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METABOLITES GENERATION VIA OXIDATION, HYDROXYLATION, ACYLATION, DECHLORINATION, DEALKYLATION & GLUCURONIDATION OF LOSARTAN BY THERMOPHILIC FUNGUS *RHIZOMUCOR PUSILLUS* NRRL 28626

G. Shyam Prasad
Department of Microbiology, Vaagdevi Degree and PG College,
Hanamkonda, Warangal-506001
Telangana State, India

K. Srisailam
University College of Pharmaceutical Sciences, Satavahana University
Karimnagar -505002, Telangana State, India

ABSTRACT

Biotransformation potential of a thermophilic fungal strain Rhizomucor pusillus NRRL 28626 was investigated using anti-hypertension drug losartan as a model drug in order to find the metabolic similarities of this fungus with that of mammals. Five metabolites viz. M1, M2, M3, M4, and M5 were detected at pH 6.0 and temp of 45ºC during four days incubation. The metabolites were detected by high-performance liquid chromatography (HPLC) and the structures were elucidated by liquid chromatography- tandem mass spectrometry (LC-MS/MS) analysis as glucuronic acid compound (M1), 3-hydroxy-N-acetyl losartan (M2), N-acetylated carboxylic acid metabolite of losartan (M4), and two novel metabolites M3 and M5. The metabolites of losartan produced were by glucuronidation, hydroxylation, acylation, dealkylation, dechlorination, and oxidation which clearly reveals that R.pusillus exhibits wide reaction diversity. The metabolites of losartan produced by this fungus are similar to those

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recorded in mammals indicating that this fungus possesses enzyme system similar to mammals. Hence, this fungus can be a suitable microbial model in drug metabolism studies.

Key words: Losartan, LC-MS/MS, HPLC, Biotransformation, *Rhizomucor pusillus*

1. Introduction

Microorganisms exist in diverse environments and experience extremes of temperature, pH, chemical composition and/or pressure which may be attributed to certain genetic and/or physiological adaptations (Cooney and Emerson 1964; Aguilar 1996; Stetter 1999). Thermophilic fungi are a small group in Eukaryota having a unique mechanism for growing at a higher temperature of up to 60°- 62°C. These fungi are found in soil and in other habitats like heaped masses of plant material, piles of agriculture and forestry products and other accumulated organic matter which provide warm, humid aerobic environments (Prasad et al. 2011).

Among the eukaryotes, only thermophilic fungi have the exceptional ability to grow at a higher temperature of 50°-60°C (Cooney and Emerson 1964) and offer many advantages in biotransformation studies over mesophiles. As the enzymes of thermophilic microorganisms are highly stable at elevated temperature which is a definite advantage for thermo catalytic activity (higher reaction rate, lower diffusional restrictions), higher stability, higher process yield (increased solubility of substrates and products and favorable equilibrium displacement in endothermic reactions), lowers viscosity and prone to less contamination problems (Mozhaev 1993). Thermostable biocatalysts are therefore very attractive. Thermophiles are an evident source of thermostable enzymes, being logical to assume that such character will present their proteins a high thermal stability (Lele et al. 2016).

Biotransformation reactions are an important route for introducing chemical functional groups into inaccessible sites of molecules, is very useful in the production of intermediates of medicinal and agricultural chemicals from both active and inactive materials. Biotransformations performed by microorganisms involve high regio and stereospecificity and require mild reaction conditions (Prasad et al. 2008).These reactions are very attractive for the preparation of metabolites of drug candidate compounds, which are required for the testing of biological activity (Fura et al. 2004) or toxicity and also as analytical standards. Biotransformation studies carried out using fungi provide an efficient and eco-friendly means of achieving large-scale

metabolite production of a wide range of drugs (Azerad 1999; Borges et al. 2009; Abourashed et al. 1999).

The biotransformation reactions mediated by the fungus can also be used as a model for mammalian drug metabolism. The advantages of a microbial system as an in vitro model for drug metabolism studies includes its low cost, ease of handling scale up capacity, and potential to reduce the use of animals (Pupo et al. 2008). The concept of using microorganisms, particularly the mesophilic filamentous fungus belonging to the genus *Cunninghamella*, as models of mammalian biotransformation has been well documented (Lisowska et al. 2006; Zhang et al. 2006). Despite many advantages, there are no reports on the use of thermophilic fungi in biotransformations for bioactive compound synthesis or as microbial models of mammalian metabolism. Thermophily in fungi is not as extreme as in eubacteria or archaea. Some species are able to grow near or above 100°C in thermal springs, solfatara fields, or hydrothermal vents (Blochl et al. 1997; Brock 1995). Because of their moderate degree of thermophily and as their habitats are not exotic, thermophilic fungi have not received much publicity and attention.

