



# Screening and evaluation of fungal resources for loratadine metabolites

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Loratadine is a selective inverse agonist of peripheral histamine H1-receptors. Microbial biotransformation gained a lot of attention for its ability to convert molecules to valuable medicinally active substances. The main objective of the present research was to investigate the ability of different fungi to biotransform the drug loratadine to its active metabolite desloratadine, because desloratadine is four times more potent, possess longer duration of action than loratadine and is effective at low doses. The screening studies were performed with selected fungi using their respective broth media and sterile incubation conditions. The drug and metabolites formed (if any) were extracted and analysed using HPLC analysis. Structural elucidation and confirmation of metabolites were by mass and proton NMR spectroscopy. Among the six fungi selected, *Cunninghamella elegans*, *Cunninghamella echinulata* and *Aspergillus niger* cultures showed extra peaks at 3.8, 3.6 and 4.1 min, respectively, in HPLC when compared with their controls, which indicated the formation of metabolites. The metabolites thus formed were isolated and their structures were confirmed as dihydroxy desloratadine, desethoxy loratadine and 3-hydroxy desloratadine by *Cunninghamella elegans*, *Cunninghamella echinulata* and *Aspergillus niger* cultures, respectively, by mass spectrometry and NMR spectroscopy. Three fungi were identified to have the ability to biotransform loratadine to its active metabolite and other different metabolites.

**Keywords.** *Aspergillus niger*; *Cunninghamella echinulate*; *Cunninghamella elegans*; desloratadine; loratadine; microbial transformation

## 1. Introduction

Loratadine, a piperidine ring derivative, is a powerful long-acting, non-sedating tricyclic antihistamine with particular fringe H1-receptor opponent movement. It is utilized in nasal and non-nasal indications of regular sensitivities and skin rashes (Haria *et al.* 1994). Loratadine is given orally, and is assimilated from the gastrointestinal tract, and has fast first-pass hepatic metabolism (Parfitt 1999). It is metabolized by isoenzymes of the cytochrome P450 system, including CYP3A4, CYP2D6, and, to a lesser extent, several others (Hibert *et al.* 1987; Barecki *et al.* 2001; Dridi and Marquet 2013). Its metabolite desloratadine is largely responsible for the anti-histaminergic effects and desloratadine's half-life is 28 h (Rele and Gurav 2012). Drug metabolism can be defined as the chemical modification of the drug which occurs in a biological environment. Drug metabolism also results in the formation of active metabolites (Parshikov *et al.* 2012) which may have activity similar or more potent than the parent compound, or may have different biological actions. Microbial transformations can be used as potential feasible methods due to their innovative and improved enzymatic systems to produce chemical compounds

which are difficult to produce by synthetic procedures (Peterson *et al.* 1952; Sedlacek 1988; Charney and Herzong 1976; Ereshefsky 1996; Bhatti and Khera 2012; Yang *et al.* 2012). From a pharmaceutical perspective, hydroxylations and glycosylations (Faber and Franssen 1993; Alarcon *et al.* 2005) are considered to be particularly useful bioconversions. They can yield new drugs, and existing drugs can be improved so as to increase activity and/or stability and decrease toxicity. In view of the above facts, the present attempt was aimed at production of active metabolite (desloratadine) or any other metabolites of loratadine using fungal cultures because desloratadine is four times more potent, possess longer duration of action than loratadine and is effective at low doses (Katchen *et al.* 1985; Hilbert *et al.* 1987; Kreutner 1987).

## 2. Materials and methods

### 2.1 Microorganisms

*Cunninghamella elegans* (NCIM-689), *Cunninghamella blackesleeana* (NCIM-691), *Cunninghamella echinulate*

(NCIM-687), *Aspergillus niger* (NCIM-589), *Aspergillus fumigatus* (NCIM-902), *Aspergillus ochraceus* (NCIM-1140) were acquired from National chemical laboratory(NCL) Pune, India.

## 2.2 Chemicals

Loratadine was a gift sample procured from Shasun pharmaceuticals, Guindy, Chennai, Tamil Nadu, India. Chemicals were purchased from S.D. Fine Chemicals, Mumbai, India. HPLC grade solvents were used for analysis.

## 2.3 Cultures

All cultures were sustained on respective agar slopes at 4°C (Azerad 1999) and sub-cultured every 6 months to maintain the viability. The suitable medium for fungal cultures is potato dextrose broth (Moody *et al.* 2002) consisted of potatochips (200 g/1000 mL, boiled for 30 min), dextrose 20 g, yeast extract 0.1 g. Prior incubation, prepared medium was sterilised by autoclaving at 121°C for 30 min (Smith and Rosazza 1975).

## 2.4 Fermentation studies

250 mL Erlenmeyer flasks containing 50 mL medium were used to implement fermentation protocol. For each bioconversion study 3 flasks were set, two were controls: drug control, which had drug added to broth medium incubated without organism, and culture control, which comprised broth medium inoculated with the respective fungi without drug. The third one was a sample consisting of both drug and culture along with medium, which were added aseptically in sterile media (Moffat 1986). All the flasks were subjected to same operating conditions: temperature was set at 28°C and agitation speed was 120 rpm for 72 h on a rotary shaker incubator. Loratadine drug solution was prepared by adding 10 mg of drug to 10 mL methanol; from this stock solution, 0.5 mL was added to respective flasks in the study so as to sustain 10 µg/mL.

## 2.5 Extraction method

The grown microbes in the flasks were inactivated by heating them in water bath at 50°C for 30 min. Then, the contents of the flasks were carefully restationed to centrifuge tubes and centrifuged at 3000 rpm for 10 min (Laboratory Centrifuge C-854/8, Remi instruments, Mumbai, India).

