Biotransformation of Silibinin (Silybin) Using Fungal Organisms

Venisetty R.K, Keshetty S and Ciddi V*

Faculty of Pharmaceutical Sciences, Kakatiya University, Warangal, Andhra Pradesh, India

ABSTRACT

Submitted: 1-12-2010 Revised: 8-3-2011 Accepted: 17-6-2011

Biotransformation of Silibinin using fungi was performed and parameters *viz.,* effect of incubation period, effect of pH, effect of temperature, effect of glucose concentration and effect of substrate concentration were studied. Of the 29 fungi studied 11 were able to metabolize Silibinin to produce one or two metabolites, which was evidenced from the absence of the new peaks in controls. Maximum biotransformation of 29% was seen with *Cunninghamella echinulata* NCIM 691. The metabolites were identified as hydroxylated (M1) and demethylated (M2) products of Silibinin by HPLC diode array and LC MS-MS analysis. These metabolites were already reported in mammalian metabolism studies. Dextrose medium 0.02% Silibinin (pH 7.0) was incubated for 8 days at 27 °C. This was found to be suitable for maximum biotransformation of Silibinin using *Cunninghamella echinulata* NCIM 691.

Keywords: Biotransformation, Metabolites, Fungi, Silibinin.

INTRODUCTION

Before approval for use in humans, a drug must undergo extensive studies to establish its efficacy and safety. An important factor in evaluation of safety and efficacy of any drug is the knowledge of how it is metabolized. Traditionally, metabolism studies are conducted on small animal models, perfused organs, cell cultures and enzyme systems. It's a universal fact that microorganisms are sources of many enzymes and they catalyze different types of chemical reactions. Monooxygenase activity in the fungi *Cunninghamella bainieri* is found to be similar to that of liver microsomal monooxygenase activity[1]. Microbial models may constitute an alternative to the use of animal models, if they can mimic the mammalian metabolism and can give some information about the metabolic fate of the drug. This methodology has the advantage of reducing the use of animals, particularly in the early phases of drug development. Microbial biotransformation studies were conducted on steroids and antibiotics since long time and are still in use. These studies have paved the way to the formalization of the so called "Microbial Models of Mammalian Metabolism" in the mid 1970s[2]. Silibinin, a flavanolignan is the chief constituent of silymarin and is used as hepatoprotective agent [3], antiproliferative agent[4] and in the treatment of Amanita poisoning[5]. About 3-8% Silibinin is excreted in urine and 80% is excreted in the bile as glucuronide and sulphate conjugates[6].

***Address for Correspondence:**

Ciddi V, Faculty of Pharmaceutical Sciences, Kakatiya University, Warangal, Andhra Pradesh, India

E-Mail: ciddiveeresham@yahoo.co.in

Major metabolites include demethylated silibinin, minor include mono-hydroxy and dihydroxy silibinin. Metabolism is mainly due to CYP3A4[7]. Till date there is no literature available on Silibinin biotransformation using fungal microorganisms.

MATERIALS AND METHODS

Chemicals

Silibinin used in the study was in amorphous form, purchased from Sigma-Aldrich, USA. The solvents were purchased from Ranbaxy Fine Chemicals Ltd., New Delhi and all other chemicals of highest available purity were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai.

Selection and procurement of microorganisms

The microorganisms used in the study were selected based on the research reports available[8,9]. Twenty nine different fungal cultures capable of producing mammalian metabolites were selected and obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTEC), Chandigarh, India and National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratories (NCL), Pune, India. The cultures were revived, subcultured and stored in refrigerator at 4 ºC. The cultures were maintained on potato dextrose agar slants as per the specifications given in NCIM and IMTEC catalogues.

Preparation of media for biotransformation

Based on the literature[8], the medium selected for biotransformation studies was Dextrose Broth. The composition of the dextrose broth (per liter) used for

biotransformation is Dextrose, 20 g; Yeast extract, 5 g; Peptone, 5 g; Sodium Chloride, 5 g; K_2HPO_4, 5 g and pH is adjusted to 7.4. The medium was poured in 50 ml conical flasks with 10 ml media in each flask. The flasks were cotton plugged, covered with aluminum foil and sterilized by autoclaving at 121° C (15lb/in^2) for 15 min.

Effect of Silibinin on media pH

The pH of the medium was recorded after addition of the drug in the concentration at which the microbial biotransformation studies could be carried out. To 10 ml of each medium 2 mg of the drug was added and observed for the changes in pH after addition of drug.