In our previous study, we reported biotransformation potentials of thermophilic fungus *Rhizomucor pusillus* NRRL 28626 using anti-helminthic drug albendazole as a model drug (Prasad et al. 2011) and recorded 4 metabolites by sulfoxidation, N-methylation and by dealkylation. In the present investigation, to further explore other reaction potentials of this fungus biotransformation was performed using losartan, an anti-hypertensive drug as a model.

2. Materials and methods

Fungal culture

The thermophilic fungus *Rhizomucor pusillus* NRRL 28626 was obtained from culture bank of Microbiology Department, Kakatiya University, Warangal. Stock cultures were maintained on yeast extract starch agar (YESA) (Yeast extract, 5.0g Starch, 30g; $MgSO_4$. $7H_2O$, 0.5 g; KH_2PO4, 1.0g; agar -agar, 20g, and distilled water 1000ml). The pH of the medium was adjusted to 6.0 with 0.1N HCl or 0.1N NaOH, stored at 4°C and sub cultured for every 3 months.

Chemicals

Losartan was obtained from Department of Pharmacy, Kakatiya University, Warangal, India. Methanol and acetonitrile were of HPLC grade obtained from Ranbaxy, New Delhi, India. Peptone, yeast extract, glucose, starch and all other chemicals were obtained from Himedia, Mumbai, India.

Fermentation

For biotransformation of losartan, fermentation was initiated in a broth containing (per liter) glucose (20 g), peptone (5.0 g), yeast extract (5.0 g), K_2HPO_4 (5.0 g), and NaCl (5.0 g). The pH of the broth was adjusted to 6.0 with 0.1 N HCl or 0.1 N NaOH. Later, the prepared media was autoclaved and cooled to room temperature before inoculation. Two-stage fermentation procedure was followed. The first stage fermentation was initiated by inoculating a 250 ml culture flask containing 50 ml of broth with a loopful of spores obtained from a freshly growing agar slant. After incubating for 48h at 120 rpm and 45°C, a 1.0 ml portion of the first-stage culture was used to inoculate a second stage 50 ml of medium in a 250 ml culture flask. The culture was incubated for 24 h before the substrate losartan (5mg) in 500µl dimethylsulfoxide was added. The flasks were incubated under similar conditions for 4 days.

Two types of controls were run simultaneously with the fermentation and analyzed in the same way. Culture controls consisting of a fermentation blank in which fungus was grown under identical conditions without substrate and substrate control comprised losartan added to the sterile medium and incubated under similar conditions and the experiment was studied in triplicates.

Extraction and identification of metabolites

At the end of 4 days incubation period, the contents of the flasks were transferred to separating funnel and extracted thrice with ethyl acetate. Later, the organic extracts were combined and evaporated using a rotary vacuum evaporator and dried over a bed of sodium sulfate. The residues thus formed were analyzed by High-Performance Liquid Chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the presence and identification of metabolites.

HPLC analysis was performed according to the method described by Vidyavathi et al. (2008) with modification to gradient elution. The samples were analyzed using Water's PDA 2595 system by injecting 10 µl of sample into the syringe-loading sample injector. The column used was Water's, Symmetry shield, C18, 4.6x250mm, 5µm. A three-component mobile phase pumped at 1ml/min containing the following: solvent A 0.01M ammonium acetate (pH 5): acetonitrile 80:20, Solvent B 0.01M ammonium acetate (pH 5): acetonitrile: 10:90 and solvent C: 0.01M ammonium acetate (pH5): methanol: 15:85.

Initial conditions were solvent A 100%. The following steps were programmed: 2-9min solvent A 90% and solvent C 10%,9-12 min solvent A 50%,solvet B 20%,solvent C30%.from 12-25min solvent A : 50%,solvent B:25% and solvent C25%.25-28 min solvent A 10%,solvent B85%,Solvent C5%.from 28-36 min solvent A10%,solvent B 85%,solvent C5%.36-46 min solvent A 100%. The metabolites of losartan were detected by absorbance at 225 nm.

