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Research Article

Fungal Biotransformation of Ramipril: A Novel and Affordable Alternative to *in-Vivo* Model of Drug Metabolism

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ABSTRACT

Biotransformation and the resulting metabolites significantly influence drug efficacy and safety. The present study aimed to establish the use of filamentous fungus *Cunninghamella elegans* in biotransforming Ramipril as a microbial model of mammalian metabolism and optimize fermentation parameters to produce maximum metabolite concentration. In the current work, ramipril microbial transformation showed mammalian metabolism characteristics, and *C. elegans* effectively bio-transformed Ramipril into its metabolite ramiprilat. The extracted metabolites were quantified employing high-performance liquid chromatography (HPLC) and characterized using mass spectroscopy (MS). The maximum metabolite concentration was observed when the media was grown in a potato dextrose agar plate for 11 days when incubated at 120rpm at 27°C. Hence this microbial model can be combined with other models to predict *in vivo* metabolism and provide metabolite benchmark values for drugs such as Ramipril.

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INTRODUCTION

Preclinical screening of the drug is required to evaluate its absorption, distribution, metabolism, excretion, and toxicology (ADME(T)) (Pekala *et al*, 2012). Interestingly, in some cases, the metabolism of a drug can lead to the formation of a pharmacologically active compound, which can ultimately be developed into an effective therapeutic agent.

Microbial biotransformation is a valuable technique for interpreting the *in vitro* drug metabolism, identifying biosynthetic routes, and predicting mammalian metabolism and toxicity (Sponchiado *et al*, 2020). One of such extensively used *in vitro* microbiological models involves using *Cunninghamella*, a filamentous fungus. *Cunninghamella* possesses unique features that make it valuable in drug metabolism research. E.g., it metabolizes drugs like indomethacin, verapamil, and pantoprazole in ways comparable to those found in mammalian enzyme systems (Ma *et al*, 2007). It has been established that the enzymes in *Cunninghamella* are identical to those that mammals use to detoxify xenobiotics. Furthermore, there is strong evidence that *Cunninghamella* is superior to other microorganisms at

predicting drug fate in mammalian species (Pekala *et al* 2012), (Prior *et al*,2010).

Ramipril (2-[N-[(S)-l-ethoxycarbonyl-3-phenylpropyl-lalanyl]-(1S,3S,5S)-2-azabicyclo[3-3-0]-octane-3-carboxylic acid, is a long-acting non-sulfhydryl drug which actively inhibits angiotensin-converting enzyme (ACE) (Levitt & Schoemaker, 2006). It is used to treat various types of hypertension and heart failure and to prolong the survival rate in individuals with clinical signs after myocardial infarction (Lu et al,2006),(Rufet al,1994)

The chemical structure of the drug is captured in Fig. 1. Following oral administration, Ramipril undergoes de-esterification in the liver to generate its active metabolite ramiprilat (Fig. 2) (Desmoulins *et al*,2008; Meisel *et al*, 1994).

