 60 µg/ml, Km and Vmax values of metabolism reactions mediated by enzymes in *Aspergillus niger* to form CM2 were 6.99 µg/ml and 0.21 µg/ml/hr respectively. These values stated that metabolism reaction which formed CM2proceeded with the maximum velocity (Vmax) of 0.21µg/ml/hr, and half maximum velocity of reaction was found at the concentration(Km) of 6.99µg/ml.

Metabolite- CM3

The enzyme kinetic studies of fungal metabolism mediated by enzymes in *Aspergillus ochreus* to form CM3 was

found that, as the incubation time was increased, the percentage metabolite formation was also increased along with increase in the substrate concentration. The percentage of metabolite CM3 formation was maximum at 72 hrs of incubation when compared with 24,36,48 and 60 hrs of incubation. Velocity of metabolic reactions for six concentrations were obtained from slope values (table no-19) of lines found in graphs plotted between incubation time and percentage of metabolite (CM3) The study of effect of substrate concentration on percentage of metabolite, CM3 formation revealed that, the maximum percentage of metabolite (CM3) was observed at 60 µg/ml substrate concentration at 72 hrs of incubation. The Michaelis–Menten kinetics was applied to biotransformation kinetics of CM3 formation because, the velocity of reaction was increased with increase in the substrate concentrations from 10 µg/ml to 60 µg/ml, Km and Vmax values of metabolism reactions mediated by enzymes in *Aspergillus ochreus* to form CM3 were 1.43 µg/ml and 0.18 µg/ml/hr respectively. These values stated that metabolism reaction which formed CM3proceeded with the maximum velocity (Vmax) of 0.18µg/ml/hr, and half maximum velocity of reaction was found at the concentration of (Km)of 1.43µg/ml.

V. CONCLUSION

It can be concluded that, *Aspergillus fumigatus*, *Aspergillus niger* and *Aspergillus ochreus* have shown the capability to transform Clobazam to its metabolites due to the presence of enzymes required for biotransformation of Clobazam. Hence, it can be demonstrated that *Aspergillus fumigatus*, *Aspergillus niger* and *Aspergillus ochreus* have potential for production of an active metabolite norclobazam as the metabolites (CM1,CM2,CM3) were derivatives of norclobazam. Formation of this active metabolite was similar to mammals. Induction and inhibition studies were confirmed that, CYP 3A4 like enzyme might be involved in the biotransformation of Clobazam by three fungi *Aspergillus fumigatus*, *Aspergillus niger* and *Aspergillus ochreus*. Thus, the present study revealed that, all the three fungi can be used as models for CYP3A4 enzyme to study pharmacological and toxicological properties and the biotransformation of other similar drugs. Enzyme kinetic studies revelaed that, metabolism mediated by enzyme present in *Aspergillus ochreus* possessed low Km value than enzymes present in *Aspergillus fumigatus* and *Aspergillus niger*. So the enzymes present in *Aspergillus ochreus* has more affinity towards Clobazam when compared with enzymes in *Aspergillus fumigatus* and *Aspergillus niger*. So maximum metabolite can be produced by *Aspergillus ochreus* with less quantity of Clobazam.