The quantification of losartan metabolites was done based on peak areas and expressed as percentages of the metabolites formed by taking total area of drug and metabolites together as 100 % as described (Srisailam et al. 2006).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed using system MDS SCIEX API-4000, Q-TRAP (Canada) with MS/MS API-4000, Q-Trap detector. Chromatographic separation was achieved by Waters column C18, 4.6×250mm, 5 µm, with enhanced mass scan (EMS) and enhanced product ion scan (EPI) detector mode. The ESI detection was set to positive mode. A temperature of 300°C and scan range of 50 amu to 800 amu was set for both EMS and EPI. The mobile phase and all other conditions were same as described for HPLC analysis. The data was acquired and processed by means of Analyst 1.4.2 software. The transformed compounds were identified from the masses of the fragmentation ions obtained in LC-MS/MS analysis, HPLC retention times and based on previous reports.

3. Results and discussion

In the present investigation, a successful biotransformation of losartan by a thermophilic fungus *R.pusillus* was reported which produced a total of five metabolites in 4 days incubation. The transformation of losartan was identified by HPLC analysis (Fig. 1) of ethyl acetate extract of test and control samples.

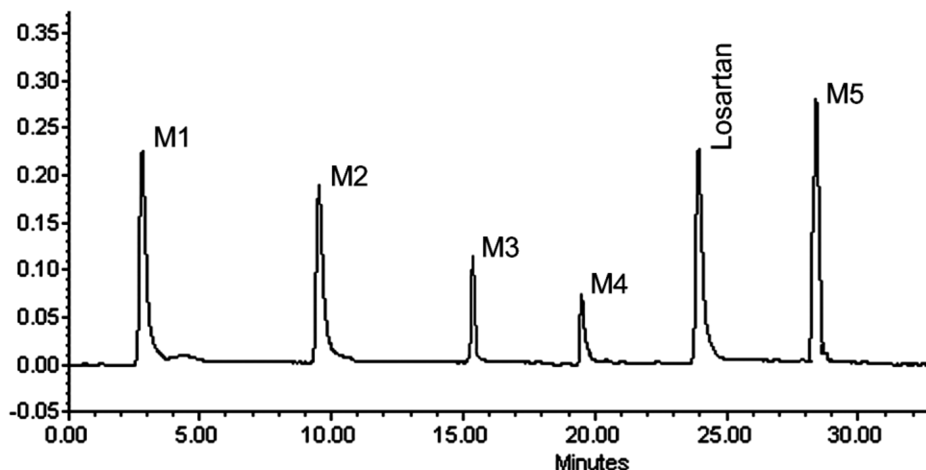

Fig.1.HPLC chromatogram showing metabolites detected in Losartan fed culture broth of *Rhizomucor pusillus* after 4 days of incubation.

The structure elucidation of the losartan metabolites were proposed from m/z values of the molecular ion (Fig.2a) and fragment ion peaks (Fig.2b) recorded in LC-MS/MS analysis, elution order and retention times of the metabolites in HPLC analysis, and based on previous reports (Vidyavathi et al. 2008; Chen et al. 1993; Stearns et al. 1995). The metabolites were quantified and designated as viz. M1 (20.6%), M2 (18.4%), M3 (8.7%), M4(7.3%), M5 (23.7%) and losartan (21.3%).

The metabolites M1, M2, M3 and M4 eluted before the parent drug in HPLC analysis indicating that they are polar than losartan and the metabolite M5 eluted after the drug which indicates that metabolite M5 is non polar than other metabolites and losartan. The retention times of losartan metabolites eluted in HPLC analysis were M1 (2.6 min), M2 (9.8 min), M3 (16.0 min), M4 (19.5 min), and M5 (28.5min) and the drug losartan was eluted 24.0 min.