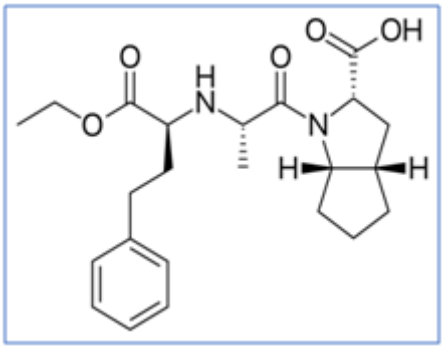

Fig. 1: Chemical Structure of Ramipril

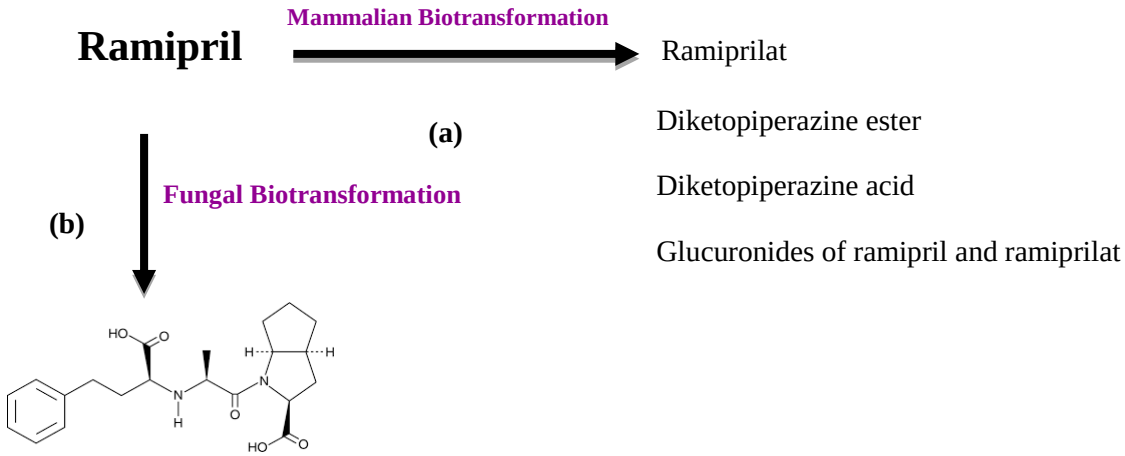

Fig. 2: Biotransformation of Ramipril by two different means (a) Mammalian (Zisaki *et al*, 2015), (b) Fungal

In light of the above facts, the present work aimed to evaluate the formation of metabolites of ramipril using filamentous fungi *Cunninghamella elegans* through robust analytical techniques and to optimize fermentation parameters to produce maximum metabolite concentration.

MATERIALS AND METHODS

Ramipril (purity≥95%), Potato dextrose agar (Himedia), DMSO (Dimethyl sulfoxide), methanol, acetonitrile and formic acid were procured from Merck, and water was purified by a Milli-Q system Millipore (Merck).

Microbial Culture and Biotransformation Conditions

Cunninghamella elegans fungal culture MTCC 9152 was procured from MTCC, Chandigarh. Stock cultures were maintained on potato dextrose agar slants at 4 °C and transferred every 6 months to maintain viability.

Biotransformation Procedures

Revival of Fungal Culture in Potato Dextrose Agar

The first stage of microbial biotransformation was carried out in a medium containing 2.4gm of potato dextrose broth dissolved in 100 mL of milli pore water. Then it was autoclaved (Equitron) at 121°C for 30 minutes and aseptically transferred into the sugar tubes. *C. elegans* fungal strain was aseptically inoculated to sugar tubes containing potato dextrose broth and incubated at 25°C until visible growth was observed. Following growth in *C. elegans* broth, it was either transferred or streaked to the potato dextrose agar plates and slants. The revived fungal culture was maintained on potato dextrose agar slants and stored at 4 °C.

Optimization of Media and Fermentation Parameter

The procedure for optimizing parameters, including temperature, incubation period, agitation speed, and drug concentration (substrate concentration), is summarized in the flow chart (Fig 3). The optimized parameters were then used in for further studies.

Extraction of Metabolites

The prepared media was autoclaved for 30 minutes at 121 °C. The inoculated broth was then incubated with fungal culture at the ideal temperature (trials were done at 20, 25, 27, and 37℃) until noticeable growth was observed (24 hrs). Next, the drug solution (25mg drug in 1mL DMSO)and 100, 500, and 1000µg/ml dilutions were made, added to the sample and drug control, and further incubated and kept in a rotary shaker (at different agitation speeds of 80, 100, 120 and 140 rpm). The

samples were collected on days 1, 5, 8, and 14. The incubated tubes were treated with methanol, centrifuged for 10 minutes at 5000 rpm, and the supernatant was collected. This supernatant was then extracted with ethyl acetate to collect the organic layer, which was then dried by air.