REFERENCES

[1] Robertson, M.M. Current Status of the 1,4- and 1,5-Benzodiazepines in the Treatment of Epilepsy: The Place of Clobazam. *Epilepsia* **1986**, *27* (Suppl. S1), S27–S41.
[2] Pernea, M.; Sutcliffe, A.G. Clobazam and Its Use in Epilepsy. *Pediatr. Rep.* **2016**, *8*, 34–38.
[3] Mahmoud, S.H.; Rans, C. Systematic review of clobazam use in patients with status epilepticus. *Epilepsia Open* **2018**, *3*, 323–330.
[4] Grigoleit, H.-G.; Hajdú, P.; Hundt, H.K.L.; Koeppen, D.; Malerczyk, V.; Meyer, B.H.; Müller, F.; Witte, P.U. Pharmacokinetic aspects of the interaction between clobazam and cimetidine. *Eur. J. Clin. Pharmacol.* **1983**, *25*, 139–142.
[5] Monjanel-Mouterde, S.; Antoni, M.; Bun, H.; Botta-Frindlund, D.; Gauthier, A.; Durand, A.; Cano, J.P. Pharmacokinetics of a Single Oral Dose of Clobazam in Patients with Liver Disease. *Pharmacol. Toxicol.* **1994**, *74*, 345–350.
[6] Theis, J.G.; Koren, G.; Daneman, R.; Sherwin, A.L.; Menzano, E.; Cortez, M.; Hwang, P. Interactions of clobazam with conventional antiepileptics in children. *J. Child Neurol.* **1997**, *12*, 208–213.
[7] De Leon, J.; Spina, E.; Diaz, F.J. Clobazam Therapeutic Drug Monitoring: A Comprehensive Review of the Literature with Proposals to Improve Future Studies. *Ther. Drug Monit.* **2013**, *35*, 30–47.
[8] Caccia, S.; Ballabio, M.; Guiso, G.; Zanini, M.G. Gas-liquid chromatographic determination of clobazam and n-desmethyl-clobazam in plasma. *J. Chromatogr.* **1979**, *164*, 100–105.
[9] Marliac, Y.; Barazi, S. Determination of unchanged clobazam in plasma by gas-liquid chromatography. *Ann. Pharm. Françaises* **1989**, *47*, 213–220.
[10] Riva, R.; Tedeschi, G.; Albani, F.; Baruzzi, A. Quantitative determination of clobazam in the plasma of epileptic patients by gas—Liquid chromatography with electron-capture detection. *J. Chromatogr. B Biomed. Sci. Appl.* **1981**, *225*, 219–224.
[11] Simonsen, K.W.; Hermansson, S.; Steentoft, A.; Linnet, K. A Validated Method for Simultaneous Screening and Quantification of Twenty-Three Benzodiazepines and Metabolites Plus Zopiclone and Zaleplone in Whole Blood by Liquid-Liquid Extraction and Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry. *J. Anal. Toxicol.* **2010**, *34*, 332–341.
[12] Mikayelyan, A.; Aleksanyan, A.; Sargsyan, M.; Gevorgyan, A.; Zakaryan, H.; Harutyunyan, A.; Zhamharyan, L.; Armoudjian, Y.; Margaryan, T. Protein Precipitation Method for Determination of Clobazam and N-Desmethylclobazam in Human Plasma by Lc-Ms/Ms. *Biomed. Chromatogr.* **2020**, *34*, e4710.
[13] Laloup, M.; Fernandez, M.D.M.R.; De Boeck, G.; Wood, M.; Maes, V.; Samyn, N. Validation of a Liquid Chromatography-Tandem Mass Spectrometry Method for the Simultaneous Determination of 26 Benzodiazepines and Metabolites, Zolpidem and Zopiclone, in Blood, Urine, and Hair. *J. Anal. Toxicol.* **2005**, *29*, 616–626.
[14] Knapp, J.; Boknik, P.; Gumbinger, H.G.; Linck, B.; Luss, H.; Muller, F.U.; Schmitz, W.; Vahlensieck, U.; Neumann, J. Quantitation of Clobazam in Human Plasma Using High-Performance Liquid Chromatography. *J. Chromatogr. Sci.* **1999**, *37*, 145–149.
[15] Bolner, A.; Tagliaro, F.; Lomeo, A. Optimised determination of clobazam in human plasma with extraction and high-performance liquid chromatography analysis. *J. Chromatogr. B Biomed. Sci. Appl.* **2001**, *750*, 177–180.
[16] Rouini, M.; Ardakani, Y.H.; Hakemi, L.; Mokhberi, M.; Badri, G. Simultaneous determination of clobazam and its major metabolite