Culture procedure

For screening experiments, two stage fermentation protocol was used[8]. First stage culture was initiated in 50 ml conical flasks containing 10 ml of sterile dextrose broth and inoculated with a loop of culture scratched from freshly grown agar slant under aseptic conditions using Laminar Flow Clean Air Workstation. The culture flasks were incubated at 180 rpm and 30 ºC in refrigerated shaker incubator (Model Innova 4230, New Brunswick Scientific Co. Inc, NJ, USA). For second stage cultures, 50 ml culture flasks containing 10 ml of the same medium were inoculated with 500 µl of 48 hr grown first stage culture and shaken under similar conditions for 48 hrs. Medium was supplemented with 0.02% Triton X 100 in order to get good dispersion of fungi[10]. All experiments were performed in triplicate.

Effect of incubation period, pH, temperature, glucose concentration and drug concentration on biotransformation of Silibinin was studied with the fungus on which maximum biotransformation was observed.

Influence of incubation period was studied by analyzing the culture broth at the end of 2, 4, 6, 8, 10, 12, 14 and16 days of incubation. Influence of pH on biotransformation of Silibinin was studied by adjusting the pH in the range of 4-11 with the help of 6N NaOH or 6N HCl. Influence of temperature on biotransformation of Silibinin was studied by incubating the inoculated flasks at different temperatures 20, 25, 27, 30, 35, and 40^0 C. Influence of dextrose concentration on biotransformation of Silibinin was studied by adding different levels of dextrose (0%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, and 3.5%). Influence of Silibinin concentration on biotransformation of Silibinin was studied by adding different concentrations of Silibinin (0.005, 0.01, 0.015, 0.02, 0.025, 0.03, 0.035 and 0.04 %) to the second stage culture.

Biotransformation

The second stage cultures (10 ml culture in 50 ml capacity conical flask) were added with 2 mg each of Silibinin in 100 µl solvent (mixture of dimethylformamide and methanol in the ratio 1: 3). Prior to addition, the drug solution was filter sterilized using sterile syringe driven filter unit. The final drug concentration in each flask will be 0.2 g/liter. Each culture was studied in quadruplicate while running suitable controls. Culture controls were composed of 100 µl of solvent and the Drug controls were composed of the sterile medium to which the same amount of the drug was added and both controls were incubated without microorganisms. The incubation was continued under similar conditions for ten days, the flasks were taken out, extracted and analyzed.

Extraction and sample preparation of Silibinin and metabolites

The cultures were taken out and extracted with 3 volumes of ethyl acetate by vortex mixing for 1 min. The combined organic phase was evaporated under reduced pressure using rotary vacuum evaporator (Buchi Labortechnik AG, Switzerland) followed by drying at room temperature in vacuum oven. The dried samples were reconstituted in 1.5 ml each of HPLC grade methanol by vortex mixing for 1 min. The samples were then taken into eppendroff tubes, centrifuged at 12,000 x g and 20 ºC for 20 min in micro refrigerated centrifuge (MICRO 17TR, Hanil Science Industrial Co., Korea). The supernatants were used for HPLC-DAD and LC-MS-MS analysis.

HPLC-DAD analysis

HPLC analysis was performed using LC-10AT (Shimadzu Corporation, Kyoto, Japan) system by injecting 20 µl of sample with Hamilton syringe into sample injector following the conditions quite close to the method described previously[11]. The column used was Luna C18, 5 µ, 250 x 4.6 mm i.d. (Phenomenex, USA). The mobile phase consisted of a mixture of acetonitrile:water (pH adjusted to 2.8 with formic acid) in 30:70 pumped isocratically at a flow rate of 1 ml/min and the drug and its metabolites were detected using diode array detector at a wavelength of 288 nm. The data analysis was performed by Class M10 software. The UV absorption spectrum of the metabolites was compared with that of Silibinin. The metabolites were quantified based on the peak areas and expressed as percentages of metabolites formed. The calculations were performed with respect to the total area of drug and metabolites together taken as 100%.

