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BIOTRANSFORMATION OF PARACETAMOL BY *CUNNINGHAMELLA ECHINULATA*

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ABSTRACT
The aim of the study was to develop a microbial model for synthesis of paracetamol metabolite for further pharmacological and toxicological studies. The metabolite of paracetamol in microbial cultures was identified, isolated and confirmed using fermentation techniques, Thin Layer Chromatography (TLC) and High Pressure Liquid Chromatography (HPLC) followed by liquid chromatography/mass spectrometry (LC/MS). Among different organisms screened, *Cunninghamella echinulata* showed an extra peak at 5.1 min in HPLC compared to its controls indicating formation of a metabolite. The metabolite was further characterized by mass spectrometry and was found to be N-acetyl-p-benzoquinoneimine (NAPQI) which is a toxic metabolite. *Cunninghamella echinulata* was able to metabolize paracetamol to its toxic metabolite by N-hydroxylation and rearrangement similar to human beings. This study has developed a model to produce toxic metabolites of other similar drugs easily for further toxicological and pharmacological studies.

Key words: *Microbial model; Metabolism; Paracetamol; N-hydroxylation; NAPQI.*

INTRODUCTION

Drug metabolism involves a series of enzymatic biotransformation of chemicals leading to formation of relatively polar substances, which are easily excreted and needed for pharmacological or toxicological evaluation of drugs. The understanding of drug metabolism plays an important role in the development of new drug entities.

Traditionally, drug metabolism studies were conducted on small animal models, perfused organs, *in vitro* enzyme systems and *in vitro* cell cultures[1,2]. Later microbial models were developed as alternative methods to study the metabolic fate of drug with advantages of reducing the number of animals utilized in research.

Microorganisms such as fungi, bacteria and yeast have been successfully used as *in vitro* models for the prediction of mammalian drug metabolism with successful applications[3,4,5]. A systematic examination of microbial hydroxylations on a variety of model organic compounds[6] followed by a comparison of O- and N-dealkylation reactions[4] propose that microbial transformation systems could closely mimic most of the phase-I transformations of drugs observed in mammals. The use of microorganisms as models of mammalian metabolism has been well documented[7,8,9,10].

In the present study, paracetamol which is a widely used over-the-counter drug with analgesic and antipyretic properties is selected which is well absorbed and extensively metabolized in the liver by cytochrome P450 2E1 enzyme, producing NAPQI [N-acetyl-p-benzo quinoneimine] which is a toxic metabolite. NAPQI is hepatotoxic because of its ability to bind covalently to sulfhydryl groups of hepatocytes causing hepatic necrosis[11,12]. The production of irreversible liver damage in over dose situations has made the study of paracetamol metabolism of toxicological importance. The mammalian metabolic pathway of paracetamol is shown in Figure 1. This study provides a tool to produce

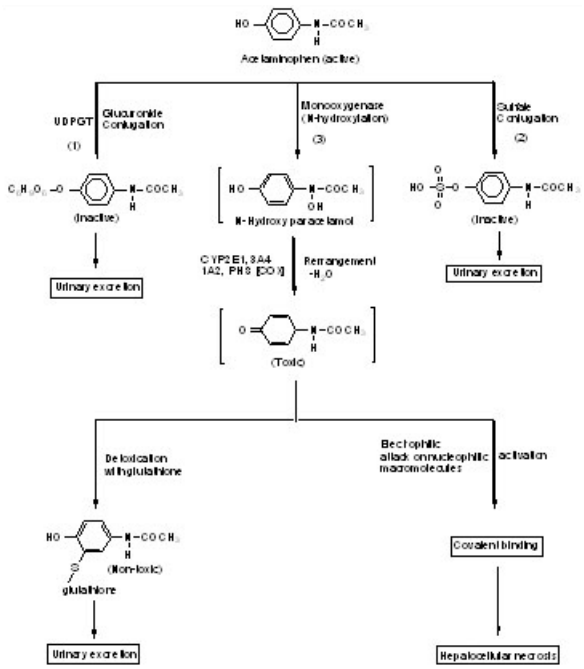

Fig 1. *Mammalian metabolic pathway of paracetamol (acetaminophen)[16,17]*

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(toxic) metabolites of other drugs easily and cheaply for further pharmacological and toxicological studies. Therefore the present study was aimed at developing a microbial model to produce metabolite of paracetamol for understanding of their actions *in vivo*, as NAPQI would be more toxic than the parent compound.

The microbial system has the advantage of collecting metabolites in larger amounts by routine fermentation techniques for further characterization as well as for pharmacological and toxicological evaluation.