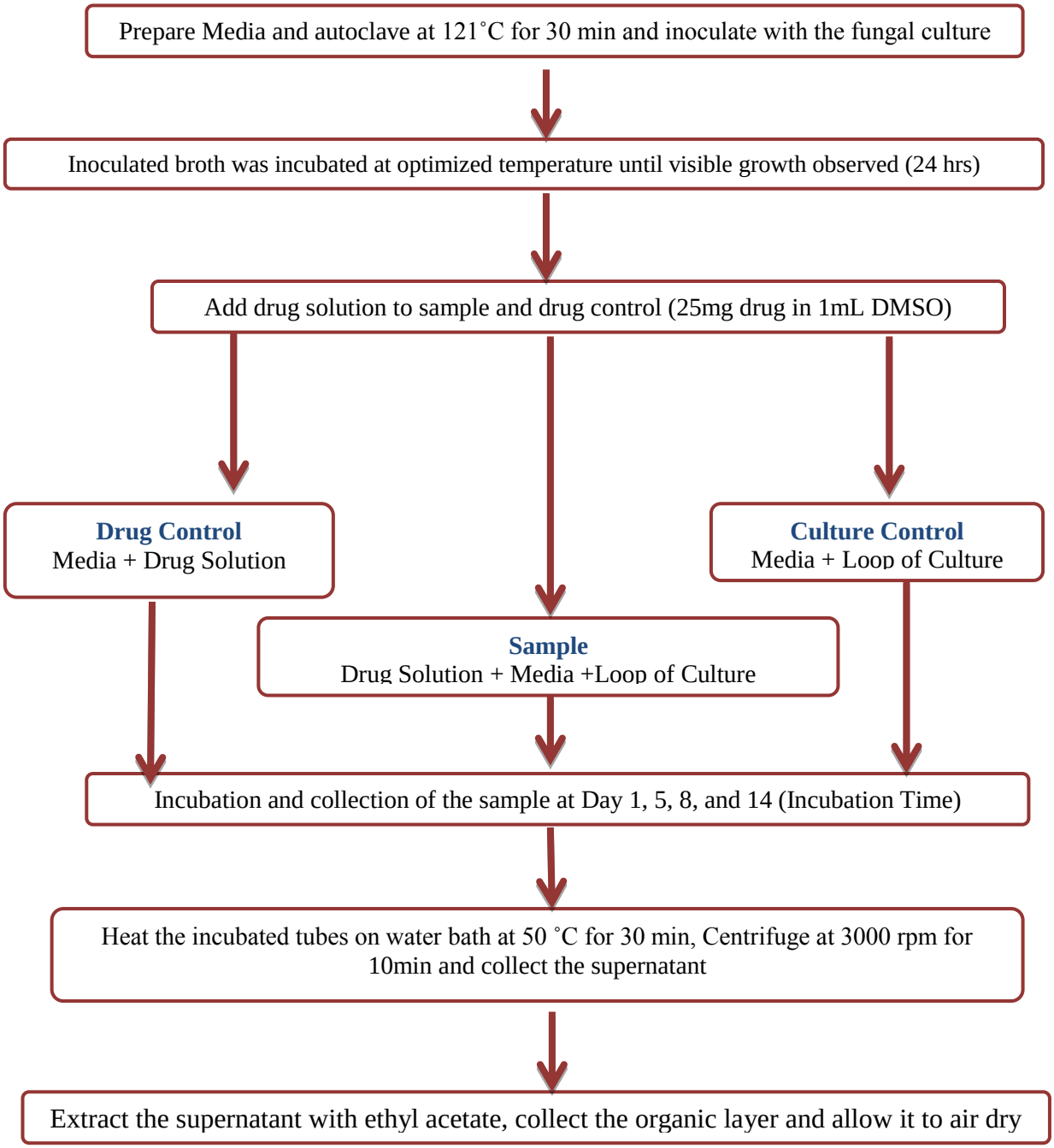

Fig 3: Flowchart Explaining the Extraction of Metabolites

HPLC estimation of Metabolites

The extracted and dried organic layer was analyzed by HPLC to determine the metabolite formed.

Chromatographic analyses were conducted on a Shimadzu LC-10A system (Kyoto, Japan) equipped with an LC-20AT pump, SPD-20AV UV-VIS variable wavelength detector, DGU-20A5 degasser, CBM-20A controller system, and SIL-20A injection valve with 100 µL loop consisting of Phenomenex C18 column (5µm, 4.6mm× 150mm) and C8 guard column (4 x 3.0 mm; Phenomenex, USA). The separation was accomplished with a mobile phase consisting of f 0.1% Formic Acid in water: Acetonitrile (10:90). The detection wavelength and run duration were set at 210 nm and 4 min, respectively. A flow rate of 1.0mL/min and an injection volume of 20µL were used.