After centrifugation the supernatants collected in separate boiling tubes from each flask were stored in refrigerator. Loratadine and the formed metabolites were extracted by treating the collected supernatant with the commixture of diethyl ether and dichloromethane in the ratio of 70:30 (Nagwa *et al.* 2014). Then, the organic layer was separated and air-dried. The dried extract was reconditioned with mobile phase for HPLC analysis. The pure drug was also analysed by HPLC and taken as a standard.

## 2.6 Analytical methods

**2.6.1 High-performance liquid chromatography:** A phenomenex luna 5µ C18(2) 100A 250×4.60 mm (Phenomenex, USA) was used for separation of drug and metabolites at a flow rate of 1 mL/min (Gajjela Ramulu *et al.* 2011), and the solvent system consisted of acetonitrile:water:methanol (1:2:1) in a HPLC system (Shimadzu, Kyoto, Japan) with LC 20 AD binary pump solvent delivery module and SPD 20AV UV detector. Sensitivity was set at 0.0001 aufs. UV detection wavelength was set at 254 nm (Soni and Sagar 2013) and run time was 20 min.

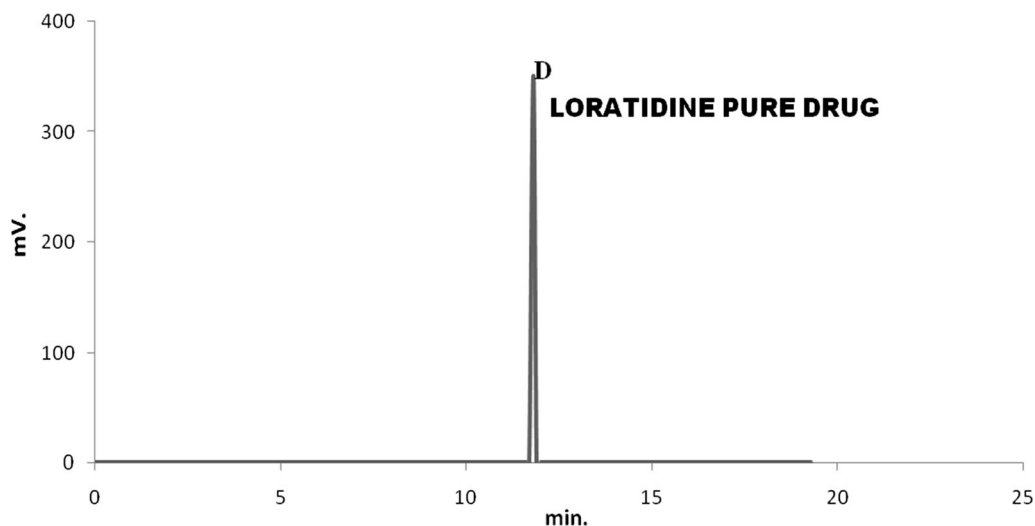
**2.6.2 Mass spectrometry:** The metabolites found in samples of cultures selected during HPLC analysis were collected and further analysed by mass spectroscopy and PNMR spectroscopy for determination of their structures. Mass spectrometer (Agilent technologies, Germany) model was API 3000MS operating 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).

**2.6.3 Proton nuclear magnetic resonance (PNMR) spectroscopy:** The structures of metabolites collected from HPLC were confirmed by PNMR spectroscopy by using BRUKER AVANCE 400 MHz [SAIF IIT, MADRAS]. Deuterated methanol was used as solvent to analyse loratadine and its metabolite (figure 1).

## 3. Results

### 3.1 Microbial screening and metabolite identification

The present research work involved screening of six different fungi for biotransformation of loratadine. Microbial screening was conducted using fermentation protocol and HPLC analysis. It is significant that the chromatograms of the blank culture controls did not show any drug peak. Blank substrate controls showed the presence of drug at retention time of 11.8 min (table 1). HPLC chromatograms of loratadine incubated with *Cunninghamella elegans*,



**Figure 1.** HPLC chromatogram of loratadine.

**Table 1.** HPLC data of loratadine from fungal culture extracts

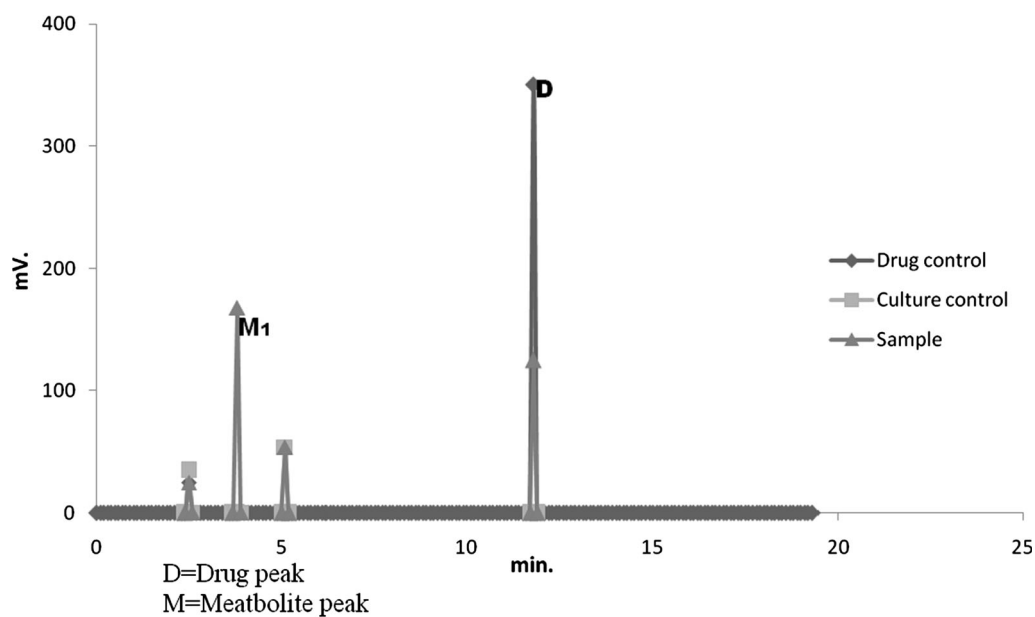
Name of the organism	Retention time in minutes			
	Drug control	Culture control	Standard	Sample
<i>Cunninghamella elegans</i> (NCIM-689)	2.5	2.5	11.8	2.5
	11.8	5.1		3.8*(M <sub>1</sub> )
				5.1 11.8
<i>Cunninghamella echinulata</i> (NCIM-687)	2.5	2.5	11.8	2.5
	11.8	5.4		3.6*(M <sub>2</sub> )
				5.4 11.8
<i>Cunninghamella blackesleeana</i> (NCIM-691)	2.5	2.5	11.8	2.5
	11.8	6.7		6.7
				11.8
<i>Aspergillus niger</i> (NCIM-589)	2.5	2.5	11.8	2.5
	11.8			4.1*(M <sub>3</sub> )
<i>Aspergillus fumigatus</i> (NCIM-902)	2.5	2.5	11.8	2.7
	11.8	4.0		4.0
				11.8
<i>Aspergillus ochraceus</i> (NCIM-1140)	2.5	2.5	11.8	2.5
	11.8			11.8