MATERIALS AND METHODS

Microorganisms

Cultures were obtained from National Chemical Laboratories, Pune, India. The cultures used in the present work were *Streptomyces griseus* (NCIM 2622), *Streptomyces rimosus* (NCIM 2213), *Aspergillus terreus* (NCIM 657), *Cunninghamella elegans* (NCIM 689), *Cunninghamella echinulata* (NCIM 691), *Saccharomyces cerevisiae* (NCIM 3090).

Chemicals

Paracetamol (acetaminophen) was obtained from Sigma, Mumbai, India. All the reagents used in the analysis were of HPLC grade and were purchased from S.D. Fine Chemicals Ltd., Mumbai, India, ethyl acetate was obtained from Merck, Mumbai, India. Deionized and glass distilled water was used for this study, culture media components were purchased from Qualigens, S.D. Fine Chemicals Ltd., Mumbai, India.

Fermentation Procedure

The experiments were carried out using respective sterile growth media which consists of peptone, sodium chloride, beef extract, distilled water and pH adjusted to 7.0-7.5 for bacteria; potato extract, dextrose, yeast extract, distilled water and pH adjusted to 5.6 for fungi and malt extract, glucose, yeast extract, peptone, distilled water and pH adjusted to 6.4-6.8 for yeast. Stock cultures were stored on agar slants prepared according to the above composition at 2-8°C[13], and transferred for every 2 months to maintain viability. The media were sterilized in an autoclave for 20 min at 121°C and 15 lb / sq.in.

Microbial metabolism studies were carried out by shake flask cultures in an incubator shaker, operating at 120 rpm at 37°C. The experiments were carried out in culture flasks (100 ml) each containing 50 ml growth medium[13,14]. Fermentations were carried out according to standard protocol[15]. The substrate (paracetamol) was prepared by dissolving 10 mg of drug in 10 ml of sterile water and 0.5ml was added to the culture medium of selected organisms at a concentration of 500 µg / ml of medium in samples and incubated in shaker. The study also maintained the substrate control to which substrate was added and incubated without microorganisms and culture controls consisted of fermentation blanks in which the micro organisms were grown under identical conditions without the substrate. The incubation was continued for 24 h to 48 h.

Extraction Procedure

The pre incubated medium was heated on water bath at 50°C for 30 min and centrifuged at 3000 rpm for 10 min at 37°C (Remi instruments Pvt. Ltd., Mumbai, India). A clear supernatant liquid was collected and extracted 5 ml of supernatant with 4 ml ethyl acetate in a cyclo mixer for 10 min by vortexing. The upper organic layer was separated and dried in vacuum dryer. Later it was reconstituted with 0.5 ml of mobile phase, water: acetonitrile – 92:8 (HPLC grade, S.D. Fine Chemicals Ltd., Mumbai, India) and centrifuged at 3000 rpm for 10 min at 37°C (R8C : Remi instruments, Mumbai, India). 20 µl portions were injected into the HPLC for analysis. Controls (Substrate and culture controls) were also prepared similarly to provide suitable blanks.

Analytical Techniques

Thin Layer chromatography

Ethyl acetate and methanol in 7:3 ratio was used as mobile phase. Silica gel coated glass plates of 7 x 3 cm were used. TLC was run until 75% of the TLC plates were occupied by the solvent front in a saturated chamber. The plates were dried under open air and the spots were observed in iodine chamber. The R_f values were calculated.

High Pressure Liquid chromatographic Procedure:

High performance liquid chromatography (HPLC) analysis was carried out using a HPLC system (Shimadzu, Kyoto, Japan) consisted of LC-10AS solvent delivery module and SPD-10A VP UV-visible spectrophotometric detector and a Wakosil II 5C-18 RS-100 a. 5 µm, 4.6 x 250 mm stainless steel column (S & E Japan). Sensitivity was set at 0.001 a.u.f.s. Mobile phase consisted of HPLC grade water and acetonitrile (92:8 % v/v) at a flow rate of 0.8 ml / min. Elution was monitored using a UV / Vis detector set at 250 nm[12].

Liquid Chromatography / Mass Spectrometry:

Mass spectral data were obtained using LC/MS (Agilent Technologies, Germany). Model was API 3000 LC-MS/MS. LC coupled to a mass spectrometer operating in the electron spray ionization (ESI) mode. Ionization was carried out in positive ion mode using ion trap detector (3.5 kV, 325°C, 210 psi) with same mobile phase.

RESULTS

Five microorganisms were screened in the present study. The microbial transformation samples were extracted and analyzed as described above. The results of TLC analysis of paracetamol and its metabolite in different extracts are given in Table 1. It was found that the spot with R_f value of 0.6 would represent paracetamol in TLC analysis. The spot with R_f value 0.4 was observed as metabolite of paracetamol in sample of *Cunninghamella echinulata* when compared to its controls. So, it was further analysed by HPLC and LCMS.