Identification of Metabolites: MS analysis

The structural elucidation of the metabolites formed was performed in UHPLC-QTOF/MS system, UHPLC (Shimadzu-Nexera x2) with a Shinpack XR ODS III column (2.0 mm × 50 mm, 1.6 µm) from Shimadzu coupled to the QTOF-MS mass analyzer (Impact II,

Bruker Daltonics). The QTOF-MS system was coupled with positive ionization operated electro spray ionization (ESI). The mobile phase consisted of acetonitrile and 0.1% formic acid in Water: Acetonitrile (10:90). The operation parameters of ESI were set as the following: capillary voltage 4000 V; end plate offset, 500 V; nebulizer pressure, 4 bar (N2); drying gas, 0.28 L/min (N2); and drying temperature, 200 °C. The QTOF-MS system recorded spectra over the range m/z 50−1000 with a scan rate of 2 Hz. Data obtained were processed with Data Analysis 4.2 Software.

RESULTS AND DISCUSSION

Optimization of media

C. elegans were grown uniformly and evenly in the Potato Dextrose Agar plate, while in the Sabouraud Dextrose Agar plate C. elegans showed uneven growth as shown in Fig. 4 and Fig. 5, respectively.

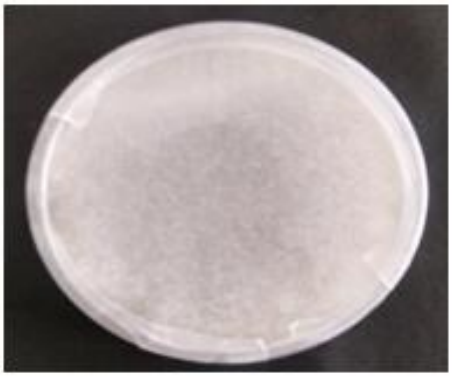

Fig. 4: Cottony white growth of *C. elegans* in Potato Dextrose Agar plate

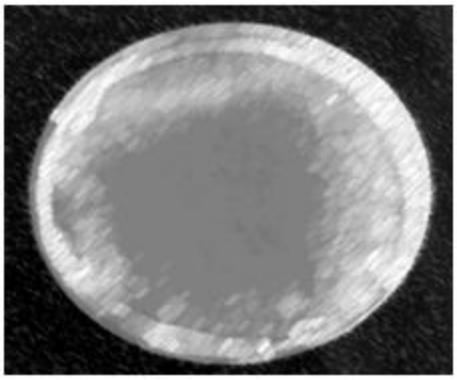

Fig. 5: Uneven white growth of *C. elegans* in Sabouraud Dextrose Agar plate

Optimization of fermentation parameters

Results of optimization of fermentation parameters showed the maximum metabolite concentration was observed when the media was grown for 11 days when incubated at 120rpm at 27°C with a maximum drug concentration of 1000µg/ml as shown in **Table 1.**

Table 1: Metabolite formed (%) in various incubation periods and agitation speed (rpm)

PARAMETERS	% METABOLITE FORMED
Incubation Period (Days)	
1	00
5	14
8	33
11	**78**
14	36
Agitation Speed (rpm)	
80	00
100	56
120	**78**
140	42
Drug concentration (µg/ml)	
100	12
500	43
1000	**79**
Temperature (°C)	
20	11
25	**76**
27	28
37	43