\*Metabolite peak.

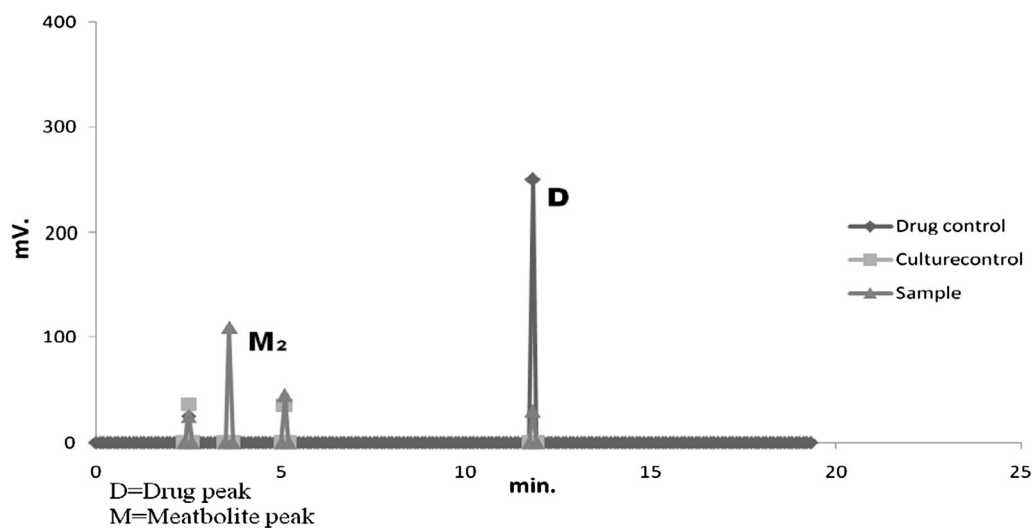
*Cunninghamella echinulata* and *Aspergillus niger* cultures have shown extra peaks at 3.8 min(M<sub>1</sub>), 3.6 min(M<sub>2</sub>) and 4.1 min(M<sub>3</sub>), respectively, compared with their two controls, as shown in figures 2–4. The presence of extra peaks revealed that the formation of metabolites. The peak at retention time of 2.5 min represents the solvent peak and peak at retention time of 5.4 represents culture contents. In the samples of

other cultures, no extra significant peaks were observed when compared with their controls. Since these three cultures biotransformed loratadine, these peaks were isolated from HPLC elute, and their structures were explored by a combination of mass and NMR spectra (table 2).

The mass spectrum of the pure drug showed a molecular ion peak at m/z 383 [M+1] which is close to the molar mass of loratadine, as shown in figure 5. The metabolite formed with *Cunninghamella elegans* – M<sub>1</sub> – showed a molecular ion peak at m/z 343.00 [M+1], which is equal to the molar mass of dihydroxy desloratadine, as shown in figure 6a. Dihydroxy desloratadine (M<sub>1</sub>) structure was further confirmed by the presence of a peak at  $\delta$  3.33 in PNMR, which represented the incidence of dihydroxy group, and the absence of peak at  $\delta$  1.25, and  $\delta$  4.13 depicted removal of carboethoxy group from the structure of that drug that is dictated in the formation of desloratadine, as shown in figures 8a and 9. The metabolite formed with *Aspergillus niger* – M<sub>3</sub> – showed a molecular ion peak at m/z 327.30[M+1], which is equal to the molar mass of 3-hydroxy desloratadine in the mass spectrum, as shown in figure 6c. 3-Hydroxy-desloratadine (M<sub>3</sub>) was confirmed by the existence of a peak at  $\delta$  5.40 in PNMR, which indicated the residence of one hydroxyl group, and absence of peaks at  $\delta$  1.25 and  $\delta$  4.13 represented removal of carboethoxy groups, as shown in figures 8c and 9. Di-hydroxy desloratadine (M<sub>1</sub>) and 3-hydroxy desloratadine (M<sub>3</sub>) might have formed in two different steps, that is, formation of desloratadine, an active metabolite by descarboethoxylation and further hydroxylation of desloratadine to dihydroxy (M<sub>1</sub>) or 3-hydroxy (M<sub>3</sub>), as shown in figure 9. The formation of dihydroxy desloratadine was also reported in *in vitro* liver microsomes (Ghosal *et al.* 2009) and mammals (Vlase *et al.* 2007). So, it indicated that



