International Journal of Multidisciplinary Research and Growth Evaluation.

Microbial screening and kinetics of metabolism of loratadine

Nandini [1*], P Sindhu [2], Juluri Sreeja [3], Salla Sri Poojitha [4], Vasavi Gorenka [5], Yogitha Singh [6]
[1-6] Department of Pharmacology, Bojjam Narasimhulu College of Pharmacy, Saidabad, Vinayak Nagar Colony, Hyderabad, Telangana, India

* Corresponding Author: **Nandini**

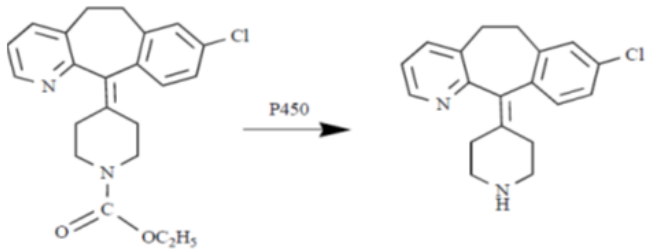
Article Info

ISSN (online): 2582-7138
Volume: 04
Issue: 01
January-February 2023
Received: 06-01-2023;
Accepted: 25-01-2023
Page No: 406-424

Abstract
The commenced research was proposed to produce known active/novel metabolites by microbial biotransformation by an easy and economic way and also to develop microbial models for CYP enzymes for drug metabolism studies. This study was also aimed at anticipation of the nature of enzyme involved in fungal biotransformation and affinity of the fungal enzyme towards substrate. It observed from this study that, *Cunnighamella elegans*, *Cunnighmella echinulata* and *Aspergillus Niger* have shown the ability to convert Loratadine to its metabolites due to the presence of enzymes essential for biotransfromation of Loratadine. Thus, the present study revealed that, *Cunnighamella elegans* can be used as model for CYP2D6 and *Aspergillus niger* can be used as model for CYP3A4 to study pharmacological and toxicological properties and the biotransformation of other similar drugs. Enzyme kinetic studies revealed that, metabolism mediated by enzymes present in *Cunnighmella echinulata* possessed low Km value than enzymes present in *Cunnighamella elegans* and *Aspergillus niger*. So the enzyme present in *Cunnighamella echinulata* has more affinity towards Loratadine when compared with other two enzymes present in *Cunnighamella elegans* and *Aspergillus niger*. So,maximum metabolite can be produced by *Cunnighmella echinulata* with less quantity of Loratadine.

Keywords: Loratadine, *Cunnighamella elegans*, *Cunnighmella echinulate*, *Aspergillus niger* CYP3A4

Introduction
Cytochrome P450s are comprised of a superfamily of enzymes responsible for the metabolism of xenobiotics, including drugs, carcinogens, and environmental chemicals as well as endobiotics which include steroids and fatty acids [1]. These enzymes are most predominant in the liver but can also be found in the intestines, lungs, and other organs and play an important role in drug disposition. In humans there are more than 20 different CYP enzymes (livers), but only six (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A) account for the metabolism of nearly all clinically useful medications. The contribution of individual cytochrome P450 enzymes to the metabolism of clinically used drugs may be crucial for understanding drug-drug interactions, drug related toxicity, and efficacy.
Loratadine (Claritin®) is a long-acting tricyclic antihistamine with selective peripheral histamine H1 -receptor antagonistic activity. Claritin® is indicated for the relief of nasal and non-nasal symptoms of seasonal allergic rhinitis and for the treatment of chronic idiopathic urticaria in patients 2 years of age or older. Following a single oral 10-mg dose to healthy male volunteers, metabolite profiles of plasma showed that LOR was extensively metabolized via descarboethoxylation to DL, oxidation and glucuronidation [2]. Major circulating metabolites included 3-hydroxy-desloratadine glucuonide (3-OH-DL-Glu), dihydroxy-DLglucuronides, and several metabolites resulting from descarboethoxylation and oxidation of the piperidine ring [2]. DL is a pharmacologically active metabolite. As shown below, DL results from decarboethoxylation of loratadine.



Loratadine (LOR) Desloratadine (DL)

Herein, we present a rapid, reproducible, simple, and sensitive HPLC, MS and H^1NMR method for quantifying Loratadine 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)).

Materials

Microorganisms

The microorganisms selected for the current study are *Aspergillus fumigates*, *Aspergillus niger*, *Aspergillus ochreus*, *Cunninghamella elegans*, *Cunninghamella echinulata* and *Cunnighamella blackesleeana*.

Chemicals

Dextrose, Yeast extract, Agar-agar were purchased from SD fine chemicals Ltd, Mumbai, India. Water, Methanol**,** Acetonitrile, Dichloromethane and Diethyl ether were purchased from Merck, Mumbai, India. Loratadine was collected from Shasun pharmaceuticals, Chennai, as gift sample.

Media preparation

As the selected six cultures were fungi (*Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus ochreus*, *Cunnighamella echinulata*, *Cunnighamella elegans*, *Cunnighamella blackesleena)*, potato dextrose broth (PDB) was used as a medium, which was suitable for fungal growth [3]. Composition of PDB medium is given in table no-1.

Table 1: Composition of PDB medium

Sl.no	Component	Quantity
1	Potato chips (Boiled for 30 min.)	20 gm
2	Dextrose	2 gm
3	Yeast extract	10 mg
4	Distilled water	Up to 100 ml

Stock culture maintenance

The viability of pure cultures was maintained by sub culturing, in which pure cultures were transferred into respective fresh agar slants for every six months and stored at 4°C [4]. 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 medium into test tubes. The prepared slants were sterilized by autoclaving at 121 °C, 15 psi for 20 min. [5]. 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 refrigerator for further use.

Screening Studies

Preparation of drug stock solutions

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 biotransformation study of each drug.

Fungal biotransformation-incubation protocol

Three different flasks were set for each culture for each drug as shown in table no-14. The prepared broth medium of 50 ml was transferred to 250 ml. Erlenmeyer flasks and 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 (table no-2) [6].

Table 2: Protocol of incubation for fungal biotransformation of drugs

Name of flask	Contents of the flask		
	Broth medium	Substrate	Culture
Blank I [Drug control	+	+	-
Blank II [Culture control]	+	-	+
Sample	+	+	+

'+' = added; '-' = Not added

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 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 drug during HPLC analysis.

Fermentation

The flasks as specified in table no- 14 for all drugs and cultures were incubated using orbital shaker incubator [CIS 24, Remi instruments, Mumbai] for 72 hrs, operated at 120 rpm, 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.

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. 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 substrate solution, inhibitor solution and 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 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 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 composed of sterile medium to which culture, inhibitor or inducer were added without substrate. Sample composed of 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 rpm, at 28°C for this study. The percentage of metabolites formed in inhibition and induction studies were calculated from the peak area of drug and metabolite obtained in HPLC analysis.

Table 3: Protocol for fungal metabolism inhibition / induction studies

Contents of the flasks	Name of the flask					
	Blank I [drug control]	**Blank II [Culture control]**	**Blank III [inhibitor/ inducer control]**	**Blank IV [Substrate +inhibitor/ inducer]**	**Blank V [inhibitor/ inducer +culture]**	**Sample [substrate +inhibitor/ inducer +culture]**
Broth medium	+	+	+	+	+	+
Substrate	+	-	-	+	-	+
Culture	-	+	-	-	+	+
Inhibitor/ Inducer	-	-	+	+	+	+

+ = added; - = Not added

Enzyme Kinetic Studies [7]

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 above studies was inoculated in labelled sample flasks containing different concentrations of 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.

Extraction Method

All incubated flaks or samples collected after predetermined incubation period in all the above studies (biotransformation, induction, inhibition and kinetic studies) were heated in 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). Supernatant was collected from all tubes and were stored in refrigerator. The extraction of drug and its metabolites was conducted by withdrawing 8 ml. of supernatant collected from each of the flask 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 analysed by HPLC after reconstitution with mobile phase, besides pure drug was also analysed by HPLC to set it as a standard.

Analytical Methods

High Pressure Liquid Chromatography [8]

Drugs and their metabolites in the extracted samples were estimated by High Performance Liquid Chromatography [HPLC] method. HPLC (Schimadzu) operating conditions used for different selected drugs. Mobile phase: Acetonitrile: Methanol: Water (HPLC grade) (0.5:0.5:1), Column: C18Phenomenex luna, Flow rate: 1ml/min., Detection wavelength: 254nm, Injection volume: 25µl, Temperature 30°C, Detector UV detector, Run time 30 min.

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

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 analysed in HPLC under same analytical conditions.

Mass spectrometry

The metabolite identified in samples of fungi screened during HPLC analysis were isolated and further analysed through Mass spectrometry to confirm its molar mass. 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 ion trap detector (3.5 kV, 325°C, 210 psi).

Proton Nuclear Magnetic Resonance spectroscopy

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

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 in relation to the peak area of drug in their respective studies.

Analysis of Kinetic Data

The data of percentage metabolite formed during kinetic studies was fitted in MS EXCEL by plotting graph between time and percentage metabolite formed at different concentrations of substrate to get velocity of

biotransformation reaction from the slope of the graph.
Then, the velocity obtained for each concentration by above method was fitted into the Michaelis–Menten equation using nonlinear regression in GraphPad Prism 5.0 [9]. Best-fit values for *K*m and Vmax were reported to assess the possible extent of affinity of fungal enzyme with the drug for better understanding of fungal biotransformation of three selected drugs.

Results

High Performance Liquid Chromatography

Six different fungi were screened to study biotransformation of Loratadine. The results of HPLC analysis of Loratadine and its metabolites in different fungal extracts are shown in figures2-8 and data is given in table no-5. No drug peak was observed in chromatograms of the blank II (culture controls). Blank I (drug controls) showed the presence of drug peak at retention time of 11.8 min. The peak at retention time of 2.5 min. depicted solvent peak and peak at retention time of 5.4 depicted as culture content peak. HPLC chromatograms of samples of *Cunnighmella elegans*, *Cunnighmella echinulata* and *Aspergillus niger* cultures have shown extra peaks at (LM1) 3.8 min., (LM2) 3.6 min. and (LM3) 4.1 min. respectively compared with their controls as represented in figure no-3,4,5 and table no. 5. No extra peaks were observed in the samples of other cultures when compared with their controls. Since, the three cultures only metabolised Loratadine, the elutes of the extra peaks were collected from HPLC and further analysed by combination of Mass and 1H NMR spectroscopy to elucidate the metabolite structure and to propose its metabolic pathway in fungi.