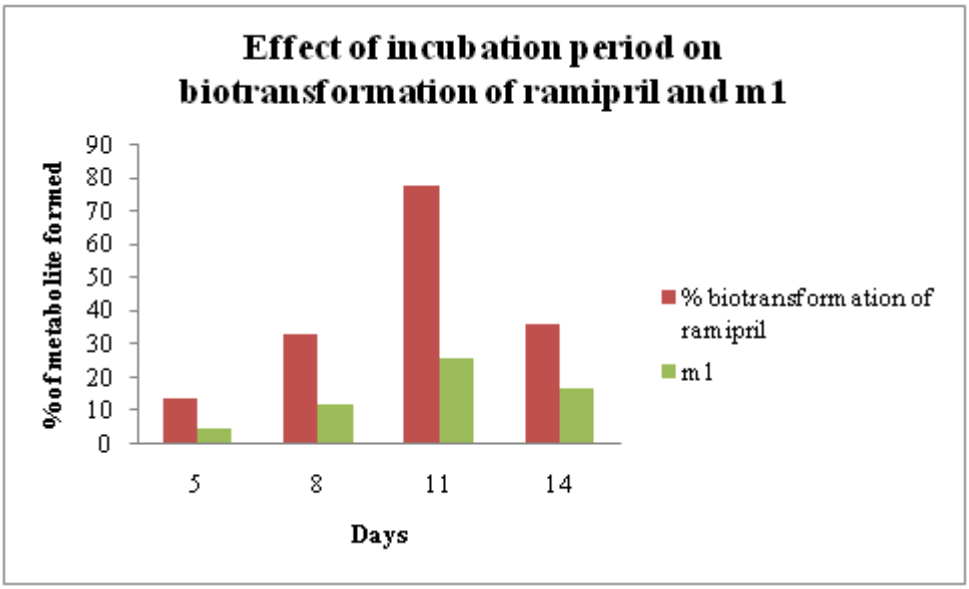

Fig. 6: Effect of Incubation Period on Biotransformation of Ramipril and m1 ***m1- Metabolite of Ramipril (Ramiprilat)***

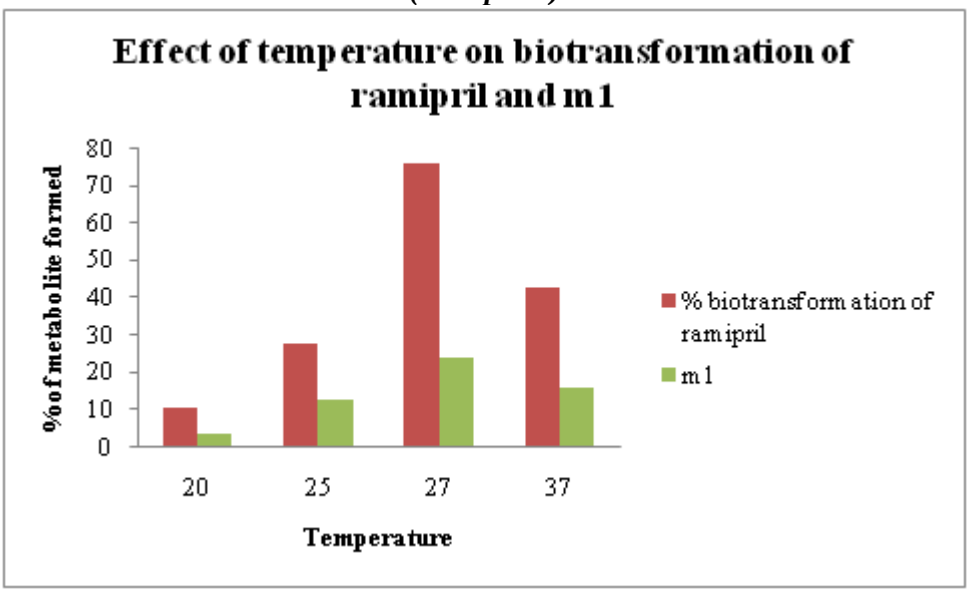

Fig. 7: Effect of Temperature on Biotransformation of Ramipril and m1 ***m1- Metabolite of Ramipril (Ramiprilat)***