Identification of metabolites

The mass spectrum of metabolite M1 showed an apparent molecular ion at m/z 600 $[M+H]^{+2}$ (addition of 177 units to parent compound losartan) which indicates conjugation of glucuronic acid to losartan resulting in the formation of a glucuronic acid conjugate of losartan. Two fragment ion at m/z 405.2 and 207 were recorded. The product ion at m/z=405 [M+H]+

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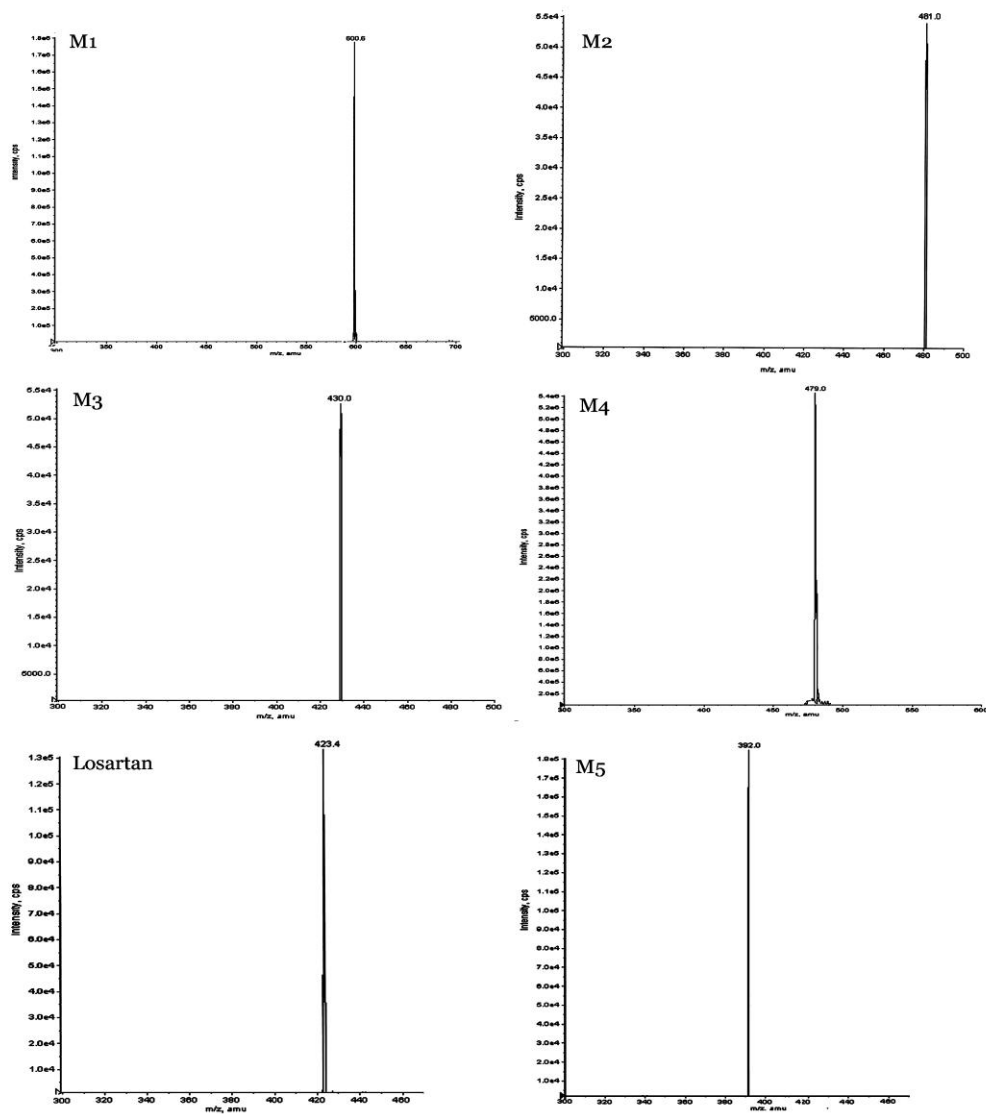

Fig.2a. LC-MS spectra of metabolites detected in Losartan fed culture broth of *Rhizomucor pusillus*.

might be arising from loss of glucuronide moiety and by dehydroxylation of diazole ring of M1. The other fragment ion observed at m/z 207 might be arising by loss of diazole ring and by cleavage of tetrazole ring. This fragment m/z 207 was also recorded by (Iwamura et al. 2011). The metabolite M1 is a glucuronic acid conjugate of losartan. Conjugation has been well recognized as an important metabolic pathway of many compounds both in mammals and in microorganisms (Bin et al. 2007). This metabolite of losartan was also recorded by many

researchers. Huskey et al. (1993) reported using liver microsomes of rats, monkeys and humans. Whereas, Krieter et al. (1995) in rat intestine while, Chen et al. (1993) reported this metabolite using mesophilic microbial culture *Streptomyces* sp. MA6751. This metabolite was reported to catalyze by UGT super family of enzymes in animals and is also detected in monkeys, rats, dogs, and humans (Zhang et al. 2006; Husky et al. 1993; Krieter et al. 1995). This metabolite was also reported to exhibits angiotensin II receptor antagonist activity and is also useful in treating hypertension, in the management of congestive heart failure and has other wide applications (Chen et al. 1993).