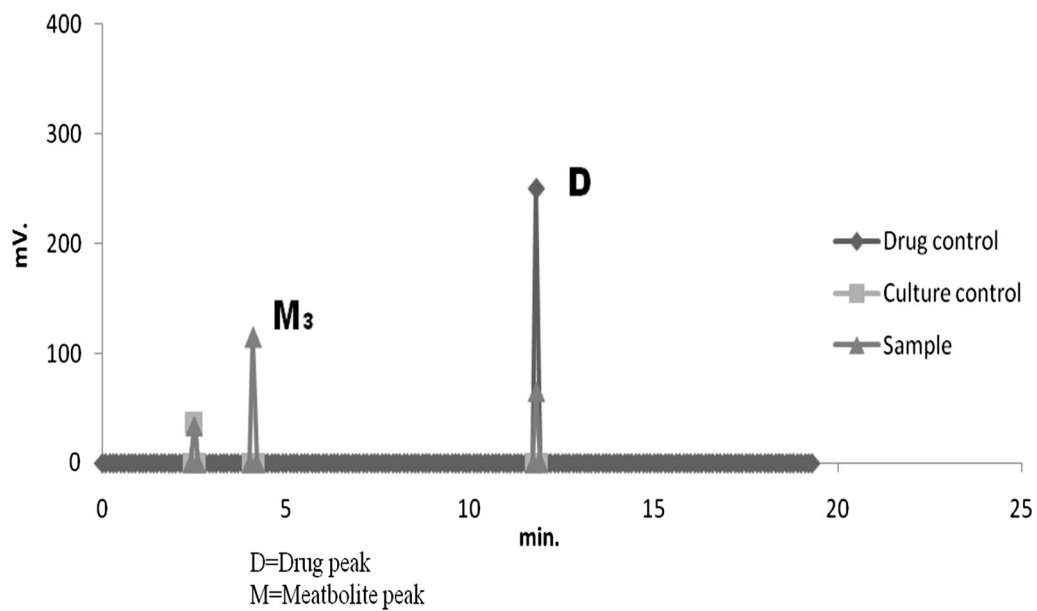
**Figure 2.** HPLC chromatogram of loratidine and its metabolite from culture extracts of *Cunninghamella elegans*.



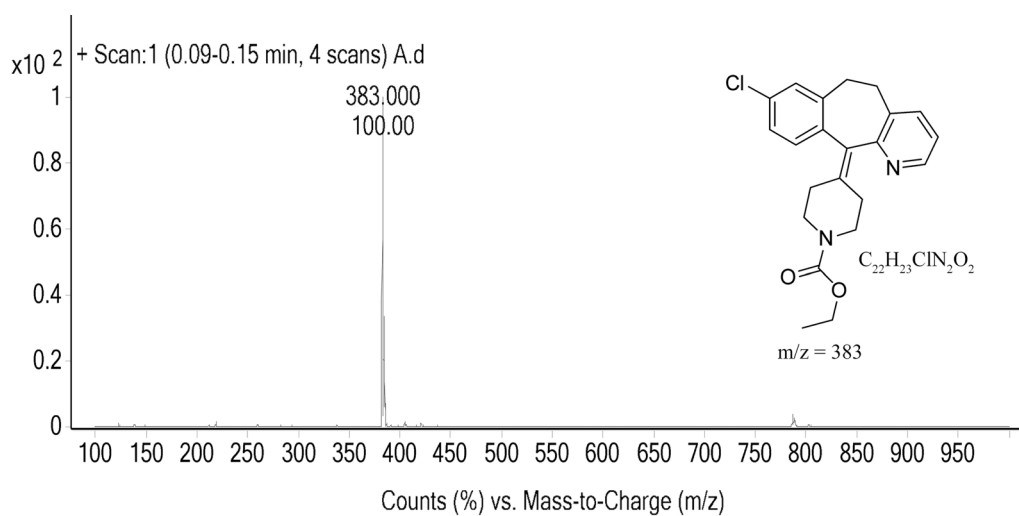
**Figure 3.** HPLC chromatogram of loratidine and its metabolite from culture extracts of *Cunninghamella echinulata*.

*Cunninghamella elegans* and *Aspergillus niger* have enzymes like mammalian CYP450 2D6 and 3A4 capable of conversion of loratidine to desloratidine, an active metabolite (figure 7). The metabolite formed with *Cunninghamella echinulata* – M<sub>2</sub> – showed a molecular ion peak at m/z 339.10[M+1], which is equal to the molecular weight