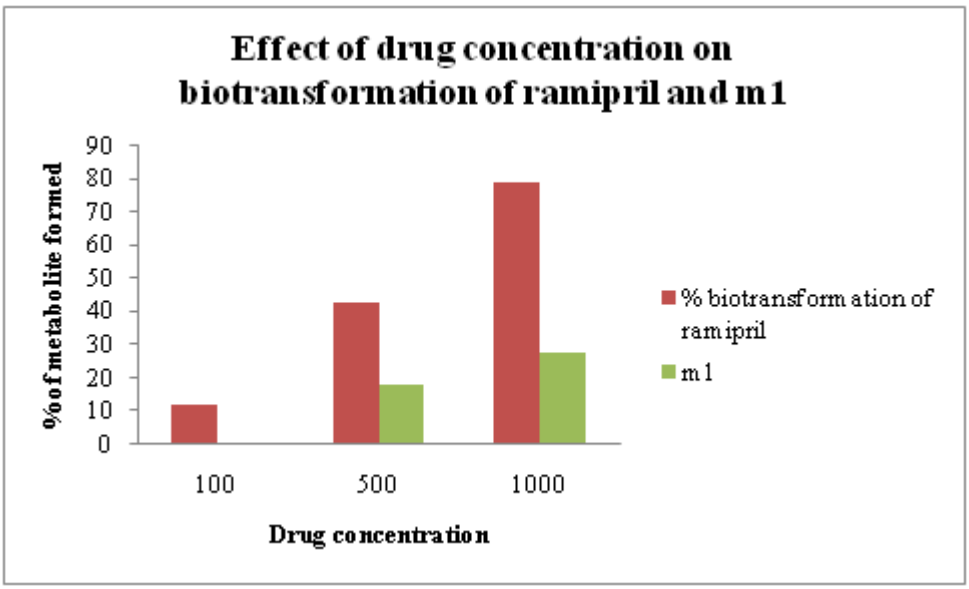

Fig. 8: Effect of Drug conc. on Biotransformation of Ramipril and m1 ***m1- Metabolite of ramipril (ramiprilat)***

HPLC Estimation of Metabolites

A representative HPLC chromatogram of ramipril and its metabolite after 8 days and 14 days of transformation by *C. elegans* is shown in Fig.3.Chromatogram (A) corresponds to the blank control. Chromatogram (B) corresponds to day 8, and chromatogram (C) corresponds to day 11.

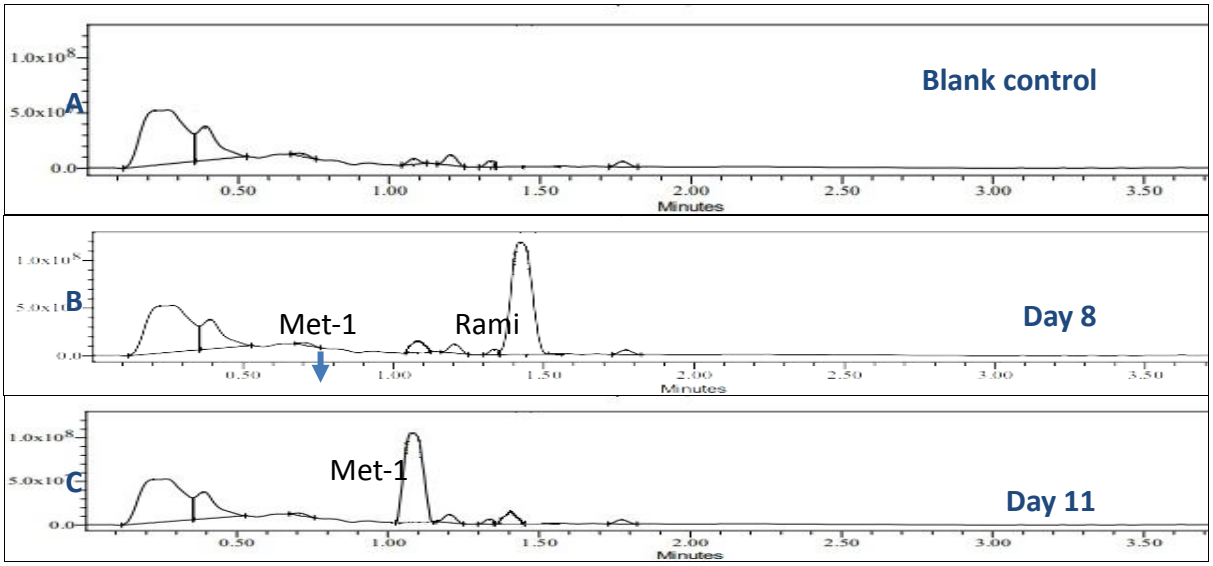

Fig. 9: A representative HPLC chromatogram of ramipril and its metabolite

After 8 days and 14 days of transformation by *C. elegans* is shown in Fig.2. Chromatogram (A) corresponds to the blank control. Chromatogram (B) corresponds to day 8, and chromatogram (C) corresponds to day 11 after biotransformation. *Met-1: Metabolite of Ramipril (Ramiprilat); Rami: Ramipril. Retention Time of Met-1: 1.08 and Rami: 1.42*

Identification of Metabolites: MS analysis- The identification of Ramipril and Ramiprilat was confirmed by comparison of the mass spectra with the authentic reference.