Table 1. *TLC data for paracetamol and its metabolite from microbial cultures*

Name of the organism	Retention factor (R_f) values			
	(Blank-I) substrate control	(Blank-II) Culture control	Pure Paracetamol	Sample
Cunninghamella echinulata (NCIM 691)	0.6	-	0.6	0.6 0.4*
Cunninghamella elegans (NCIM 689)	0.6	-	0.6	0.6
Saccharomyces cerevesiae (NCIM 2622)	0.6	-	0.6	0.6
Streptomyces griseus (NCIM 2213)	0.6	-	0.6	0.6
Streptomyces rimosus (NCIM 3090)	0.6	-	0.6	0.6
Aspergillus terreus (NCIM 657)	0.6	-	0.6	0.6

** - Metabolite spot.*

The results of HPLC analysis of paracetamol and its metabolite in different extracts are given in Table 2.

Table 2 . *HPLC data for paracetamol and its metabolite from microbial culture extracts*

Name of the organism	Retention time in minutes			
	(Blank-I) substrate control	(Blank-II) Culture control	Pure Paracetamol	Sample
Cunninghamella echinulata (NCIM 691)	2.3	2.3	2.3	2.3
	--	2.9	--	2.8
	--	--	--	5.1*
	8.8	--	8.8	8.8
	--	--	--	--
Cunninghamella elegans (NCIM 689)	2.3	2.3	2.3	2.3
	3.5	3.5	--	3.5
	8.8	--	8.8	8.8
Saccharomyces cerevesiae (NCIM 2622)	2.3	2.3	2.3	2.3
	4.2	4.2	--	4.2
	8.8	--	8.8	8.8
Streptomyces griseus (NCIM 2213)	2.3	2.3	2.3	2.3
	3.5	3.5	--	3.5
	8.8	--	--	--
			8.8	8.8
Streptomyces rimosus (NCIM 3090)	2.3	2.3	2.3	2.3
	3.5	3.5	--	3.6
	8.8	--	8.8	8.8
Aspergillus terreus (NCIM 657)	2.3	2.3	2.3	2.3
	4.7	4.8	--	4.8
	8.8	--	8.8	8.8

** - Metabolite peak.*

Two peaks were seen in all extracts with retention times of 2.3 min. and 8.8 min. It was found that the peak at 2.3 min represents broth constituent whereas the peak at 8.8 min corresponds to paracetamol by comparing the controls and sample. An additional peak at retention time of 5.1 min was observed in sample of *Cunninghamella echinulata* when compared to its controls (Table 2 and Fig. 2), indicating metabolite of paracetamol was formed, which was isolated by collecting from elute of HPLC and the structure was confirmed by LC/MS as shown in Figs.3 and 4 for paracetamol and its metabolite respectively. The mass spectrum of pure paracetamol showed a molecular ion peak at m/z 152 and fragment ion peak at m/z 110 (Fig. 3). The mass spectrum of metabolite revealed a molecular ion peak at m/z 150 and fragment ion peak at m/z 107 (Fig. 4). The fragmentation pattern of paracetamol and its metabolite was compared and shown in Fig. 5. The proposed metabolic pathway of paracetamol by *Cunninghamella echinulata* is shown in Fig. 6.

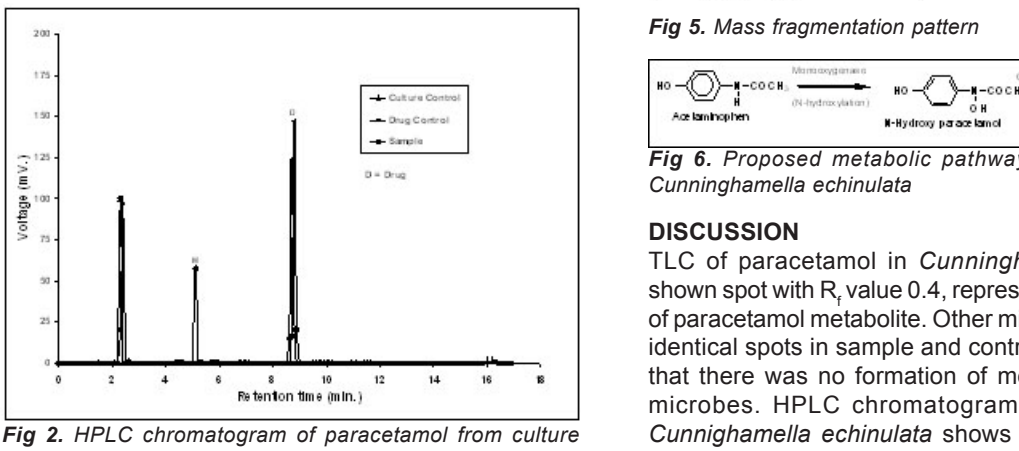

Fig 2. *HPLC chromatogram of paracetamol from culture extracts of Cunninghamella echinulata.*