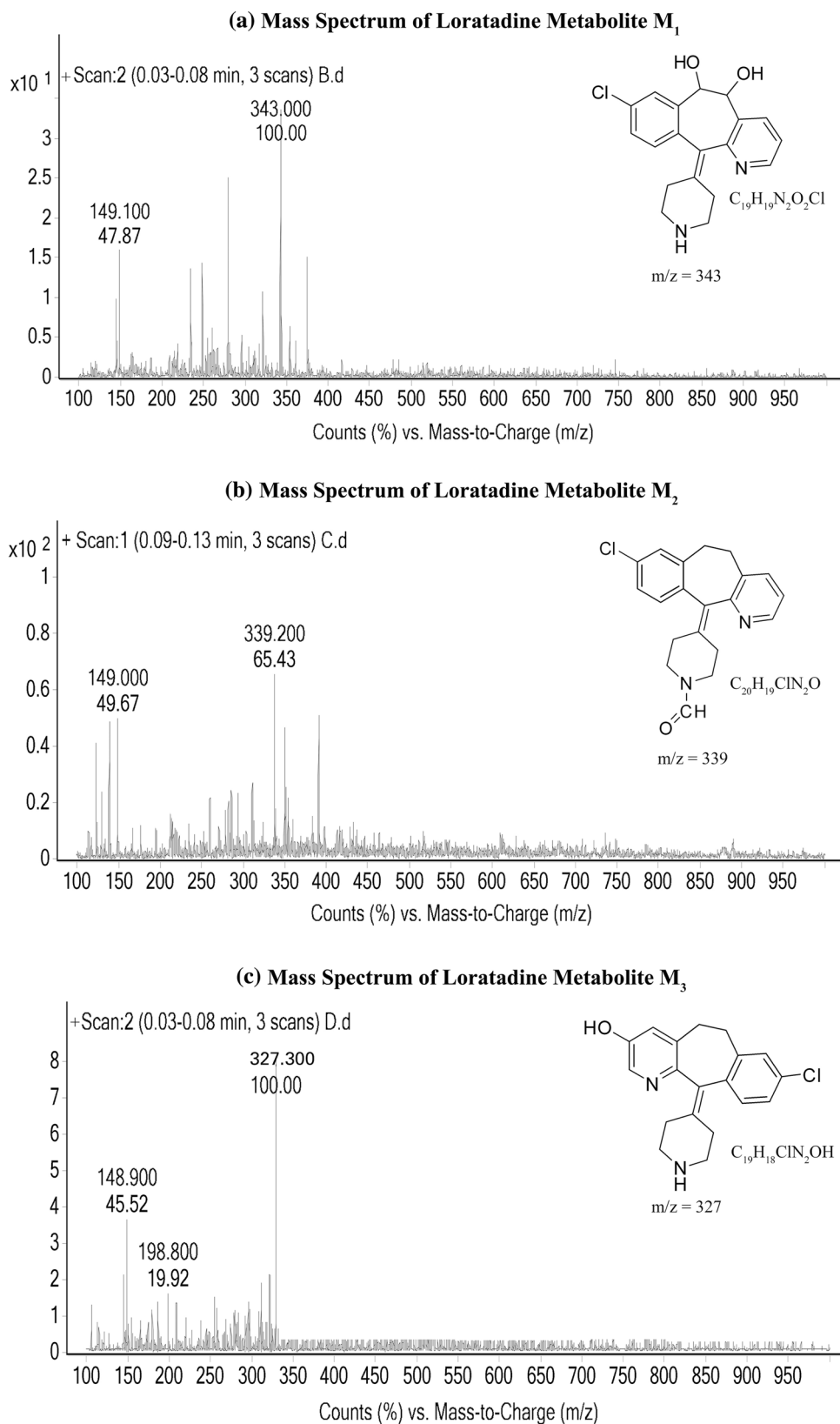
of desethoxy loratidine in the mass spectrum figure 6b. Desethoxy loratidine has a peak at  $\delta$  8.02 in PNMR that illustrated the presence of –CHO group, as shown in figures 8b and 9. It is a novel metabolite as it was not found in literature; it indicates that, this is a new derivative of loratidine formed by *Cunninghamella echinulata* in present study.



**Figure 4.** HPLC chromatogram of loratadine and its metabolite from culture extracts of *Aspergillus niger*.



**Figure 5.** Mass spectrum of loratadine.



**Figure 6.** Mass spectrum of loratadine metabolite (a)M<sub>1</sub>, (b)M<sub>2</sub> and (c)M<sub>3</sub>.

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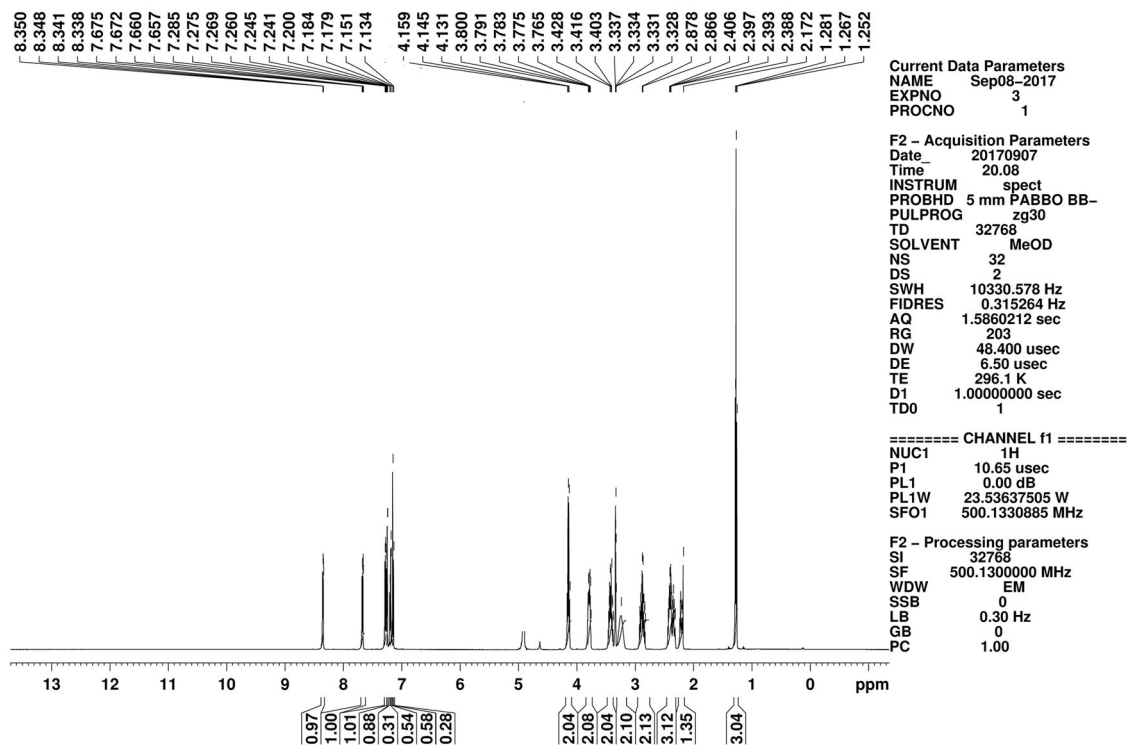


Figure 7. NMR spectrum of loratadine.