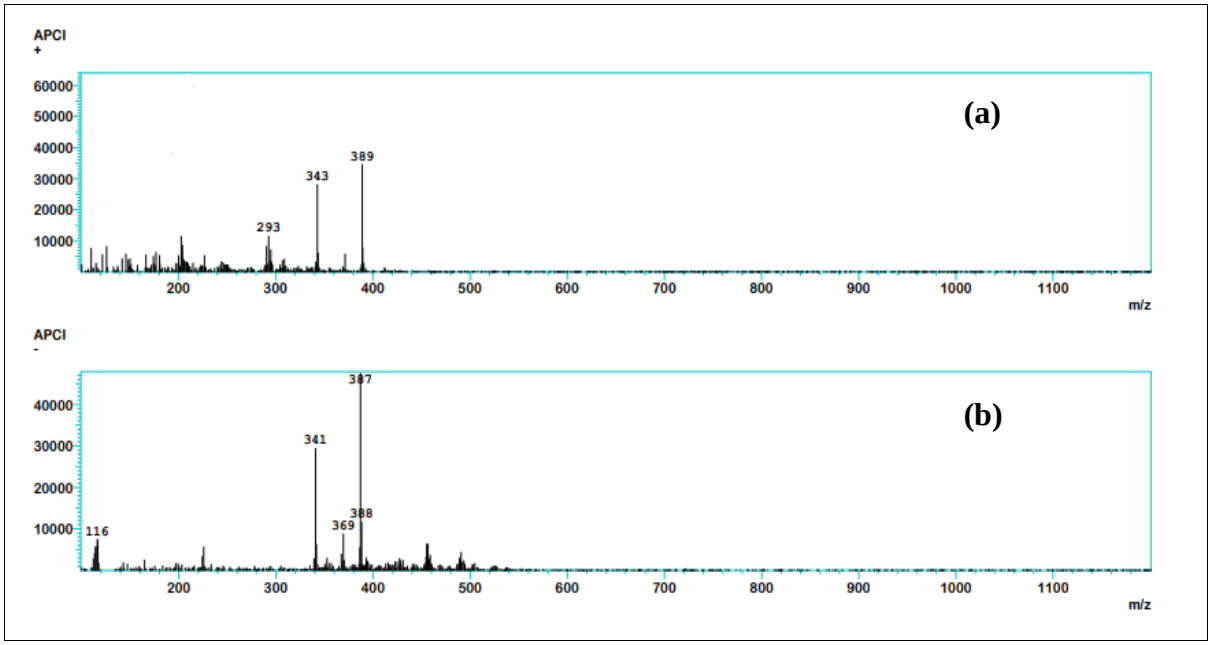

Fig. 10: Mass spectrum of ramipril (a) and its metabolite, ramiprilat (b)

This study reported successful Ramipril biotransformation by *Cunninghamella elegans* fungal culture MTCC 9152 to Ramiprilat. The biotransformation of Ramipril to Ramiprilat by *Cunninghamella elegans* was affected by media, incubation period, RPM, temperature, drug concentration, and agitation speed. The study revealed uneven growth of *C. elegans* observed in Sabouraud Dextrose Agar. While, in potato dextrose agar, the fungal cottony white uniform growth was observed. So,

for further studies, Potato dextrose agar was selected as the suitable media for fungal growth.

The incubation period was finalized based on the maximum amount of metabolite formed on day 11 (78%), and on day 14, the metabolite concentration decreased, indicating the degradation of the metabolite formed.

For incubation of the samples, they were kept in rotary it was observed that the maximum percentage of metabolite was formed at an agitation speed of 120 rpm.

The HPLC chromatograms on days 8 and 11confirmed that the drug got converted to its metabolite, as the concentration of Ramipril decreased with an increase in its metabolite concentration. Further, the mass spectra of the Ramiprilat confirmed its identity based on reference spectrum data available.

CONCLUSION

While models derived from animal tissue enable fine dissection of metabolic processes and identification of the essential enzymes involved, reactions scale-up to identify the actual metabolites can prove to be highly costly and raise ethical questions. As a result, this technique proved superior to mammalian or chemical synthesis, both of which are time-consuming and fraught with ethical issues.

Although microbial biotransformation has long been utilized as a less expensive option for reaction scale-up for metabolite, it also yields non-mammalian metabolites, requiring extensive chromatographic comparison necessary to identify the intended products.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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