Table 5: 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)**	2.5	2.5	-	2.5
	-	-	-	**3.8*(LM1)**
	-	5.1		5.1
	11.8	-	11.8	11.8
***Cunnighamella echinulata* (NCIM-687)**	2.5	2.5		2.5
	-	-	-	**3.6*(LM2)**
	-	5.4	-	5.4
	11.8	-	11.8	11.8
Cunnighamella blackesleeana (NCIM-691)	2.5	2.5	-	2.5
	-	6.7	-	6.7
	11.8		11.8	11.8
***Aspergillus niger* (NCIM-589)**	2.5	2.5	-	2.5
	-	-	-	**4.1*(LM3)**
	11.8	-	11.8	11.8
Aspergillus fumigatus (NCIM-902)	2.5	2.5	-	2.5
	-	4.0	-	4.0
	11.8	-	11.8	11.8
Aspergillus ochreus (NCIM-1140)	2.5	2.5	-	2.5
	11.8	-	11.8	11.8

"*" – Metabolite peak

Table 6: Percentage of Loratadine metabolites formed during kinetic studies

Con. µg/ml	**Incubation time (hrs.)**														
	24			36			48			60			72		
	LM1 (%)	**LM2 (%)**	**LM3 (%)**	**LM1 (%)**	**LM2 (%)**	**LM3 (%)**	**LM1 (%)**	**LM2 (%)**	**LM3 (%)**	**LM1 (%)**	**LM2 (%)**	**LM3 (%)**	**LM1 (%)**	**LM2 (%)**	**LM3 (%)**
10	0.84	0.08	0.91	1.69	1.56	1.49	2.4	2.51	2.5	7.8	7.2	7.97	9.0	8.02	8.4
20	0.95	0.08	1.0	1.70	1.72	1.50	2.6	2.5	2.5	8.0	7.6	8.4	10.6	10.4	11.3
30	0.96	0.09	1.08	1.70	1.75	1.66	2.6	2.5	2.6	8.8	7.9	8.6	12.3	10.5	13.1
40	1.04	0.10	1.15	1.80	1.83	1.76	2.8	2.6	2.77	9.13	8.6	11.6	15.6	11.8	17.9
50	1.11	0.10	1.08	1.94	2.0	1.72	3.0	2.9	2.67	9.96	9.4	11	17.4	12.5	14.0
60	1.16	0.11	0.99	2.22	2.2	1.62	3.04	2.9	2.4	10.0	9.46	7.9	18.8	13.8	13.4

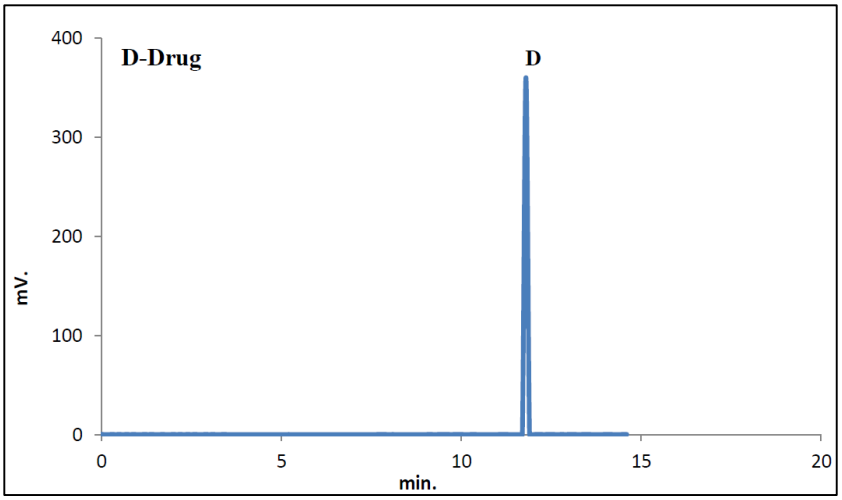


Fig 2: HPLC chromatogram of Loratadine

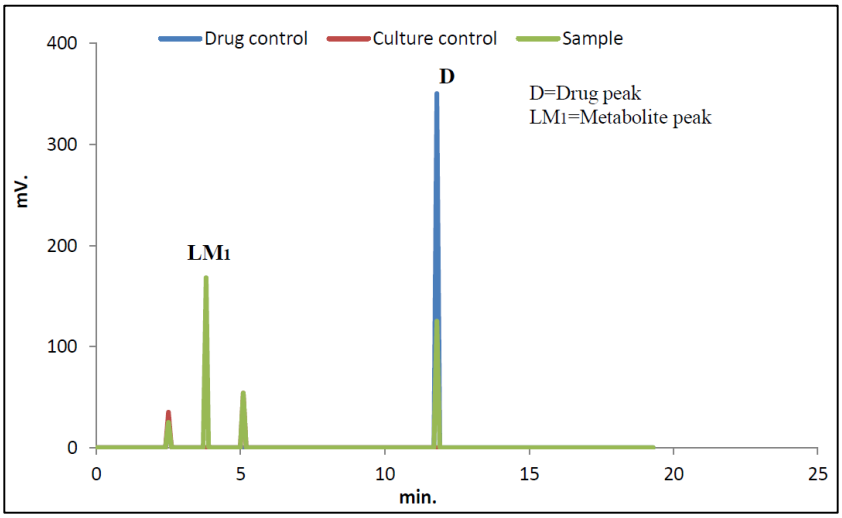


Fig 3: HPLC chromatogram of Loratadine from culture extracts of *Cunnighamella elegans*

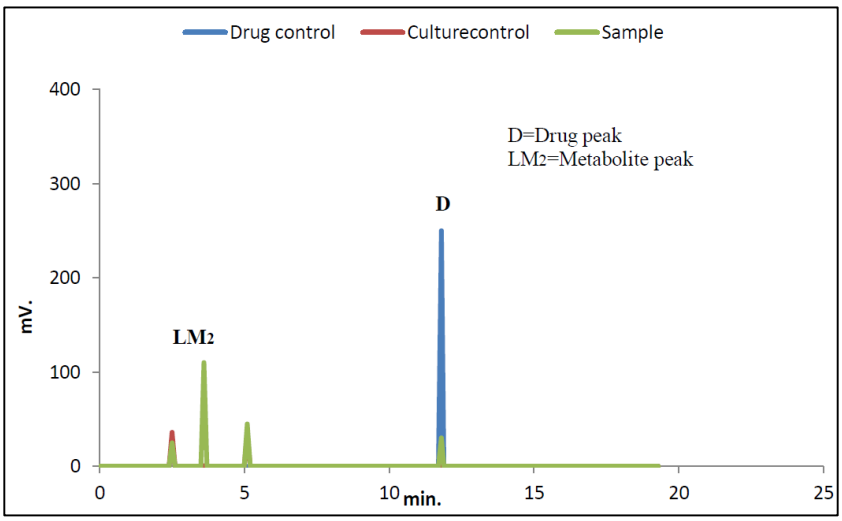


Fig 4: HPLC chromatogram of Loratadine from culture extracts of *Cunnighamella echinulata*

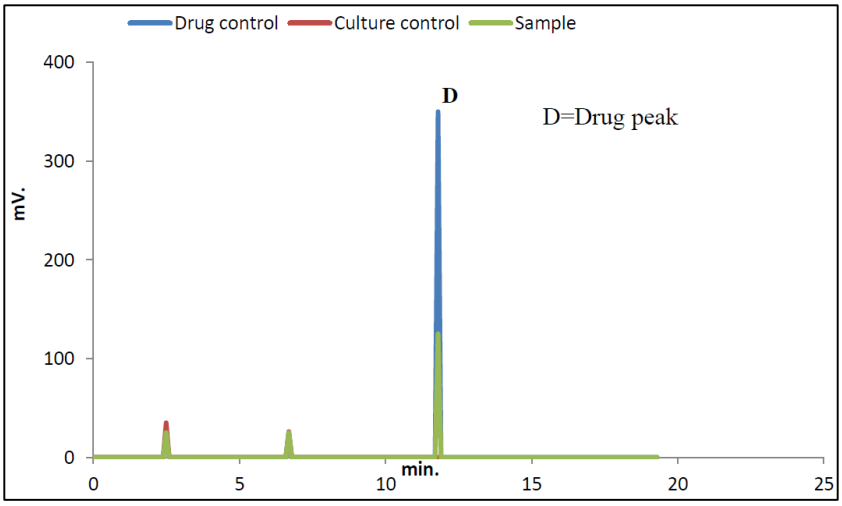


Fig 5: HPLC chromatogram of Loratadine from culture extracts of *Cunnighamella blackesleena*

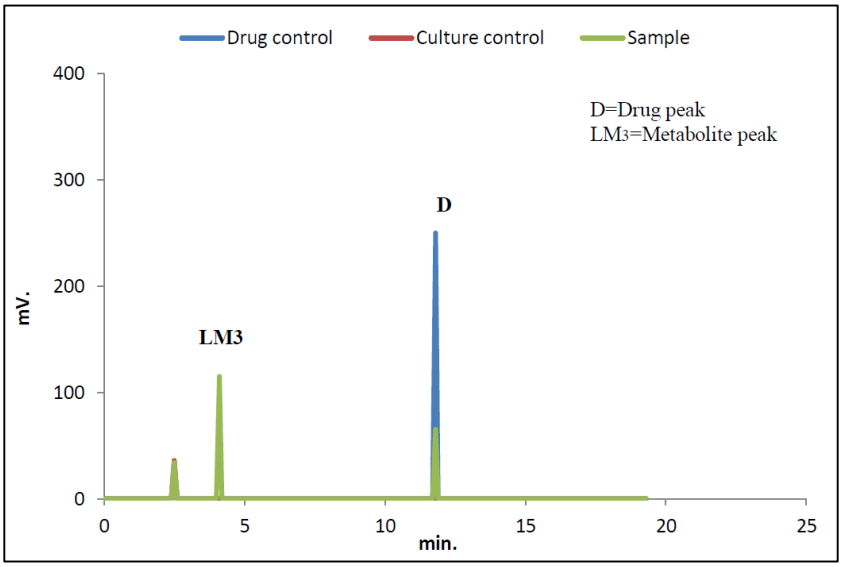


Fig 6: HPLC chromatogram of Loratadine from culture extracts of *Aspergillus Niger*

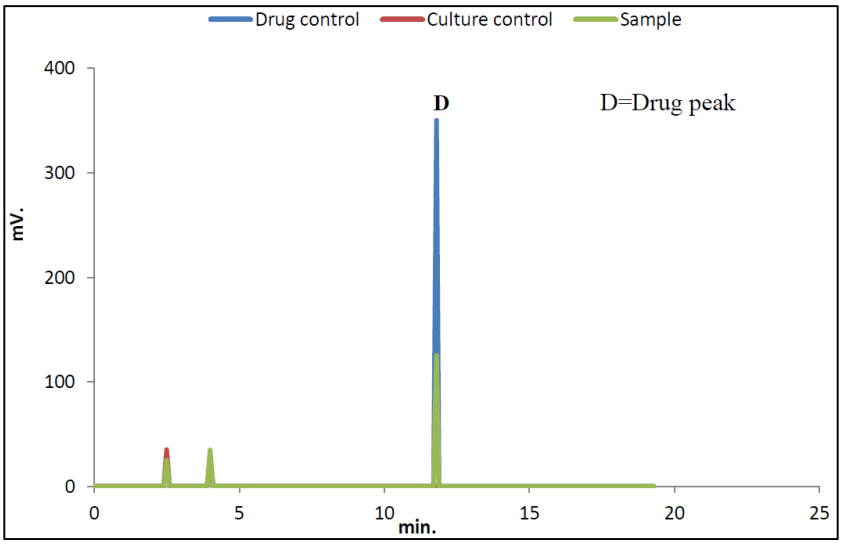


Fig 7: HPLC chromatogram of Loratadine from culture extracts of *Aspergillus fumigatus*.