in human plasma by a rapid HPLC method. *J. Chromatogr. B* **2005**, *823*, 167–171.
[17] Ratnaraj, N.; Goldberg, V.; Lascelles, P.T. Determination of clobazam and desmethylclobazam in serum using high-performance liquid chromatography. *Analyst* **1984**, *109*, 813–815.
[18] Pistos, C.; Stewart, J.T. Direct injection HPLC method for the determination of selected benzodiazepines in plasma using a Hisep column. *J. Pharm. Biomed. Anal.* **2003**, *33*, 1135–1142.
[19] Tomasini, J.-L.; Bun, H.; Coassolo, P.; Aubert, C.; Cano, J.-P. Determination of Clobazam, n-desmethylclobazam and their hydroxy metabolites in plasma and urine by high-performance liquid chromatography. *J. Chromatogr. B Biomed. Sci. Appl.* **1985**, *343*, 369–377.
[20] Kunicki, P. Simple and sensitive high-performance liquid chromatographic method for the determination of 1,5-benzodiazepine clobazam and its active metabolite *N*-desmethylclobazam in human serum and urine with application to 1,4-benzodiazepines analysis. *J. Chromatogr. B Biomed. Sci. Appl.* **2001**, *750*, 41–49.
[21] Akerman, K.K. Analysis of clobazam and its active metabolite norclobazam in plasma and serum using HPLC/DAD. *Scand. J. Clin. Lab. Investig.* **1996**, *56*, 609–614.
[22] Dusci, L.J.; Hackett, L.P.; Dusci, L.J.; Hackett, L.P. Simultaneous Determination of Clobazam, N-Desmethyl Clobazam and Clonazepam in Plasma by High Performance Liquid Chromatography. *Ther. Drug Monit.* **1987**, *9*, 113–116.
[23] Brachet-Liermain, A.; Jarry, C.; Faure, O.; Guyot, M.; Loiseau, P. Liquid Chromatography Determination of Clobazam and Its Major Metabolite N-Desmethylclobazam in Human Plasma. *Ther. Drug Monit.* **1982**, *4*, 301–306.
[24] Zilli, M.A.; Nisi, G. Simple and sensitive method for the determination of clobazam, clonazepam and nitrazepam in human serum by high-performance liquid chromatography. *J. Chromatogr. B Biomed. Sci. Appl.* **1986**, *378*, 492–497.
[25] Moody J.D, James P. Freeman, Peter P. Fu and Cerniglia C.E, Biotransformation of Mirtazapine by *Cunninghamella elegans*. *Drug Metbolism Disposition*, 2002; 30[11]: 1274–1279.
[26] Azerad R. Microbial models for drug metabolism. *Advances in Biochemistry and Biotechnology*, 1999; 63:169–218.
[27] Smith RV, Rosazza JP. Microbial models of mammalian metabolism. *Journal of Pharmaceutical Sciences* 1975; 64:1737–1758.
[28] Moffat, A.C. In; Clarke's Identification and isolation of drugs. Jackson, J.V.; Moss,M.S; Widoop, B. Green field ES ed., 2edn, Pharmaceutical Press, London. 1986; 170.
[29] Yuh Lin, Ping Lu, Cuyue Tang, Qin Mei, Grit Sandig, A. David Rodrigues, Thomas H. Rushmore, And Magang Shou , Substrate Inhibition Kinetics For Cytochrome P450-Catalyzed Reactions, *Drug Metabolism and Disposition*, 2001; 29(4)1:368-374.
[30] Magang shou, Qin mei, Michael W. Ettore, JR, Renke Dai., Thomas A. Baillie and Thomas H. Rushmore, Sigmoidal kinetic model for two co-operative substrate-binding sites in a cytochrome P450 3A4 active site : an example of the metabolism of diazepam and its derivatives, *Biochemical Journal*,1999; 340: 845-853.
[31] Giraud C, Tran A, Rey E, et al. In vitro characterization of clobazam metabolism by recombinant cytochrome P450 enzymes: importance of CYP2C19. *Drug Metabolism and Disposition.* 2004; 32:1279–1286.