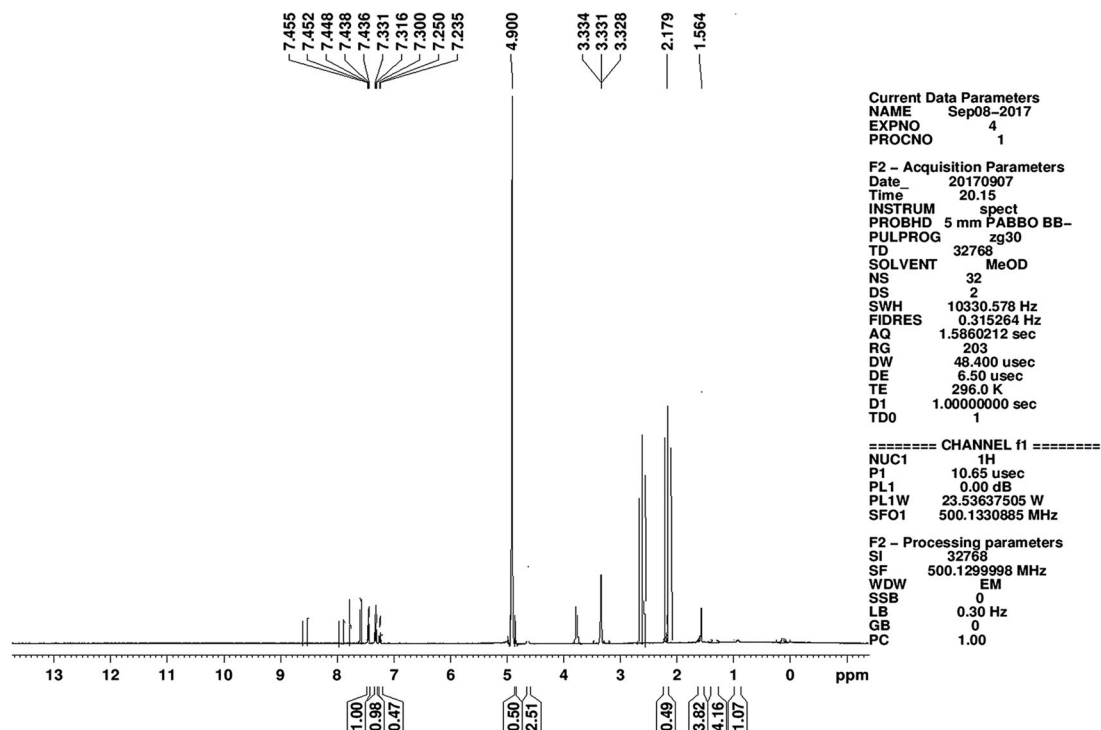
#### 4. Discussion

Six fungal cultures were screened in the present study to evaluate the ability of biotransformation of loratadine, which is a long-acting tricyclic antihistaminic drug and substrate for CYP 3A4 and CYP 2D6 (Yumibe *et al.* 1996). Among six cultures, *Cunninghamella elegans*, *Cunninghamella echinulata* and *Aspergillus niger* have shown a potential to metabolize the loratadine as per HPLC analysis. The metabolites formed by *Cunninghamella elegans* and *Aspergillus niger* were dihydroxy ( $M_1$ ) and mono-hydroxyl ( $M_3$ ) desloratadine respectively, as per their mass and PNMR spectra. Desloratadine is an active metabolite of loratadine found in mammals (Ramanathan *et al.* 2007). This illustrated that the above-mentioned two fungi are capable of converting loratadine to its active metabolites in the present study. The formation of dihydroxy and mono-hydroxyl derivatives of desloratadine was consistent with results of loratadine biotransformation found in *in vitro* liver microsomes (Ghosal *et al.* 2009) and mammals (Vlase *et al.* 2007). The metabolite formed by *Cunninghamella echinulata* was desethoxy loratadine ( $M_2$ ) as per mass and PNMR studies, which is a novel metabolite found in the study.

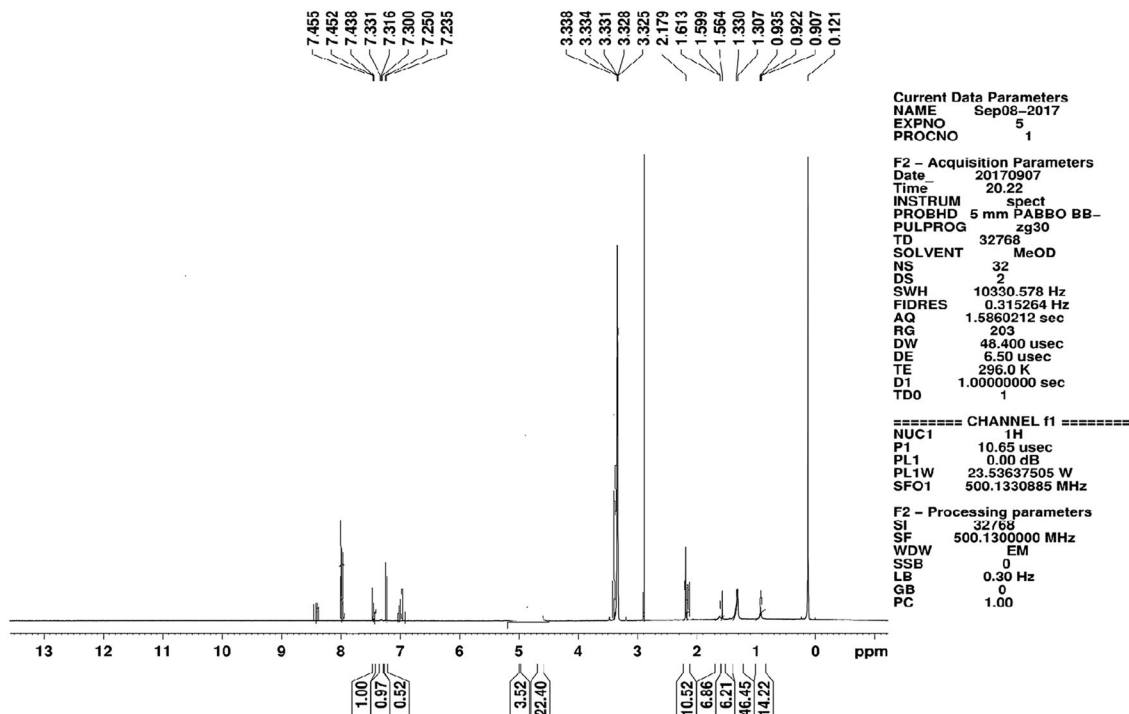
Hence, based on acquired results, the present exploration established the procreation of metabolites similar to the metabolites produced in mammals. Metabolites were produced by dihydroxy descarboethoxylation (dihydroxydesloratadine,  $M_1$ ) and hydroxy descarboethoxylation (3-hydroxy desloratadine,  $M_3$ ) reactions with *Cunninghamella elegans* and *Aspergillus niger* cultures respectively. The novel metabolite found was by desethoxylation (desethoxyloratadine,  $M_2$ ) reaction. Thus, it demonstrated that the fungi have potential to biotransform the drug loratadine to its metabolites. Among three cultures, *Cunninghamella elegans*, and *Aspergillus niger* were found as microbial resources for production of an active metabolite desloratadine as an intermediate product, and *Cunninghamella echinulata* formed the novel metabolite of loratadine. Biological activities of the novel metabolites will be evaluated in further studies, but it can be expected to have similar activity to desloratadine. So, these fungal cultures can be used as sources for production of desloratadine and reciprocal models of CYP3A4 and 2D6 for anticipation of biotransformation that occurred in mammals to produce active metabolites or novel derivatives of drugs for further pharmacological and toxicological evaluation.