Metabolite M2 eluting at 9.8 min gave m/z 481 [M+H]+ (addition of 58 units to losartan) which was supported by fragment ions m/z 337, 278, 311 and 241.The fragments ion 337 might be arising by diazole ring cleavage of M2 and fragment ion 278 by benzyl cleavage of the fragment 337 which supports N-acetylation of hydroxy losartan. The fragment ion peak at m/z 241 suggests hydroxylation of an alkyl group of losartan. Based on this assumption the metabolite was identified as 3-hydroxy-N-acetyl losartan. N-acetylation of hydroxy losartan resulted in metabolite M2 formation. The N-acetylation reactions catalyzed by N-acetyl transferase in mammals are well known. This metabolite of losartan using mesophilic bacteria was reported earlier (Vidyavathi et al. 2008). Chen et al. (1993) in his earlier studies recorded 3-hydroxy losartan which was not recorded in the present study using *R.pusillus* which might be due to the instantaneous conversion of 3-hydroxy losartan to 3-hydroxy-N-acetyl losartan at high temperature. A Similar type of N-acetylation reactions using microbial cultures was reported previously (Foster et al. 1998; Milliken et al. 2004; Huang et al. 1994).

The metabolite M3 had a retention time of 16.0 min and showed a molecular ion at m/z 430 $[M+H]^+$ with major fragment ion at m/z 337, 278,262 and 112. The fragment ion 337 might be arising by diazol ring cleavage of M3 and the fragment 278 by the loss of propyl group of cleaved diazole ring of fragment m/z=337. The other fragment 262 might be derived from further dealkylation of 278 fragment. The fragment with m/z=112 corresponds to tetrazole ring of M3. Based on the fragmentation pattern (Fig.3) the metabolite was identified as a novel metabolite of losartan. This metabolite was assumed to be produced by sequential oxidation and acylation of demethylated and dechlorinated parent compound losartan. This type of demethylation and dechlorination of chlorinated hydroquinones using bacteria was reported earlier (Milliken et al. 2004).