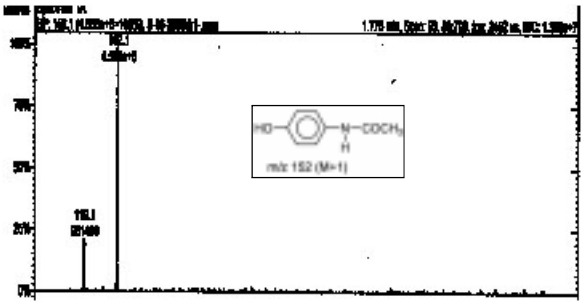

Fig 3. *Mass spectrum of pure paracetamol.*

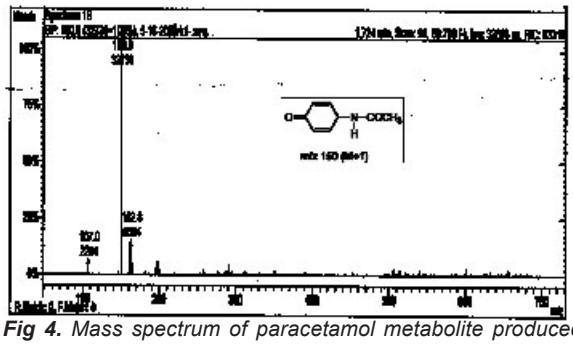

Fig 4. *Mass spectrum of paracetamol metabolite produced by Cunninghamella echinulata*

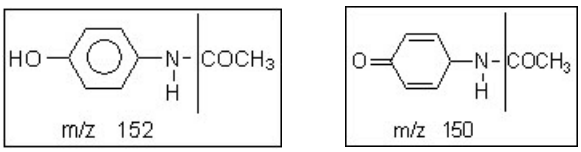

Fig 5. *Mass fragmentation pattern*

Fig 6. *Proposed metabolic pathway of paracetamol by Cunninghamella echinulata*

DISCUSSION

TLC of paracetamol in *Cunninghamella echinulata* shown spot with R_f value 0.4, representing the formation of paracetamol metabolite. Other microbes have shown identical spots in sample and controls which indicated that there was no formation of metabolites by those microbes. HPLC chromatogram of the sample of *Cunnighamella echinulata* shows an additional peak than its controls compared to other organisms which

represents that paracetamol was transformed to its metabolite (Fig. 2). In case of other organisms, they have shown identical peaks in sample and controls which indicated that the organisms analyzed could not metabolize the drugs. The mass spectrum of paracetamol exhibited a molecular ion peak at m/z 152 (M+1) (Fig.3) which was supported by fragment ion at m/z 110 (Fig. 3). The molecular ion of m/z 150 (M+1) in mass spectrum of metabolite of paracetamol by *Cunninghamella echinulata* represents the NAPQI i.e. N-acetyl-p-benzoquinoneimine as its metabolite. The formation of above metabolite was supported by fragment ion peak at m/z 107 (Fig. 4). The phase-I metabolite of paracetamol in human beings is also NAPQI[11,16,17] which is a toxic metabolite due to its ability to bind to micromolecules of liver both in animals and human beings, thus paracetamol is a well documented example of a hepatotoxin[15]. It was observed that *Cunninghamella* contains species of importance in medical mycology and in biotechnological processes. They possess cytochrome P450 monooxygenase systems analogous to those in mammals and phase-II drug metabolism enzymes[18],[19]. They have the ability to metabolize a wide variety of xenobiotics using both Phase-I (oxidative) and Phase-II (conjugative) biotransformation mechanisms[20]. It was well documented that *Cunninghamella echinulata* has the ability to metabolize various drugs such as amphetamine[21], bisoprolol[22], bornaprine[23], papavarine[24], praziquantel[25] to their respective metabolites which are similar to mammalian metabolites. So, it can be concluded that *Cunninghamella echinulata* may metabolize paracetamol to its toxic metabolite NAPQI as reported in human and animals.

It was also found that the microorganisms could metabolize the drugs by N-hydroxylation, based on the reports of N-hydroxylation of N-methyl carbazole (a component of tobacco smoke) by *Cunninghamella echinulata*[26-29]. Thus NAPQI may also be found in *Cunninghamella echinulata* through N-hydroxylation as in N-methyl carbazole and rearrangement to NAPQI like in human.

On the basis of the above results and discussion, the present investigation suggested that *Cunninghamella echinulata* was able to metabolize paracetamol to NAPQI, a hepatotoxic metabolite of paracetamol by N-hydroxylation and rearrangement similar to human beings and animals. Thus it can be concluded that *Cunninghamella echinulata* can be used as a microbial model for producing toxic metabolite, NAPQI and such toxic metabolites of other drugs easily for further toxicological and pharmacological studies.

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