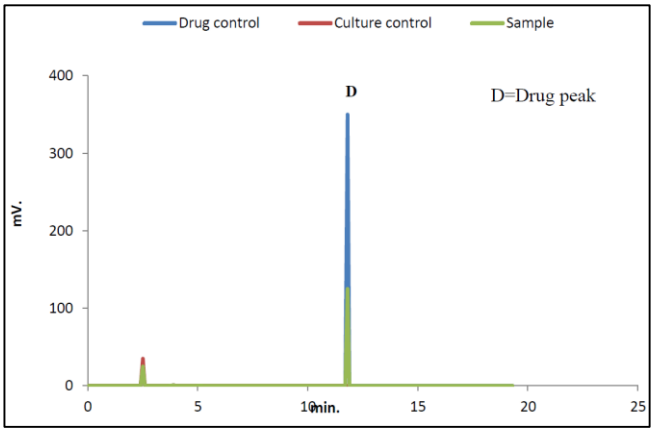


Fig 8: HPLC chromatogram of Loratadine from culture extracts of *Aspergillus ochreus*.

ii. Mass spectrometry

Mass spectrum of Loratadine and metabolites and their fragmentation patterns are shown in figures 9-15. Mass spectrum of drug was compared with mass spectra of metabolites (LM1, LM2 and LM3). The mass spectrum of Loratadine has shown a molecular ion peak at m/z 383(M+1) which is equal to the molecular weight of Loratadine as in figure no9.

Metabolite-LM1

The mass spectrum of LM1, a metabolite produced by *Cunninghamella elegans* has shown a molecular ion peak at m/z 343.0 [M+1] that is equal to the molecular weight of dihydroxy desloratadine as displayed in figure no-10. The structure of metabolite LM1 is also supported by its fragments at m/z 279,307,308 and 325 as per mass fragmentation pattern shown in figure no.11.

Metabolite- LM2

The mass spectrum of LM2, a metabolite produced by *Cunnighamella echinulata* has shown a molecular ion peak at m/z 339.10[M+1], which is equal to the molecular weight of desethoxy loratadine as shown in figure no-12 and its mass fragments at m/z 259,294 and 304 in mass fragmentation pattern are also supported the structure of LM2 as shown in figure no-13.

Metabolite-LM3

LM3, a metabolite produced by *Aspergillus niger* has shown a molecular ion peak at m/z 327.30[M+1] which is equal to the molar mass of mono hydroxy desloratadine in mass spectrum shown in figure no-14. The metabolite LM3 structure is supported by its mass fragments at m/z 274,292 and 298 in mass fragmentation pattern as shown in figure no-15.