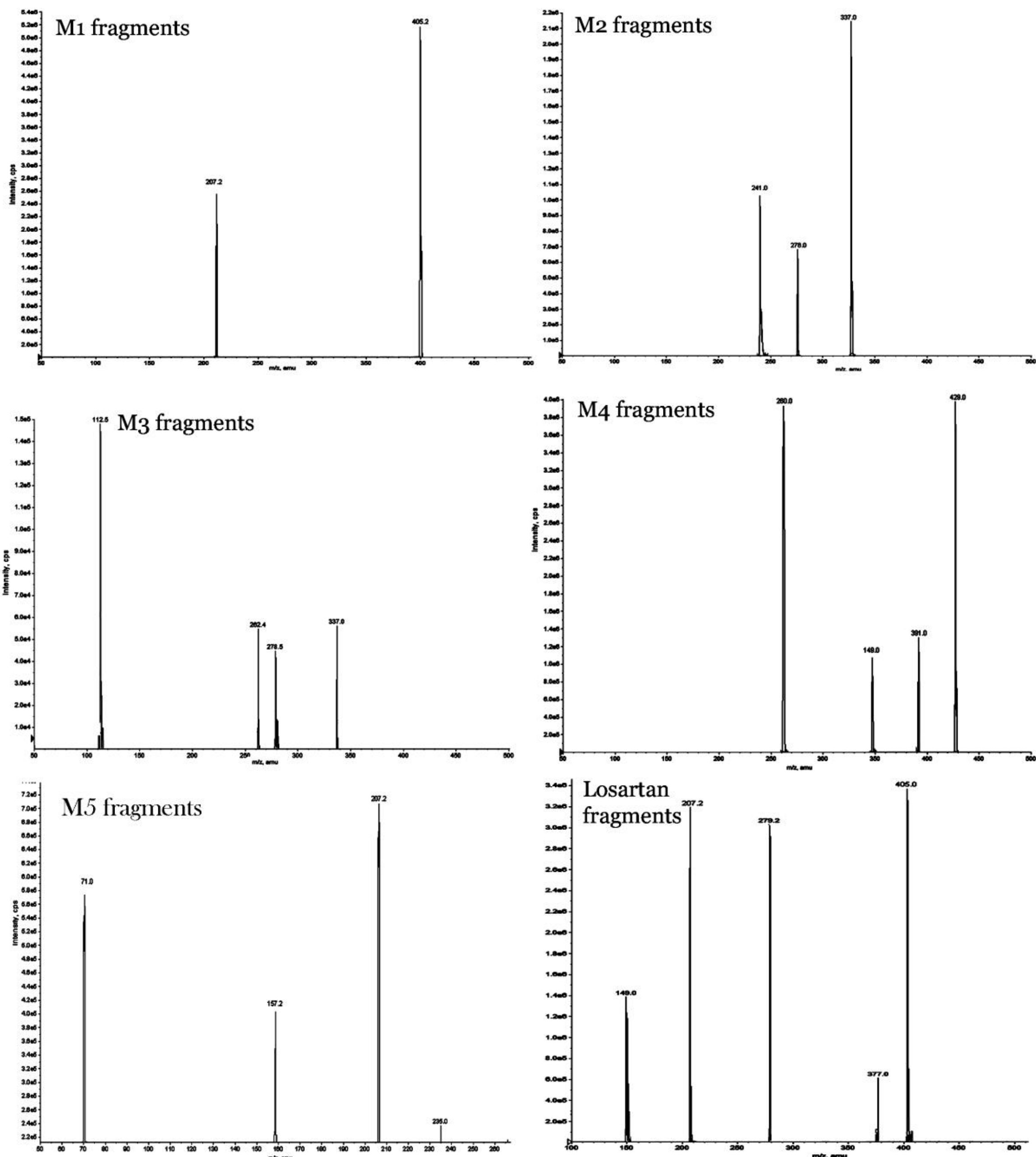

Fig.2b. Fragmentation peaks of losartan and its metabolites recorded in LC-MS/MS analysis

The metabolite M4 produced by *R.pusillus* eluting at 19.5 min showed molecular ion at m/z 479 [M+H]+ supported by fragments 429, 391,260 and 149. This indicates the formation of

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the N-acetylated carboxylic acid metabolite, arising by oxidation and acylation of parent compound losartan.

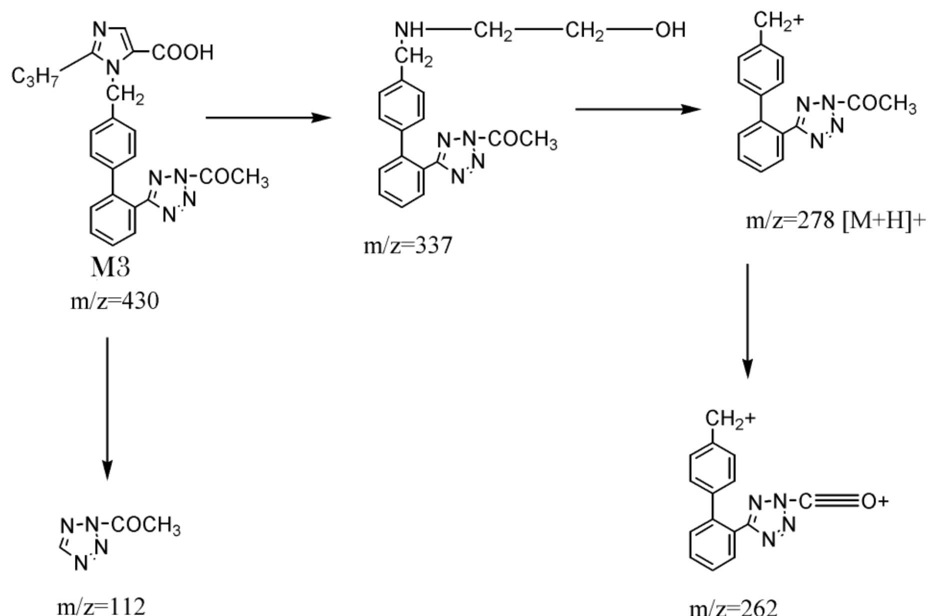

Fig.3 Mass fragmentation pattern of metabolite 3(M3)