(a) NMR Spectrum of Loratadine Metabolite M<sub>1</sub>

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(b) NMR Spectrum of Loratadine Metabolite M<sub>2</sub>

C-SAMPLE-2.....Keerthana

Figure 8. NMR spectrum of loratadine metabolite (a)M<sub>1</sub>, (b)M<sub>2</sub> and (c)M<sub>3</sub>.



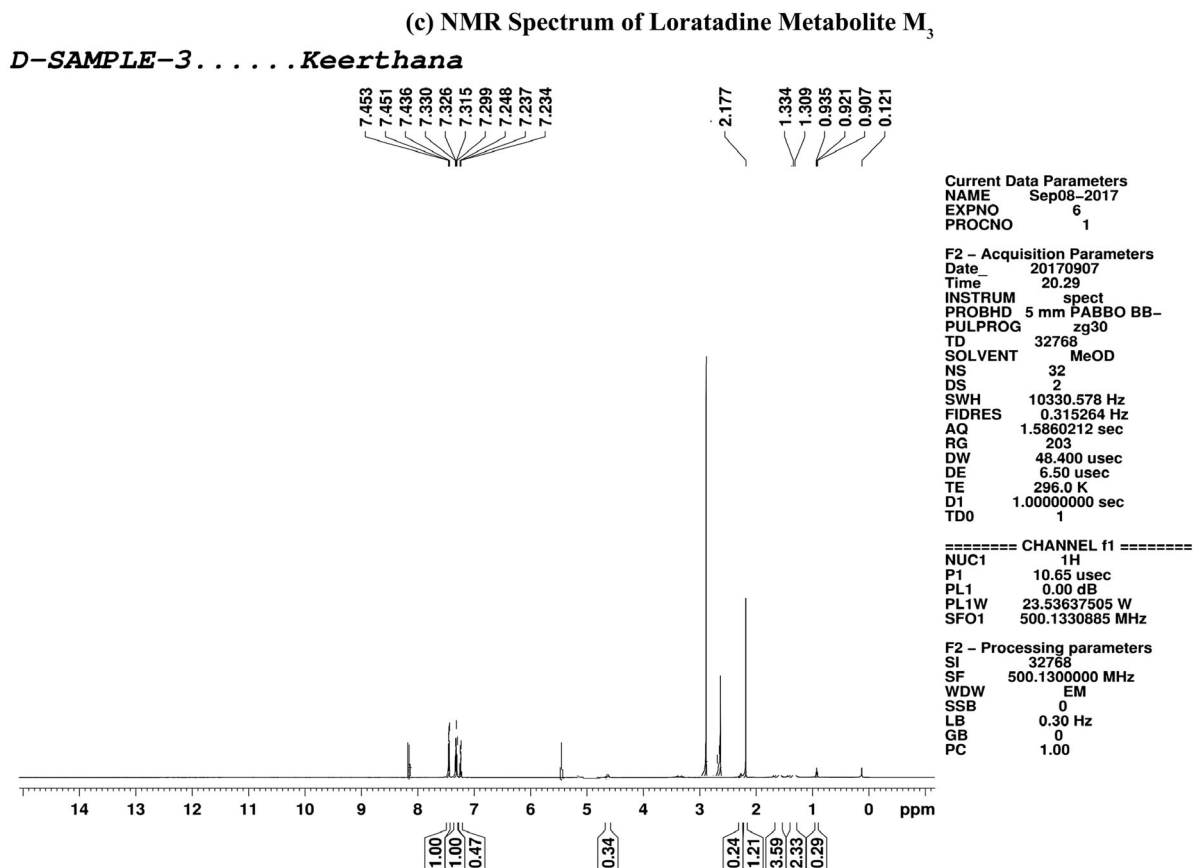


Figure 8. continued

**Table 2.** <sup>1</sup>H NMR Proton assignment of drug and metabolites

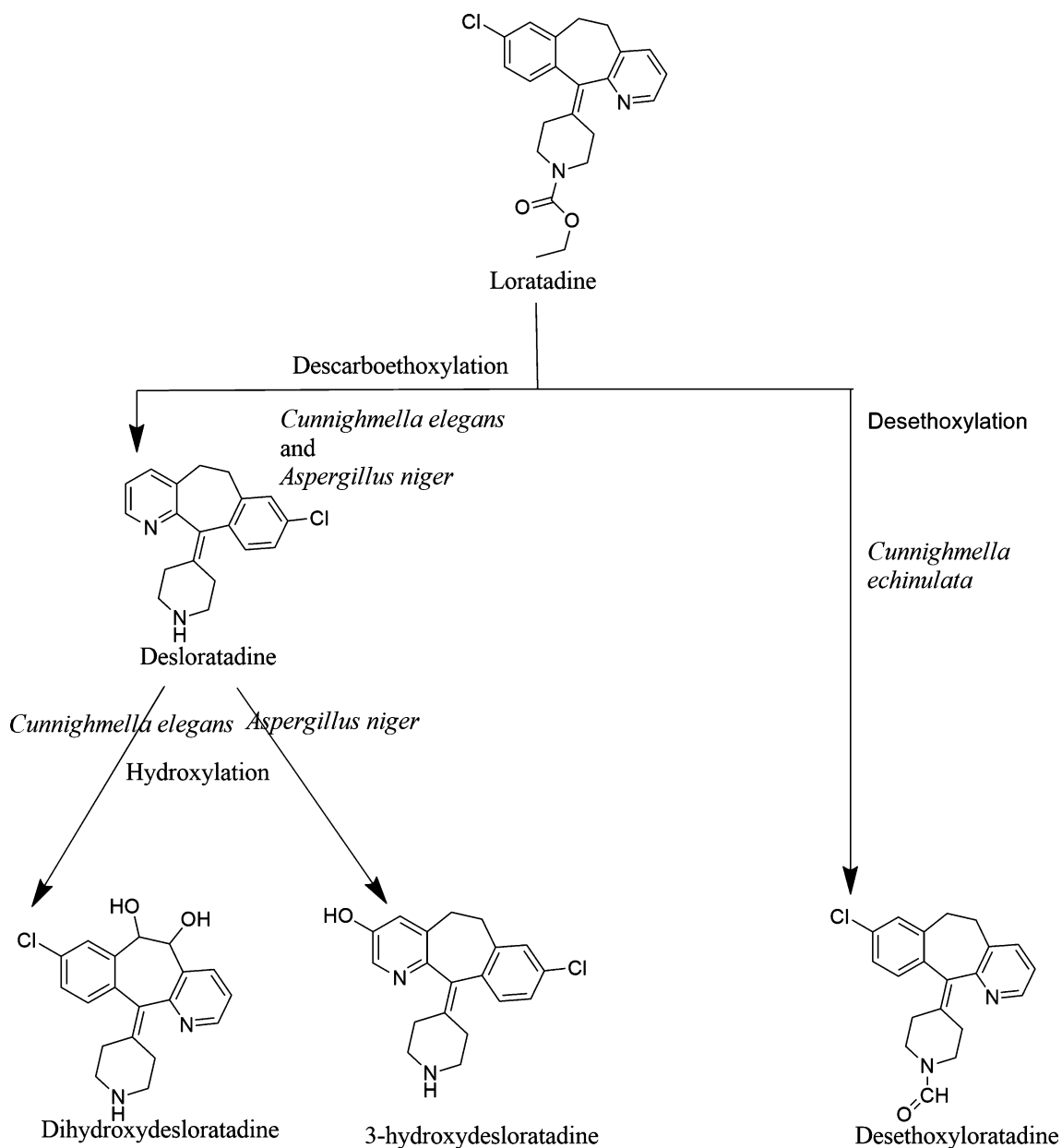
COMPOUND	<sup>1</sup> H NMR proton assignment
Loratadine	1.25-1.28(t,3H,CH <sub>3</sub> ), 2.17-2.40(m,4H,CH <sub>2</sub> ), 2.86-2.87(t,4H,CH <sub>2</sub> ), 3.32-3.42(t,2H,CH <sub>2</sub> ), 3.76-3.80(t,2H,CH <sub>2</sub> ), 4.13-4.15(t,2H,OCH <sub>2</sub> ), 7.23-7.455(m,5H,CH), 8.43(s,1H,CH).
M <sub>1</sub>	<b>1.564(s, H,NH)</b> , 2.179(t,4H,CH <sub>2</sub> ), 2.59(t,4H, CH <sub>2</sub> ), <b>3.32-3.33(t,2H,OH)</b> , <b>4.9(s,2H,CH)</b> , 7.23-7.45(m,5H,CH), 8.41(d,1H,CH).