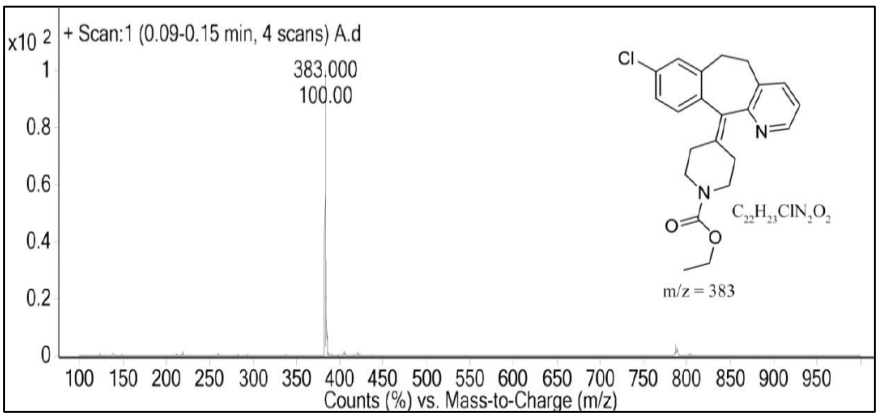


Fig 9: Mass spectrum of Loratadine

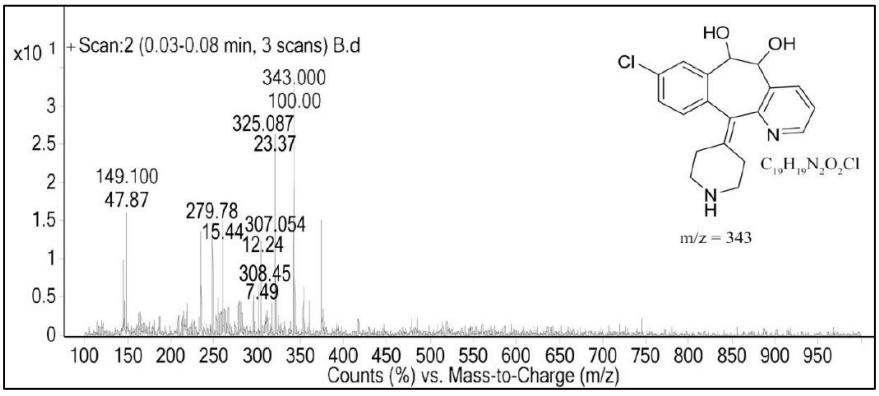


Fig 10: Mass spectrum of Loratadine metabolite, LM1

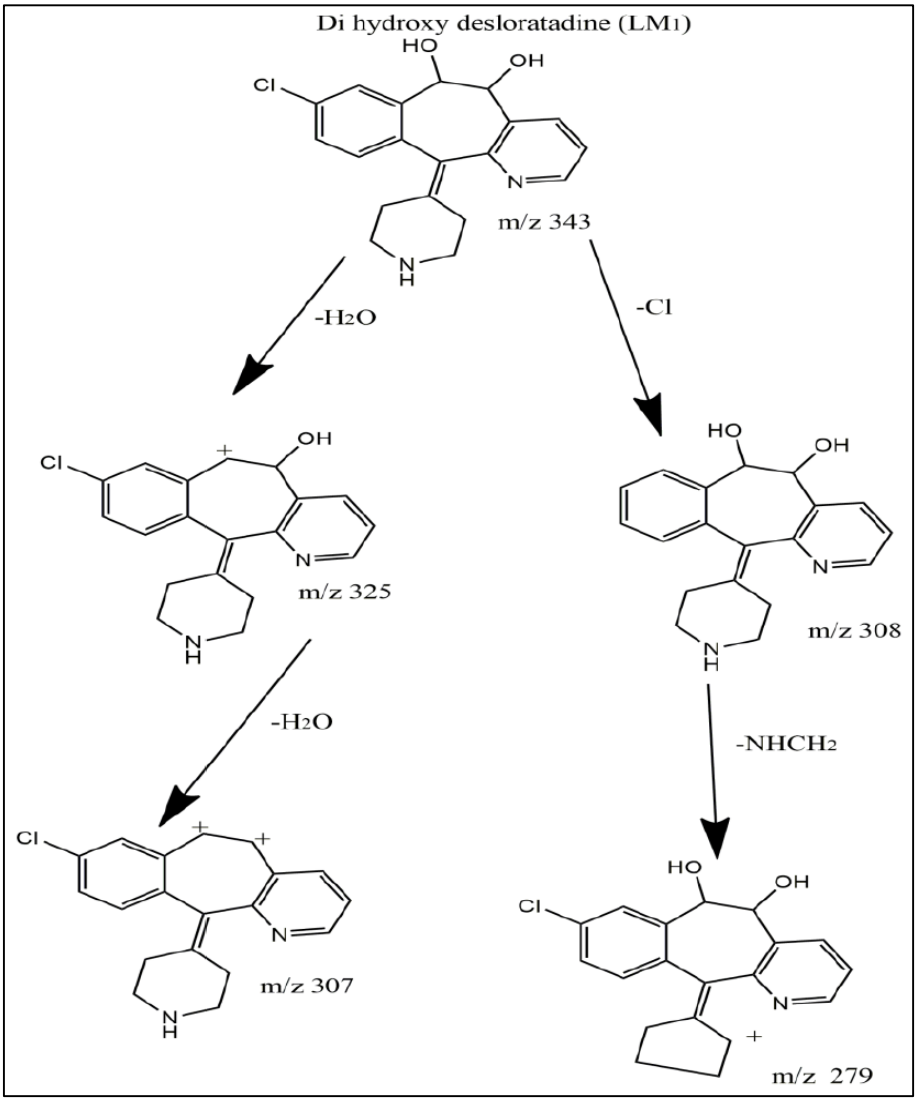


Fig 11: Mass fragmentation pattern of Loratadine metabolite, LM1

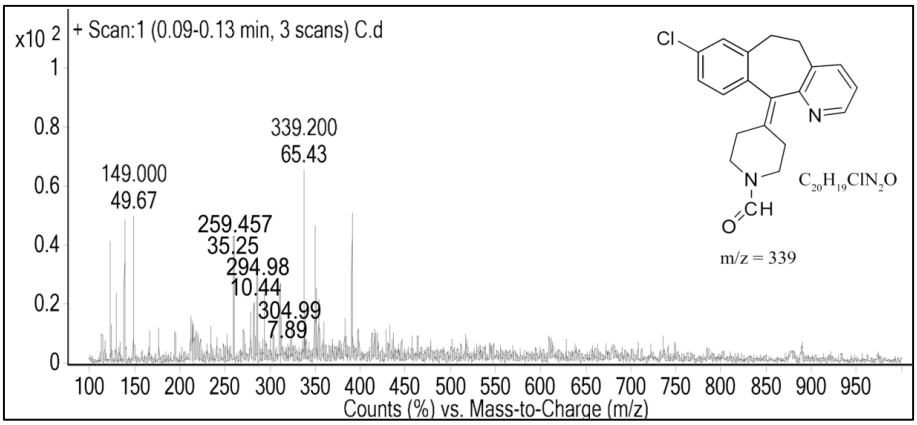


Fig 12: Mass spectrum of Loratadine metabolite, LM2

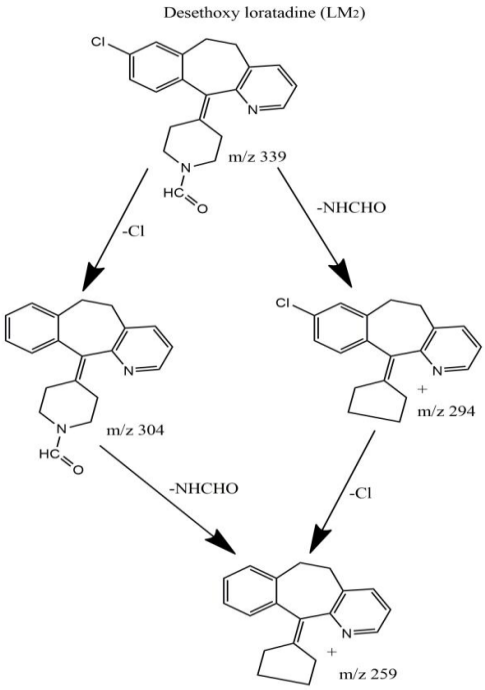


Fig 13: Mass fragmentation pattern of Loratadine metabolite, LM2

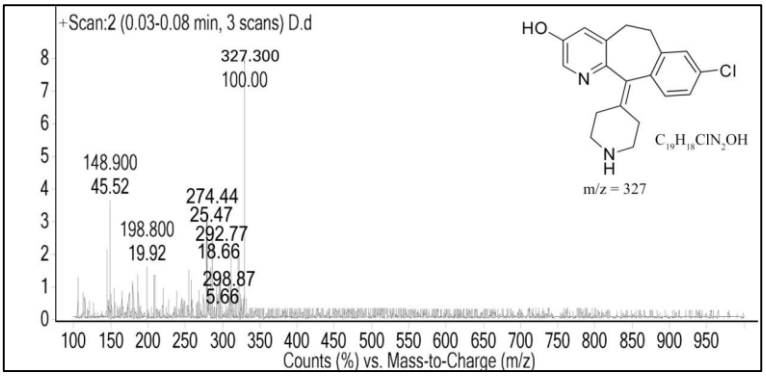


Fig 14: Mass spectrum of Loratadine metabolite, LM3

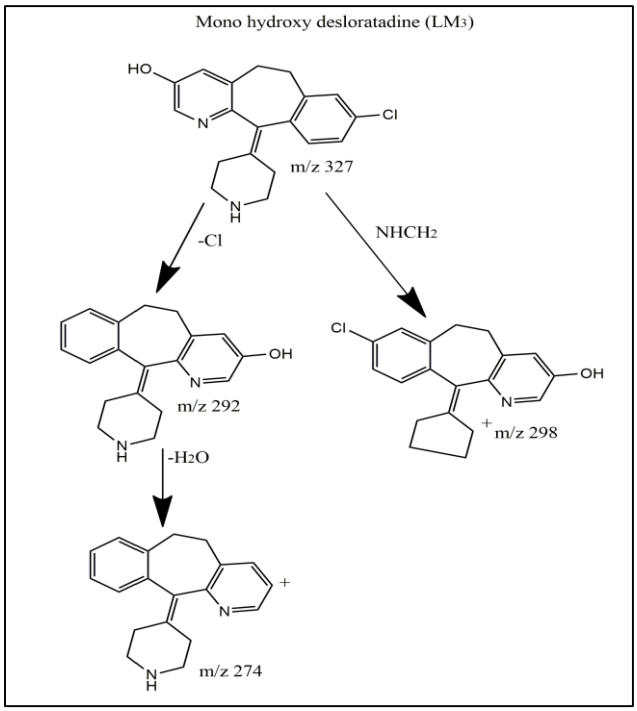


Fig 15: Mass fragmentation pattern of Loratadine metabolite, LM3

iii. [1]HNMR Spectroscopy

The structures of LM1, LM2 and LM3 were further confirmed by 1H NMR spectroscopy. 1H NMR spectrum of Loratadine shown in figure no-16 was used to compare with 1HNMR spectra of metabolites.

Metabolite-LM1

Dihydroxy desloratadine, the structure of LM1 was confirmed by appearance of a peak at δ 3.33 in its 1H NMR spectrum (figure no-17) represented the presence of dihydroxy group and absence of peaks at δ1.25 and δ 4.13 indicated removal carboethoxy group from the structure of Loratadine and the formation of desloratadine.

Metabolite-LM2

Structure of LM2 as desethoxy loratadine was confirmed by observation of a peak at δ 8.02 in 1HNMR spectrum dictated the presence of –CHO group as shown in figure no-18.

Metabolite-LM3

Mono hydroxy desloratadine, the structure of LM3 was confirmed by presence of a peak at δ 5.40 in its 1H NMR spectrum (figure no-19) stated the incidence of one hydroxy group and absence of peak at δ1.25 and δ 4.13 illustrated removal carboethoxy group which indicated the formation of desloratadine.

1H NMR proton assignment of Loratadine and its three fungal metabolites (LM1, LM2 and LM3) is shown in table no-7.