The fragment ion peaks indicate oxidation of primary alcoholic group to a carboxylic acid. The fragment 429 might be arising from dechlorination and demethylation (from butyl side chain) of M4. The fragment m/z 391 is decarboxylation and deacylation product of M4. The other fragment 260 might be arising by dealkylation, dacylation and by loss of tetrazole ring of M4. Another fragment ion at m/z=149 might be arising by dechlorination, dehydroxylation and by benzylic cleavage of M4. The metabolite M4 might be produced by N-acetylation and carboxylation of parent compound losartan. This metabolite was also recorded in the mammalian metabolic pathway of losartan catalyzed by CYP3A4 (Yun et al. 1995). This metabolite was also recorded previously (Vidyavathi et al. 2008) in culture broth of mesophilic bacteria *Proteus vulgaris*, which clearly indicates that similar enzyme system exists in mammals, bacteria, and fungi.

Metabolite M5 eluted at 28.5 min in the present study showed a molecular ion peak at m/z 392[M+H]+ (decrease of 44 units from carboxylic acid metabolite) with m/z= 235, 207.2,157 and 71 as fragments. The fragment ion with m/z 235 might be arising from the loss of diazole ring from M5 and the fragment with m/z= 207 by tetrazole ring cleavage of fragment ion with m/z =235. The fragment ion with m/z 207 was also recorded by Iwamura et al. (2011).The fragment with m/z=157 corresponds to diazole ring of M5. The metabolite M5 might be produced by decarboxylation of a carboxylic acid metabolite of losartan. Based on the fragmentation pattern (Fig. 4) the metabolite was identified as another novel metabolite of losartan.

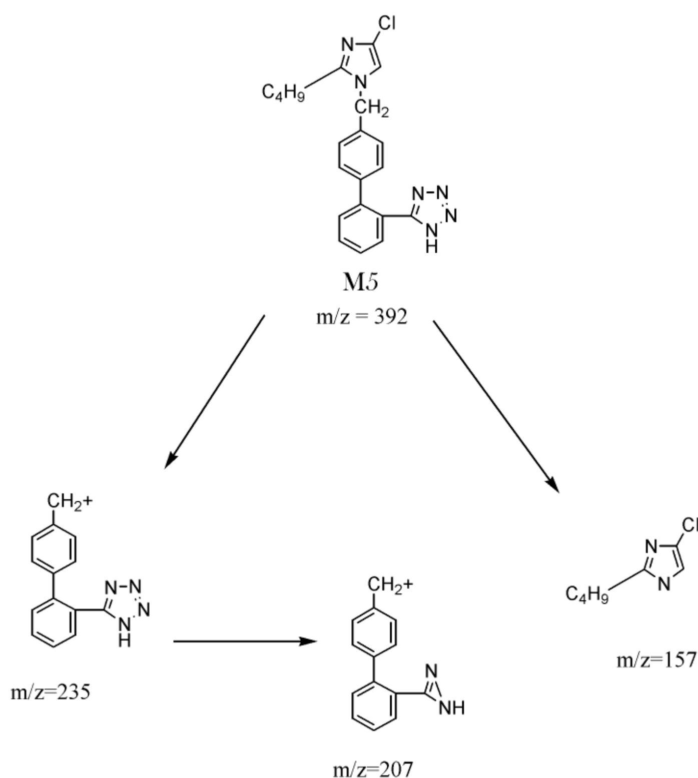

Fig.4 Mass fragmentation pattern of metabolite 5(M5)

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The compound eluting at 24.0min was identified as losartan by comparison of the retention time and mass fragments of pure drug. Losartan has generated a molecular ion [M+H]+ at m/z 423 with fragment ion peaks at m/z 405, 337, 279 and 149 in its mass spectrum. The proposed metabolic pathway of losartan in culture broth of *R.pusillus* was presented in fig.5.