M <sub>2</sub>	2.17(s,4H, CH <sub>2</sub> ), 2.88(m,4H, CH <sub>2</sub> ), 3.325-3.33(m,4H, CH <sub>2</sub> ), 7.23-7.45(m,5H,CH), <b>8.02(m,1H,CHO)</b> , 8.43(m,1H,CH).
M <sub>3</sub>	<b>1.30(s,1H,NH)</b> , 2.17(s,4H,CH <sub>2</sub> ), 2.59(m,4H,CH <sub>2</sub> ), 2.88(d,4H,CH <sub>2</sub> ), <b>5.40(s,1H,OH)</b> , 7.23-7.45(m,4H,CH), 8.21(s,1H,CH).

\*Bold face indicates that additional peaks observed in NMR spectra of metabolites compared to NMR spectrum pure drug.

## 5. Conclusion

The present study revealed that *Cunninghamella elegans* and *Aspergillus niger* were able to transform loratadine to its metabolites, dihydroxy desloratadine and 3-hydroxy-desloratadine, similar to that the mammals.

*Cunninghamella echinulata* was able to transform the drug in to desethoxy loratadine, which is a novel metabolite. These observations were confirmed by results of mass spectrometry and PNMN studies. Hence, these can be used as microbial models for mammalian biotransformation studies.



**Figure 9.** Proposed metabolic pathway of loratadine.

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