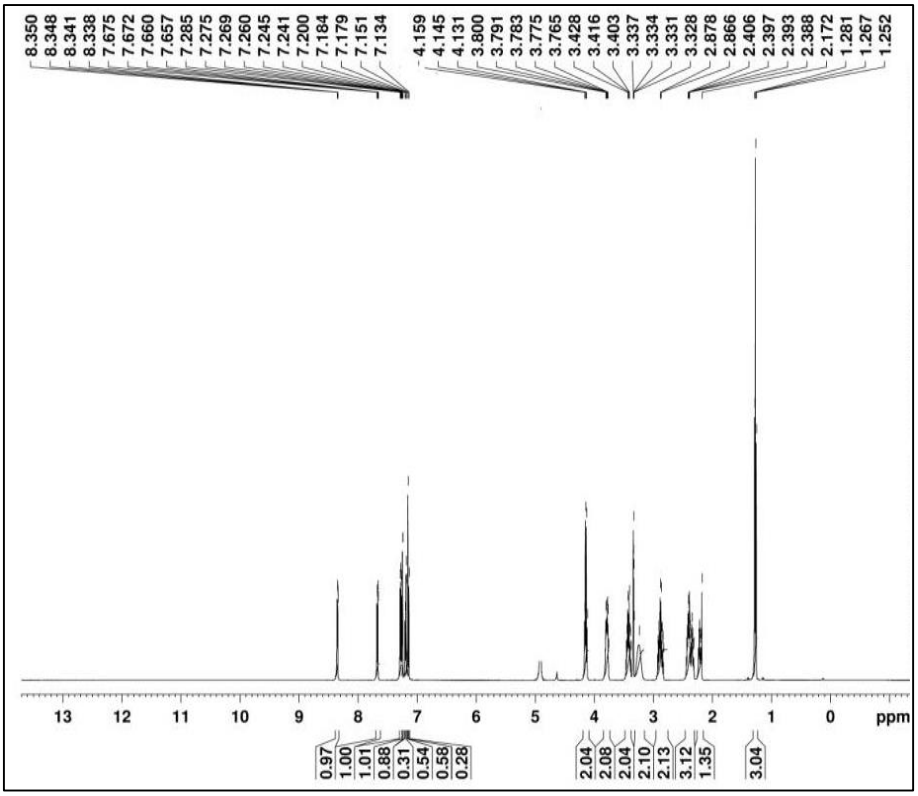


Fig 16: [1]HNMR spectrum of Loratadine

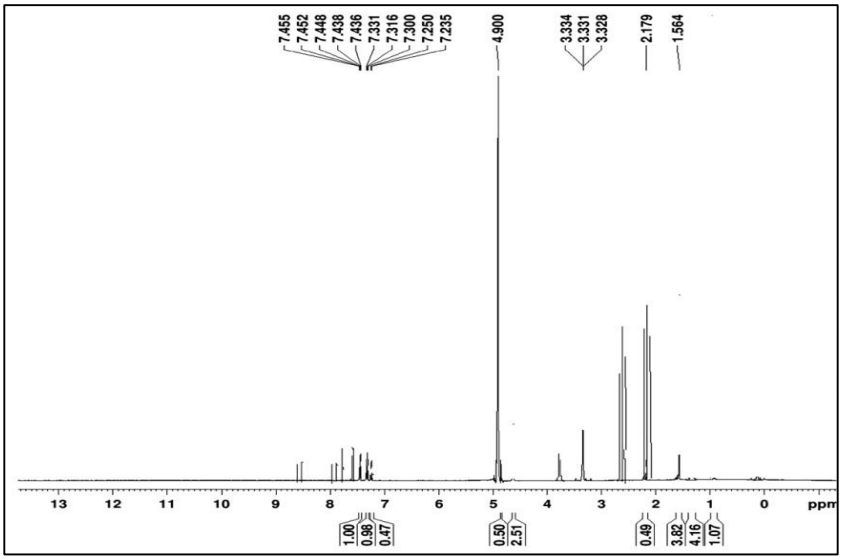


Fig 17: [1]HNMR spectrum of Loratadine metabolite, LM1

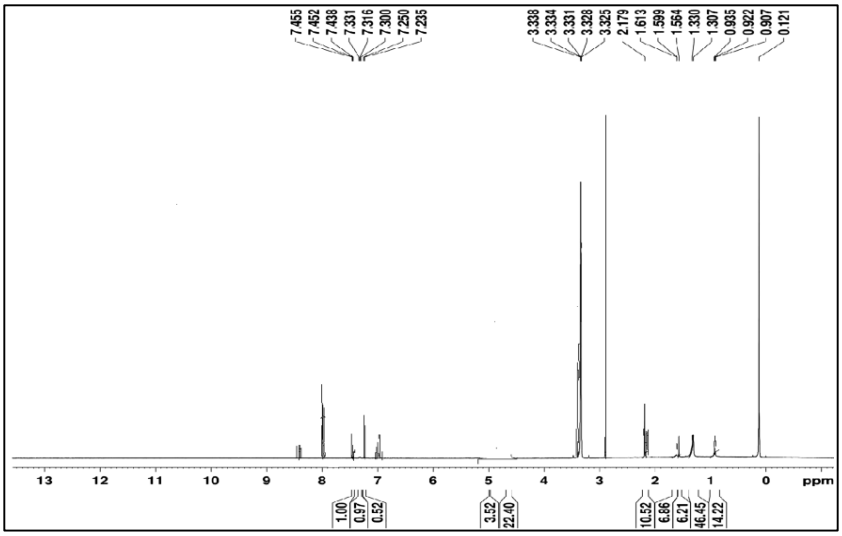


Fig 18: [1]HNMR spectrum of Loratadine metabolite, LM2

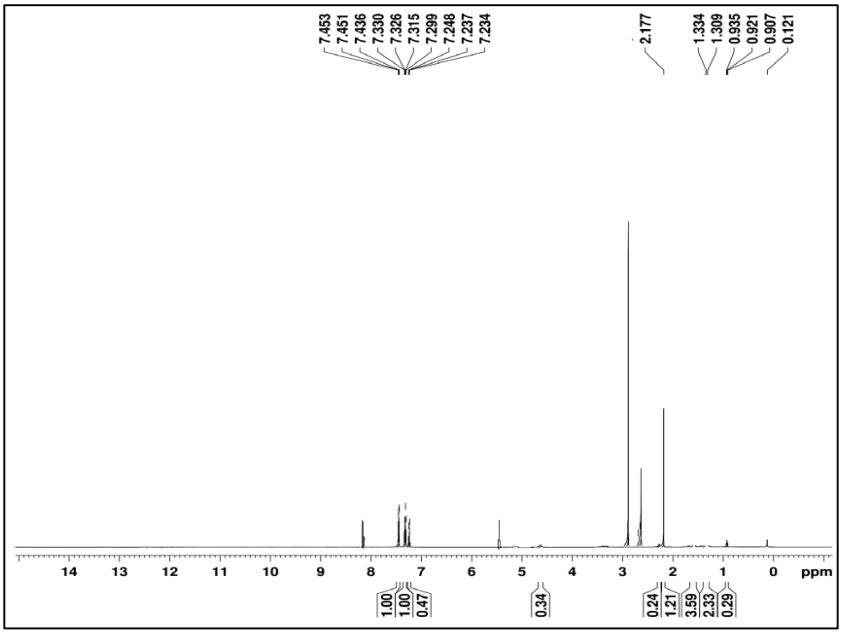


Fig 19: [1]HNMR spectrum of Loratadine metabolite, LM3

Table 7: 1HNMR Proton assignment of Loratadine and its metabolites

Compound name	1H NMR proton assignment	Figure no
Loratadine	1.25-1.28(t,3H,CH3), 2.17-2.40(m,4H,CH2), 2.86-2.87(t,4H,CH2) , 3.32-3.42(t,2H,CH2), 3.76-3.80 (t,2H,CH2), 4.13-4.15(t,2H,OCH2), 7.23-7.455(m,5H,CH), 8.43(s,1H.CH).	16
LM1	**1.564(s, H,NH),** 2.179(t,4H,CH2), 2.59(t,4H, CH2), **3.32-3.33(t,2H,OH),4.9(s,2H,CH),** 7.23-7.45(m,5H,CH),8.41(d,1H,CH).	17
LM2	2.17(s,4H, CH2), 2.88(m,4H, CH2), 3.325-3.33(m,4H, CH2), 7.23-7.45(m,5H,CH), **8.02(m,1H,CHO),** 8.43(m,1H,CH).	18
LM3	**1.30(s,1H,NH),** 2.17(s,4H,CH2), 2.59(m,4H,CH2), 2.88(d,4H,CH2), **5.40(s,1H,OH),** 7.23-7.45(m,4H,CH), 8.21(s,1H,CH).	19

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

Induction and Inhibition Studies of Fungal Metabolism

Induction and inhibition studies were conducted to find the nature of enzyme involved in fungal biotransformation of Loratadine. CYP 3A4 inducer (Carbamazepine) and inhibitor (Fluoxetine) were used to conduct induction and inhibition studies of metabolism for LM1 and LM3 formed by two fungi, *Cunnighamella elegans* and *Aspergilus niger* respectively, because, the formation of known active metabolite was found as an intermediate with these two fungi only. The metabolite (LM2) formed by *Cunnighamella echinulata* was a novel metabolite, so it is not possible to predict CYP 450 inducer and inhibitor to conduct induction and inhibition studies. The results of HPLC analysis of Loratadine metabolism during induction and inhibition studies by two fungi in presence of inducer and inhibitor are shown in table no-8.

Table 8: HPLC data of induction and inhibition studies of microbial metabolism of Loratadine

Name of the organism and metabolite	Type of study	Name of drug	Peak area (mV/s)		% metabolite formed
			Drug	Metabolite	
Cunnighamella elegans LM1	Biotransformation study	Loratadine	1007432	81321	8.07
	Induction study	Carbamazepine (as CYP 3A4 inducer)	1004934	81316	8.06
	Inhibition study	Fluoxetine (as CYP 3A4 inhibitor)	1007652	82961	8.2
Aspergillus niger LM3	Biotransformation study	Loratadine	256746	24554	9.5
	Induction study	Carbamazepine (as CYP 3A4 inducer)	296632	43261	14.5 *
	Inhibition study	Fluoxetine (as CYP 3A4 inhibitor)	73462	0	0 *
Cunnighamella elegans LM1	Biotransformation study	Loratadine	1007432	81321	8.07
	Induction study	Carbamazepine (as CYP 3A4 inducer)	1068942	183402	17.1 *
	Inhibition study	Fluoxetine (as CYP 3A4 inhibitor)	934761	7560	0.8 *

* Significant change in percentage of metabolite.

Metabolite - LM1

No significant change in the area of peaks of metabolite LM1 from culture extracts of *Cunnighmella elegans* in the presence of CYP 3A4 inducer (Carbamazepine) and inhibitor (Fluoxetine) was found. So the induction and inhibition studies with *Cunnighmella elegans* were further conducted by CYP2D6 inducer and inhibitor because Desloratadine (an active metabolite found to be mediated by both CYP3A4 and CYP2D6 in mammals) was formed as an intermediate product by *Cunnighamella elegans* (figure no-28). The study found that, increase in the percentage of metabolite LM1 formation from 8.07% to 17.1% in the presence CYP2D6 inducer (Dexamethasone) and decrease in the percentage of metabolite formation from 8.07% to 0.8% in presence of CYP 2D6 inhibitor (Paroxetine). It indicated that CYP2D6 like enzyme might be involved in biotransformation of Loratadine by *Cunnighamella elegans*. HPLC chromatograms of induction and inhibition studies of Loratadine metabolism by *Cunnighamella elegans* are shown in figures 20-23.

Metabolite-LM3

The percentage of metabolite formed by *Aspergillus niger* was increased from 9.5 % to 14.5% in presence of CYP 3A4 inducer and decreased from 9.5% to 0% in presence of CYP 3A4 inhibitor. It indicated that CYP3A4 like enzyme might be involved in biotransformation of Loratadine by *Aspergillus niger*. So, further induction and inhibition studies were not performed with CYP2D6 inducer or inhibitor. HPLC chromatograms of induction and inhibition studies of Loratadine metabolism by *Aspergillus niger* are shown in figures 24 and 25.

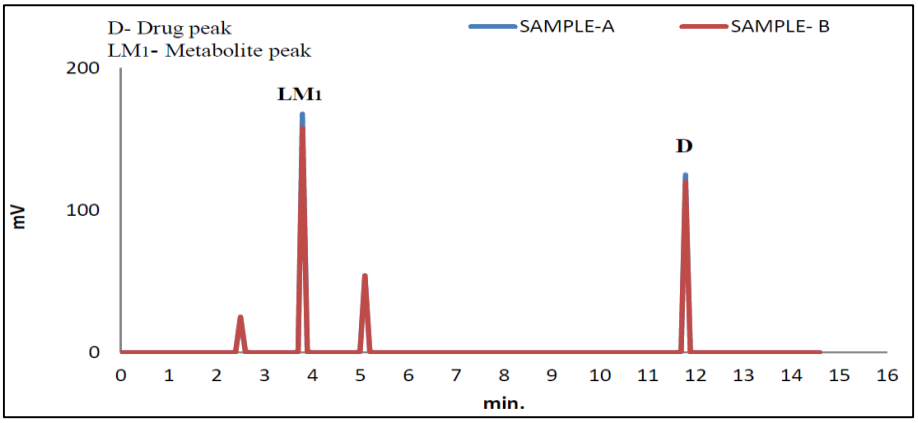


Fig 20: HPLC chromatogram of Loratadine in the presence of Carbamazepine from culture extracts of *Cunnighamella elegans* (CYP3A4 induction study)

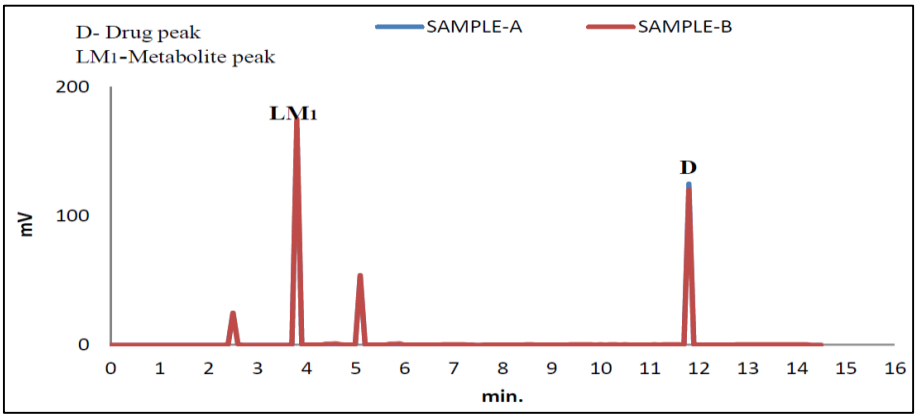
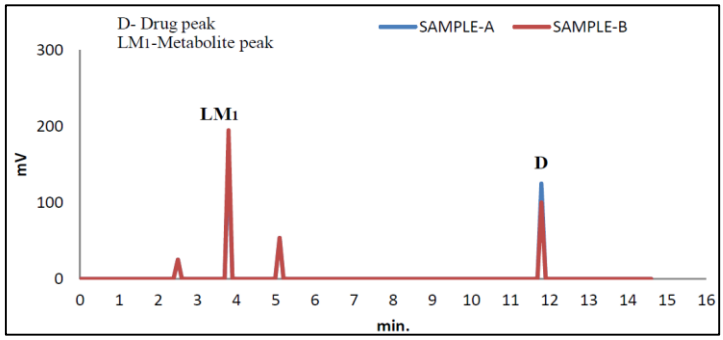
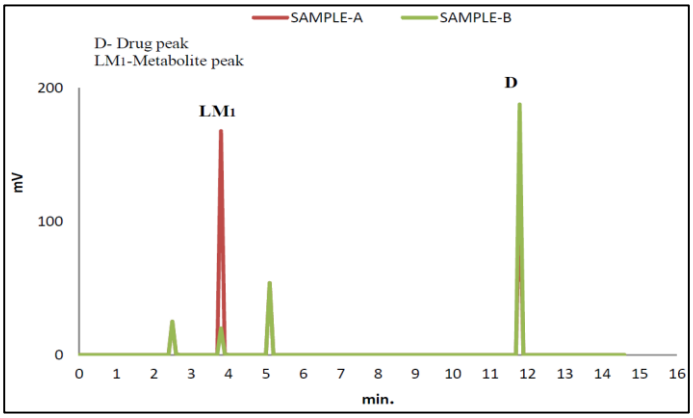


Fig 21: HPLC chromatogram of Loratadine in the presence of Fluoxetine from culture extracts of *Cunnighamella elegans* (CYP 3A4 inhibition study)



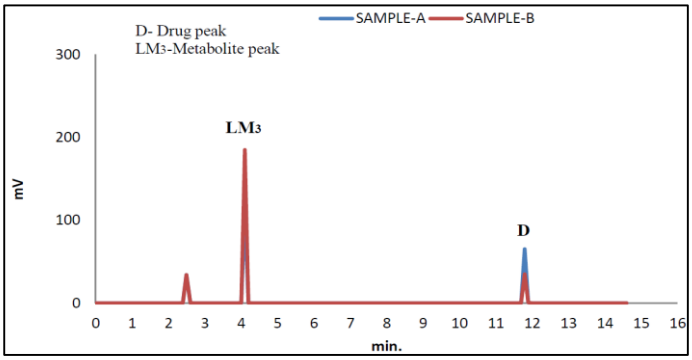
*Sample-A-without inducer *Sample-B- with inducer.