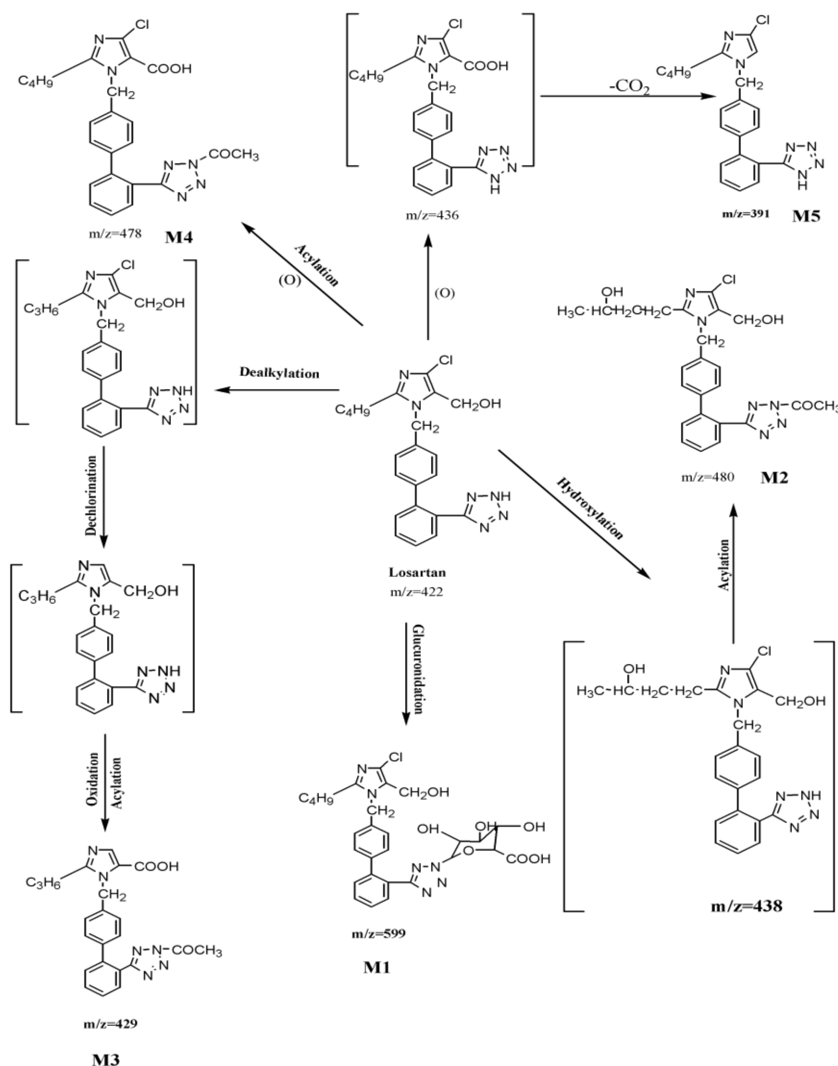

Fig.5 Proposed metabolic pathway of losartan in culture broth of *Rhizomucor pusillus*

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Carboxylic acid compound of losartan was not recorded in culture broth of *R.pusillus* but, was reported in mesophilic bacteria *Proteus vulgaris* (Vidyavathi et al. 2008) and in mammalian metabolism of losartan (Stearns et al.1995) indicating that the produced carboxylic acid metabolite was not stable in culture broth of *R.pusillus* but instantaneously converted to metabolite M5. Milliken et al. (2004) recorded similar type of decarboxylation reaction using microbial cultures.

In our previous study (Prasad et al. 2011) with *R.pusillus,* a total of 4 metabolites of albendazole were recorded by sulfoxidation (M1, M2), N-methylation (M3) and a novel metabolite by dealkylation (M4). In the present investigation, the same fungus *R.pusillus* transformed losartan to 5 metabolites including two novel metabolites (M3, M5) by oxidation, hydroxylation, acylation, dechlorination, dealkylation & glucuronidation of losartan which clearly states that this thermophilic fungus *R.pusillus* NRRL 28626 has the ability to catalyze diverse reactions compared to mesophilic fungi. Similarly, the metabolites of losartan produced by the fungus are similar to metabolites of losartan reported in mammals which clearly states that similar type of enzyme system exists in mammals and this fungus.

4. Conclusion:

It is evident from our investigation that thermophilic fungus *Rhizomucor pusillus* NRRL 28626 produced a total of five metabolites of losartan both novel and previously reported in mammals. As the metabolic pattern of the fungus is similar to mammals this fungus can be used as a microbial model in drug metabolism studies. Further, desired metabolites can be produced in large quantities; isolated and biological activity studies can be performed. In addition, the fungus has produced two novel metabolites of losartan. Hence, this fungus can be employed for producing compounds of novel structures with possible novel biological activities.

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Conflict of interest:

The authors declare that there is no conflict of interest

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