Fig 22: HPLC chromatogram of Loratadine in the presence of Dexamethasone from culture extracts of *Cunnighamella elegans* (CYP 2D6 induction study)



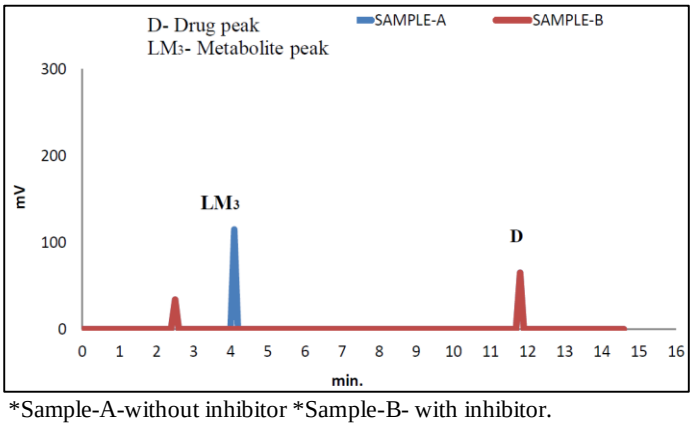
*Sample-A-without inhibitor *Sample-B- with inhibitor.

Fig 23: HPLC chromatogram of Loratadine in the presence of Paroxetine from culture extracts of *Cunnighamella elegans* (CYP 2D6 inhibition study)



*Sample-A-without inducer *Sample-B- with inducer.

Fig 24: HPLC chromatogram of Loratadine in the presence of Carbamazepine from culture extracts of *Aspergillus niger* (CYP3A4 induction study)



*Sample-A-without inhibitor *Sample-B- with inhibitor.

Fig 26: HPLC Chromatogram of Loratadine in the presence of Fluoxetine from culture extracts of *Aspergillus niger* (CYP3A4 inhibition study)

Enzyme Kinetic Studies

Enzyme kinetic studies were conducted with three organisms *Cunninghamella elegans*, *Cunnighamella echinulata* and *Aspergillus niger* for LM1, LM2 and LM3 respectively. 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 and analyzed by HPLC. The results of HPLC analysis obtained in the form of peak area were used to calculate percentage metabolite formed at different time points for each concentration. For each concentration of substrate, a graph was plotted between incubation time vs percentage metabolite formed. Six plots were obtained for six concentrations of substrate for each metabolite. Slope values obtained from each plot were considered as velocity of the metabolic reaction by respective fungi.

Effect of incubation period on percentage of 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. The graphs were plotted between incubation time and percentage metabolite formation to know the effect of incubation time on metabolite formation.

Metabolite-LM1

As the incubation time was increased the percentage of metabolite (LM1) formation was also increased up to 72 hrs of incubation, with all six concentrations of substrate as represented in figure no-26.

Metabolite-LM2

In case of formation of metabolite (LM2) by *Cunnighmella echinulata*, as the incubation time was increased, the percentage metabolite formation was also increased with six concentrations of substrate. Graphs were plotted between percentage of metabolite LM2 vs incubation time are represented in figure no-38. Effect of incubation time on percentage metabolite LM2 formation at six different concentrations of substrate is shown in figure no-27 and table no-6.

Metabolite -LM3

As the incubation time was increased the percentage metabolite LM3 formation was also increased with six concentrations of substrate. Graphs were plotted between incubation time vs percentage of metabolite LM3 are represented in figure no-40. Effect of incubation time on percentage metabolite LM3 formation for six different concentrations of substrate is shown in figure no-28 and table no-6.

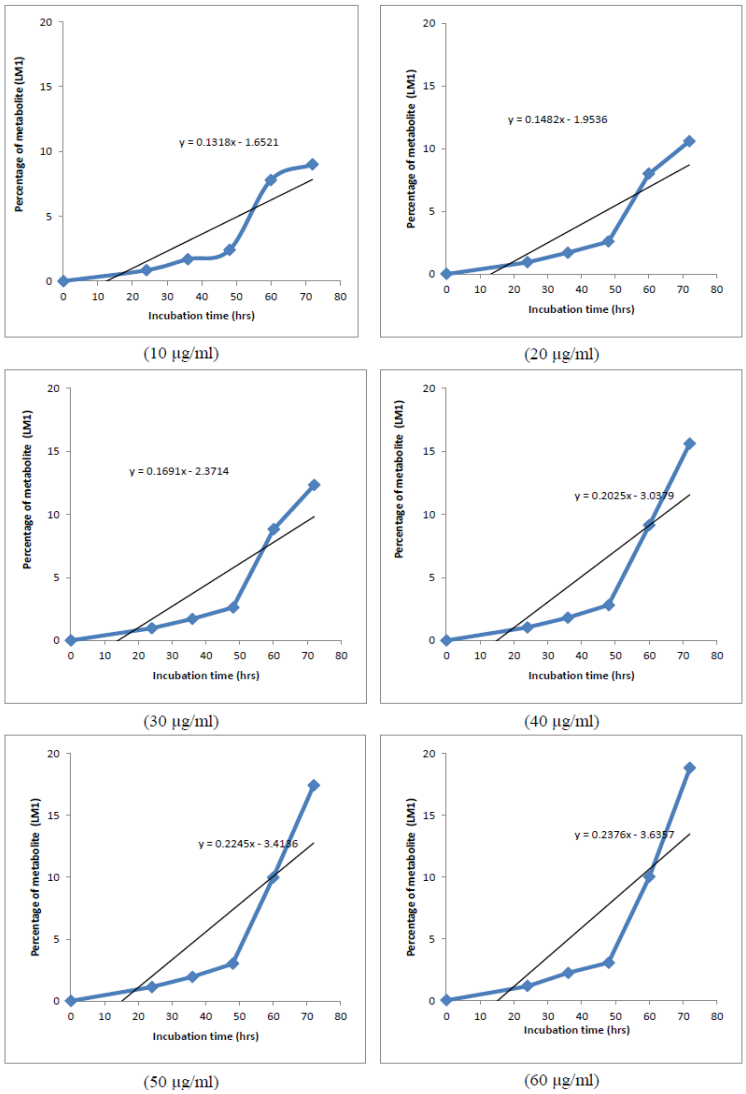


Fig 26: Percentage of metabolite, LM1 formed at different incubation time points with different concentrations of Loratadine

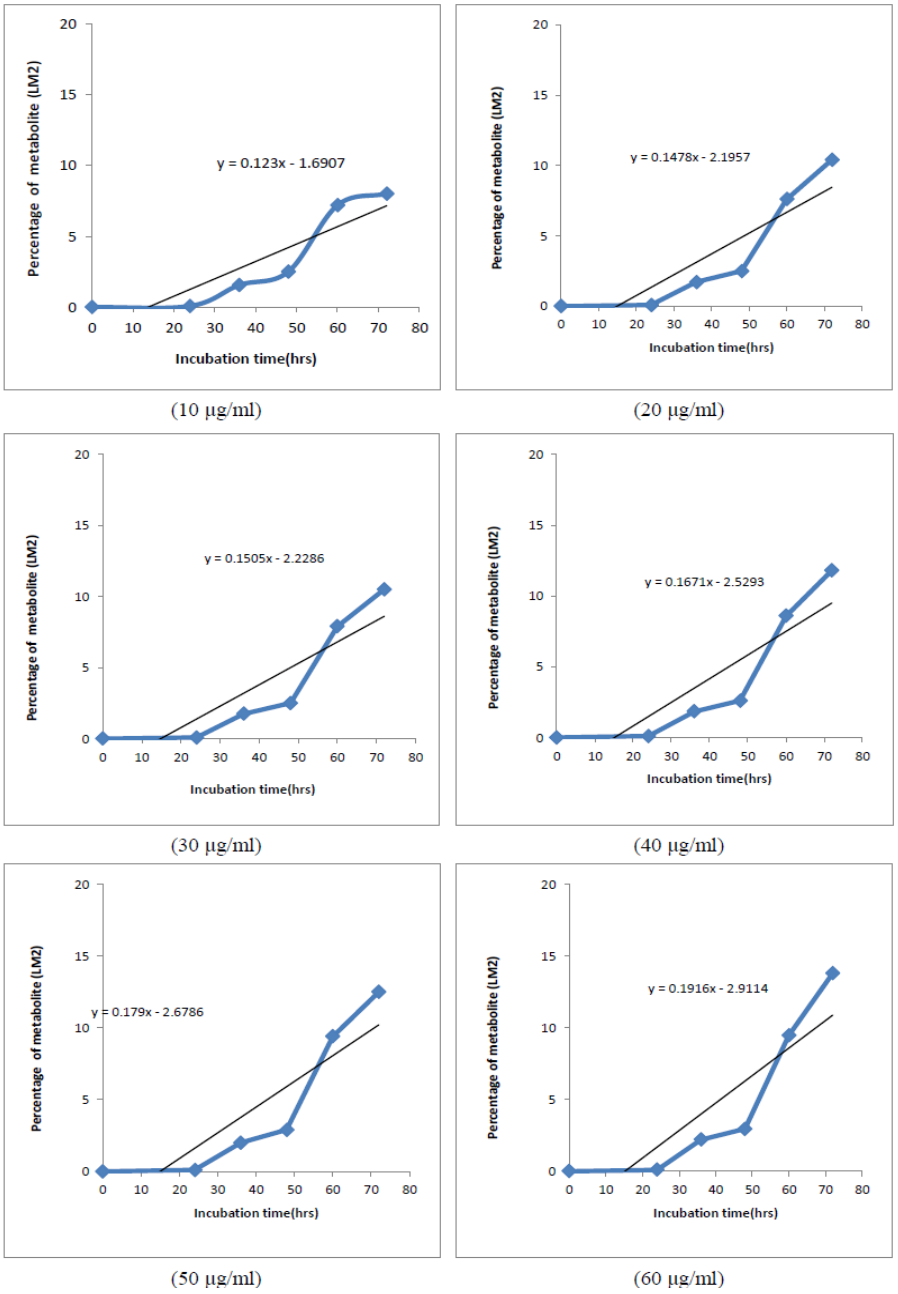
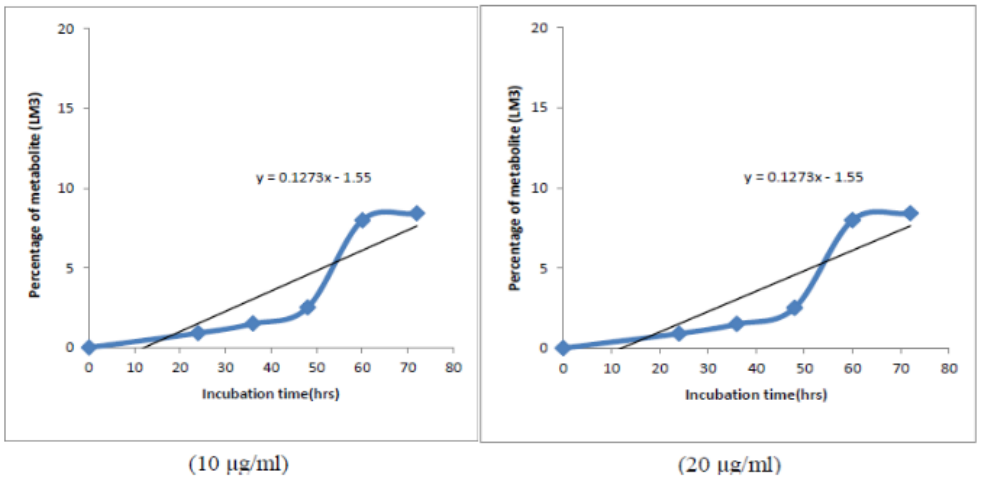


Fig 27: Percentage of metabolite, LM2 formed at different incubation time points with different concentrations of Loratadine



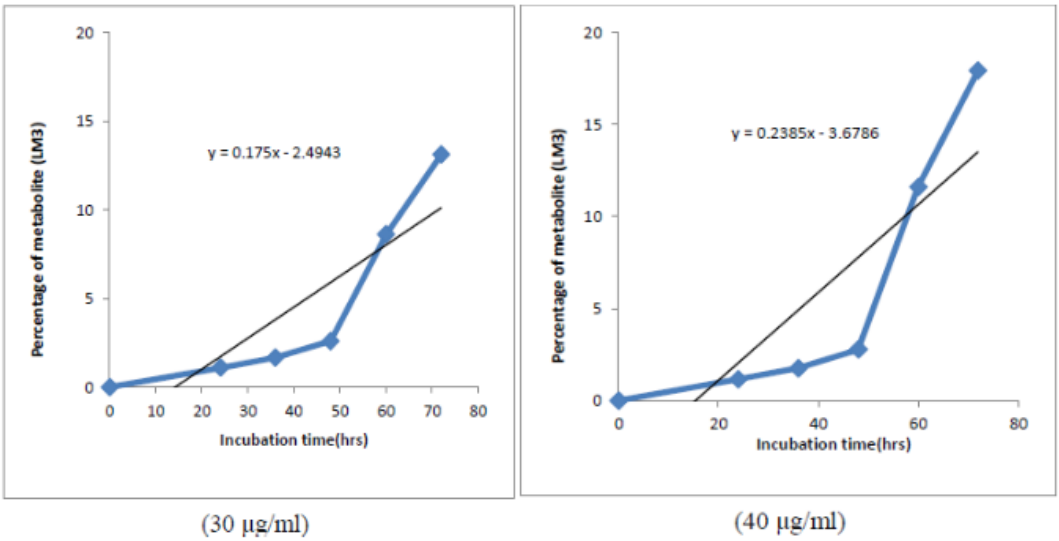


Fig 28: Percentage of metabolite, LM3 formed at different incubation time points with different concentrations of Loratadin

Slopes values were obtained from above plots for three metabolites at six different concentrations which are considered as velocity of metabolic reactions by respective organisms as represented in table no-9.

Table 9: Slope values (velocity) of Loratadine metabolites for six different concentrations

Substrate concentration (µg/ml)	Slope values or velocity (µg/ml/hr)		
	LM1	LM2	LM3
10	0.131	0.123	0.127
20	0.148	0.147	0.127
30	0.169	0.150	0.175
40	0.202	0.167	0.238
50	0.224	0.179	0.198
60	0.237	0.191	0.173

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 metabolite formation. Effect of substrate concentration on percentage of three metabolites (LM1, LM2 and LM3) after incubation of 72 hrs is represented in figure no-29, 30 and 31. After 72 hrs of incubation, formation of LM1 and LM2 were increased with the substrate concentrations from 10 to 60 µg/ml.

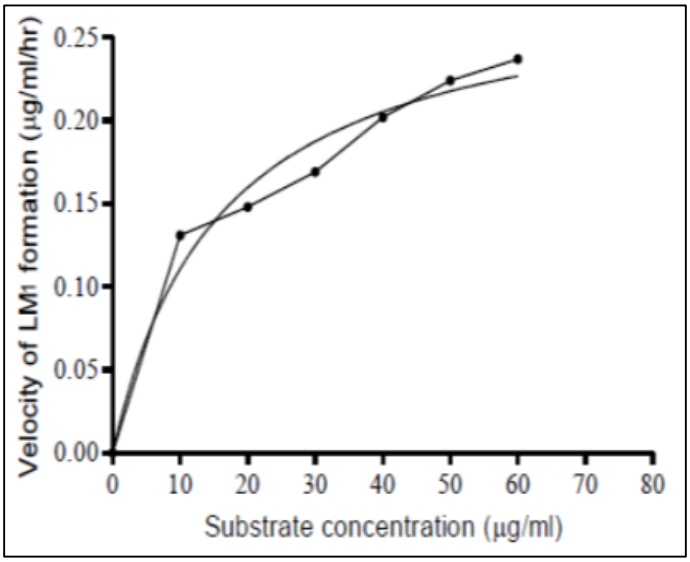


Fig 29: Michaelis-Menten plot of LM1

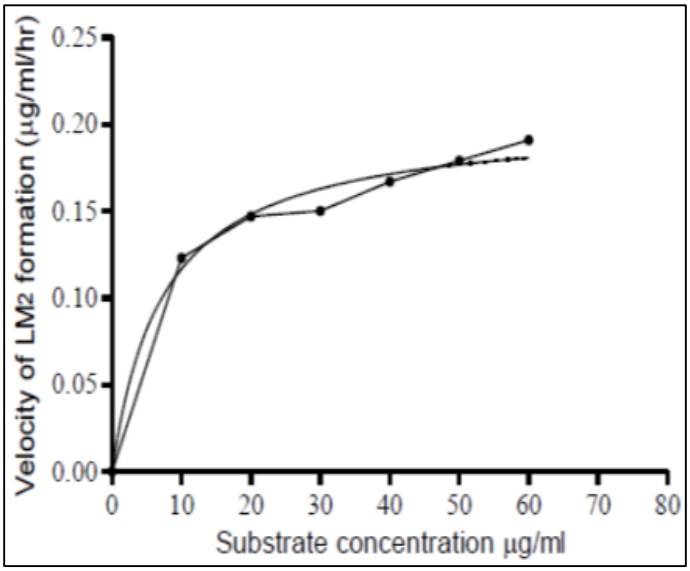


Fig 30: Michaelis-Menten plot of LM2

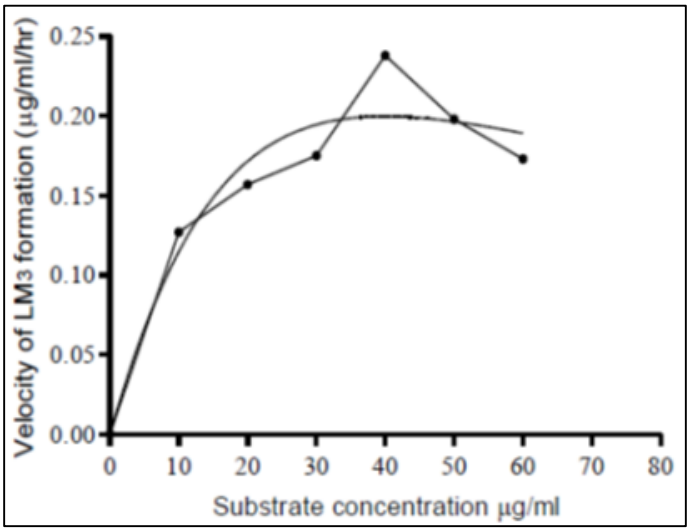


Fig 31: Substrate inhibition plot of LM3

Table 10: Kinetic parameters of Loratadine metabolism by three organisms

Kinetic Parameters	LM1 (by *Cunnighamella elegans)*	LM2 (by *Cunnighamella echinulata)*	LM3 (by *Aspergillus niger*)
Km (µg/ml)	15.96	7.47	39.68
Vmax (µg/ml/hr)	0.28	0.20	0.59
Ki (µg/ml)	-	-	40.11

Discussion

Screening Studies

The potential of fungi for their capacity to biotransform the drug Loratadine, a CYP 2D6 and CYP3A4 substrate [10] to its active metabolite was determined using six fungal cultures. The similar enzymatic systems like CYP 450 enzymes essential for biotransformation of Loratadine may be present in fungi, hence any one of these may have ability to biotransform Loratadine to its active metabolite, desloratadine. The conversion of Loratadine to its active metabolite was examined in the current study using fermentation method and HPLC analysis. Among six, the samples of *Cunnighmella elegans*, *Cunnighmella echinulata*, *Aspergillus niger* have shown extra peaks in HPLC compared to their respective controls might be due to its metabolites. Hence, these were considered as the fungi able to biotranform Loratadine to its metabolites LM1, LM2 and LM3 respectively. While, samples of other organisms were correlated with thier controls, identical peaks were found in samples and controls indicated no biotransformation and metaboite formation by other organisms.

Metabolite- LM1

The sample of *Cunnighamella elegans* has shown an extra peak at retention time of 3.8min. when compared withits controls. The extra peak indicated the formation of Loratadine metabolite (LM1). The structure of metabolite, LM1 was assesed by mass spectrometry and 1H NMR spectroscopic studies.

Metabolite- LM2

The sample of *Cunnighamella echinulata* has shown an extra peak at retention time of 3.6min.whencompared withits controls. The extra peak denoted the formation of Loratadine

metabolite (LM2) by this fungi. The structure of metabolite, LM2 was elucidated by mass spectrometry and 1H NMR spectroscopic studies.

Metabolite- LM3

The sample of *Aspergillus niger* has shown an extra peak at retention time of 4.1 min.when compared with its controls. The extra peak indicated the formation of Loratadine metabolite LM3 by *Aspergillus niger*. Mass spectrum of Loratadine metabolite [LM3] formed by *Aspergillus niger* has shown 56 Da lower molecular weight than that of Loratadine which suggested the loss of carboethoxy group from parent drug structure and addition of one hydroxy group to parent drug structure. The structure of metabolite LM3 was also supported by the mass fragments at m/z 298, 292 and 274. 1H NMR spectrum of LM3 has shown peak at δ 5.40 represented the presence of one hydroxy group and absence of peak at δ1.25 and δ 4.13 denoted removal carboethoxy group from parent drug structure.

Loratadine is extensively metabolised to an active metabolite 'desloratadine' in human by *in-vitro* liver microsomes [11] and other mammals similar to metabolites acquired in case of *Cunnighamella elegans* and *Aspergillus niger* in the present study. Whereas the metabolite LM2, was a novel metabolite as it was not found earlier in existing literature; it represented that, this is a new derivative of Loratadine produced by *Cunninghamella echinulata* in the present work.

Microbial metabolism induction and inhibition studies

Two different CYP enzymes 3A4 and 2D6 are involved in mammalian metabolism of Loratadine to form desloratadine. Hence, whether the enzymes like these (CYP3A4 and 2D6) are involved in fungal metabolism of Loratadine or not was examined by induction and inhibition studies. The enzymes involved in biotransformation of Loratadine by two fungi, which were able to form desloratadine or its derivatives (LM1 and LM3) were confirmed by induction and inhibition studies.

In present study, a CYP 3A4 inducer [Carbamazepine], inhibtor [Fluoxetine], a CYP 2D6 inducer [Dexamethasone] and inhibitor [Paroxetine] were used during induction and inhibition studies to cofirm the type of specific enzymes involved in biotransformation of Loratadine by *Cunnighamella elegans* and *Aspergillus niger*. Indcution and inhibition studies were condcuted for LM1 and LM3 and calculated the percentage of metabolite formed during induction and inhibition studies.

Metabolite- LM1

The percentage of metabolite (LM1) formation was not influenced in case of samples incubated with *Cunnighamella elegans* in presence of inducer and inhibitor. It stated that CYP 3A4 like enzyme might not be involved in the microbial biotransformation of Loratadine by *Cunnighamella elegans*. So, the induction and inhibition studies were further conducted with CYP2D6 inducer and inhibitor. The percentage of metabolite formation was increased in presence of CYP 2D6 inducer and decreased in the presence of CYP 2D6 inhibitor. It indicated that CYP 2D6 like enzyme might be involved in the microbial biotransfromation of Loratadine by *Cunnighamella elegans* to form 'LM1' a derivative of its active metabolite desloratadine.

Metabolite- LM3

In case of samples incubated with *Aspergillus niger* the percentage metabolite (LM3) formation was increased in the presence of CYP 3A4 inducer and decreased in the presence of CYP 3A4 inhibitor. It indicated that CYP 3A4 like enzyme might be involved in the microbial biotransformation of Loratadine by *Aspergillus niger*. The results of CYP 2D6 and CYP 3A4 induction and inhibition studies of micobial metabolism of Loratadine by *Cunnighamella elegans* (LM1) and *Aspergillus niger* (LM3) confirmed the involvement of CYP 2D6 and CYP 3A4 like enzymes respectively in fungal biotransformation of Loratadine similar to mammals.

Enzyme Kinetic Studies

The enzyme kinetic studies are crucial to trace out the affinity of fungal enzyme towards the substrate by estimating kinetic parameters like Km and Vmax. Kinetics of microbial metabolism 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 diffrent incubation periods for each concentration.

Metabolite- LM1

Based on the results, it was noticed that, as the incubation time was increased the percentage metabolite (LM1) formation was also increased along with increase in the substrate concentration in the metabolism of Loratadine by *Cunnighamella elegans*. Velocity of the metabolism reaction was obtained from slope values of lines found in graphs plotted between incubation time and percentage of metabolite (LM1) for each concentation of substrate. The study of effect of incubation time on percentage of metabolite (LM1) with six different concentrations of substrate has showed that, the percentage of metabolite LM1 formation was maximum after 72 hrs of incubation when compared with 24,36,48 and 60 hrs of incubation. The study of effect of substrate concentration on percentage metabolite LM1 formation revealed that, the maximum percentage of metabolite (LM1) was observed at 60 µg/mlsubstrate concentration after 72 hrs of incubation. Then, Michaelis-Meneten kinetics was applied to data of velocity of metabolism reaction vs substrate concentration inorder to estimate Km and Vmax. Km and Vmax values of metabolism reactions mediated by enzymes in *Cunnighamella elegans* to form LM1 were 15.96µg/mland 0.28µg/ml/hrrespectively. These values stated that metabolism reaction which formed LM1proceeded with the maximum velocity (Vmax) of 0.28 µg/ml/hr, and half maximum velocity of reactionwas found at the concentration (Km) of 15.96 µg/ml.

Metabolite- LM2

The enzyme kinetic studies of fungal metabolism mediated by enzymes in *Cunnighamella echinulata* to form LM2 was found that, as the incubation time was increased the percentage metabolite formation was also increased along with increase in the substrate concentration in the metabolism of Loratadine. 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 (LM2). The study of effect of incubation time on percentage of metabolite (LM2) with six different concentrations of substrate has showed that, the

percentage of metabolite LM2 formation was maximum after 72 hrs of incubation when compared with 24,36,48 and 60 hrs of incubation. The study of effect of substrate concentration on percentage metabolite LM2 formation revealed that, the maximum percentage of metabolite (LM2) was observed at 60 µg/mlsubstrate concentration after 72 hrs of incubation. The Michaelis–Menten kinetics was applied to kinetics of LM2 formation because, the velocity of reaction was increased with increase in the substrate concntrations from 10 µg/ml to 60 µg/ml, Km and Vmax values of metabolism reactions mediated by enzymes in *Aspergillus niger* to form LM2 were 7.47 µg/ml and 0.20 µg/ml/hrrespectively. These values stated that metabolism reaction which formed LM2proceeded with the maximum velocity (Vmax) of 0.20µg/ml/hr, and half maximum velocity of reaction was found at the concentration (Km) of 7.47µg/ml.

Metabolite- LM3

Intrestingly in case of micobial metabolism mediated by enzyme in *Aspergillus niger* to form LM3 as the incubation time was increased the percentage metabolite formation was increased. Velocity of the metabolism reaction was obtained from slope values of lines found in graphs plotted between incubation time and percentage of metabolite (LM3) for each concentation of substrate. The study of effect of incubation time on percentage of metabolite (LM3) with six different concentrations of substrate has showed that, the percentage of metabolite LM3 formation was maximum after 72 hrs of incubation when compared with 24,36,48 and 60 hrs of incubation. The study of effect of substrate concentration on percentage metabolite LM3 formation revealed that, the maximum percentage of metabolite (LM3) was observed at 40 µg/mlsubstrate concentration after 72 hrs of incubation (figure no-42). As substrate concentartion was increased, further decrease in the metabolite formation was observed after a concentration of 40 µg/ml. It indicated that, the percentage of metabolite (LM3) was decreased with increased concentration of 50 and 60µg/ml, might be due to some inhibitory effects. Hence, Michaelis-Meneten kinetics could not be applied to this results and substrate/product inhibition kinetics was applied to estimate kinetic parameters like Ki, Km and Vmax. These values of metabolism reactions mediated by enzymes in *Aspergillus niger* to form LM3 were 40.11 µg/ml, 39.68µg/ml and 0.59µg/ml/hrrespectively. It stated that, metabolism reaction which formed LM3proceeded with the maximum velocity (Vmax) of 0.59µg/ml/hr, and half maximum velocity of reaction was foundat the concentration of (Km)39.68µg/mland metabolite (LM3)formation was inhibited at40.11 µg/ml(Ki) concentration of Loratadine.

Conclusion

It can be concluded that, *Cunnighamella elegans*, *Cunnighmella echinulata* and *Aspergillus niger* have shown the ability to convert Loratadine to its metabolites due to the presence of enzymes essential for biotransfromation of Loratadine. Hence, it can be demonstrated that *Cunnighamella elegans* and *Aspergillus niger* have potential for production of an active metabolite, desloratadine as their metabolites were derivatives of desloratadine. Formation of this active metabolite was similar to mammals. Thus, the present study revealed that, *Cunnighamella elegans* can be used as model for CYP2D6 and *Aspergillus niger* can be used as model for CYP3A4 to study pharmacological and toxicological properties and the biotransformation of other similar drugs. Enzyme kinetic studies revealed that, metabolism mediated by enzymes present in *Cunnighmella echinulata* possessed low Km value than enzymes present in *Cunnighamella elegans* and *Aspergillus niger*. So the enzyme present in *Cunnighamella echinulata* has more affinity towards Loratadine when compared with other two enzymes present in *Cunnighamella elegans* and *Aspergillus niger*. So,maximum metabolite can be produced by *Cunnighmella echinulata* with less quantity of Loratadine.

References

1. Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, *et al*. P450 superfamily; update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics. 1996; 6:1-42.
2. Ramanathan R, Reyderman L, Kulmatycki K, Su AD, Alvarez N, Chowdhury SK, Alton KB, *et al*. Disposition of loratadine in healthy volunteers. Xenobiotica. 2007; 37(7):753-769.
3. Moody JD, James P Freeman, Peter P Fu, Cerniglia CE. Biotransformation of Mirtazapine by *Cunninghamella elegans*. Drug Metbolism Disposition. 2002; 30(11):1274-1279.
4. Azerad R. Microbial models for drug metabolism. Advances in Biochemistry and Biotechnology. 1999; 63:169-218.
5. Smith RV, Rosazza JP. Microbial models of mammalian metabolism. Journal of Pharmaceutical Sciences. 1975; 64:1737-1758.
6. Moffat AC. 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.
7. 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.
8. Rajeev Soni, Gali Vidya Sagar. Design and development of quick dissolving tablet containing Loratadine by direct compression Method. International Journal of Pharmaceutical, Chemical and Biological Sciences. 2013; 3(3):771-800.
9. Magang shou, Qin mei, Michael W Ettore, JR, Renke Dai, Thomas A Baillie, 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.
10. Dorra Dridi, Pierre Marquet. Kinetic parameters of human P450 isofor involved in the metabolism of the antiallergic drug, loratadine. International Journal of Biology and Biological Sciences. 2013; 2(2):019-027.
11. Ghosal A, Gupta S, Ramanathan R, *et al*. Metabolism of loratadine and further characterization of its in vitro metabolites. Drug Metabolism Letters. 2009; 3(3):162-170.