LC-MS-MS analysis

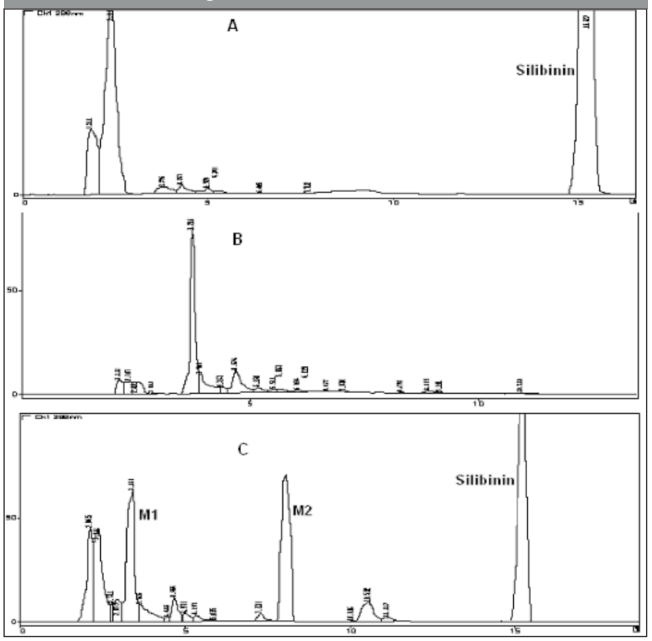
The m/z values of drug and metabolites and their fragmentation ions were recorded by LC-MS-MS using Perkin Elmer Sciex API mass spectrometer set in negative mode. The API-4000 LC-MS/MS was operated under the

multiple reaction-monitoring mode (MRM) using the electrospray ionization technique. The separation was performed on X-Terra MS C-18 column (4.6 mm x 50 mm, 5 µ). The mobile phase consisted of a mixture of acetonitrile:water (pH adjusted to 2.8 with acetic acid) in 70:30 ratio, was isocratically pumped at a flow rate of 1 ml/min. 50 µl of the sample was injected using autosampler device. The vaporizer temperature and the discharge current were set at 350º C and 5.0kV respectively. The fragments were scanned in the m/z range of 100 to 700 and the data were processed with Sciex Analyst software. The metabolites were identified basing on pattern of UV spectra in HPLC-DAD and m/z values of the fragmentation products obtained in LC-MS-MS analysis.

RESULTS

There was no significant change in the medium pH after the addition of the drug. In the drug control (i.e., drug in medium) no additional peak is seen in HPLC analysis indicating that the substrate Silibinin was stable in the medium and the recoveries of Silibinin from these media were greater than 94.1%. HPLC analysis of the culture extracts showed that 11 out of 29 cultures were able to metabolize Silibinin to produce one or two metabolites evidenced from the absence of these new peaks in drug control as well as culture controls. Metabolites of Silibinin produced by fungal organisms are represented in Table 1. Representative HPLC chromatographs are shown in Fig. 1. Silibinin was eluted at ~15 min and the metabolites produced, designated as M1 and M2 were eluted at 3.1 min and 7.9 min respectively. All these new peaks had shown similar UV spectral pattern (from 190 to 370 nm) to that of Silibinin.

Fig. 1: Representative HPLC chromatograms A) Drug control, B) Culture control and C) Biotransformation using *Cunninghamella echinulata* NCIM 691.



The metabolites M1, M2 and Silibinin have shown the deprotonated molecular ions at m/z values 497, 467 and 481 respectively. Fragmentation pattern of Silibinin and its metabolites M1 and M2 were shown in Fig. 2. The ionization spectrum of the m/z 481 pertaining to Silibinin (Mol. Wt. 482) showed product ions with m/z 301 (fragment I) and 179 (fragment II). The structures of fragments pertaining to m/z 301 and 179 are shown in Fig. 3. M1 gave the deprotonated molecular ion at m/z 497 and the ionization spectrum of the m/z 497 showed product ions at m/z 317 and 179. In the present study, 9 cultures have produced this metabolite. M2 gave the deprotonated molecular ion at m/z 467 and the ionization spectrum of the m/z 467 showed product ions at m/z 301 and 165. In the present study, 7 cultures have produced this metabolite. Biotransformation was maximum (31 %) with *Cunninghamella echinulata* NCIM 691 when incubated for 8 days. Concentration of metabolites M1 and M2 formed varied with different days of incubation and were found to be maximum (14 and 17 %) when incubated for 8 days (Fig. 7). After 8 days of incubation the concentration of metabolites remained almost constant or decreased slightly.

Biotransformation of Silibinin could take place over a wide range of pH from 4.0 to 10.0. Maximum biotransformation occurred at pH 7.0 (30%) and the metabolites M1 and M2 were also formed significantly (14 and 16% respectively) when compared to other pH conditions (Fig. 8). At different range of pH studied the formation of M2 was more when compared to M1. At pH 4.0 and 10.0 metabolite, M1 formation was not observed and at pH 11.0 biotransformation of Silibinin was not seen.

Fig. 2. Representative LC MS-MS chromatograms A) Drug control, B) Culture control and C) Biotransformation using *Cunninghamella echinulata* NCIM 691.

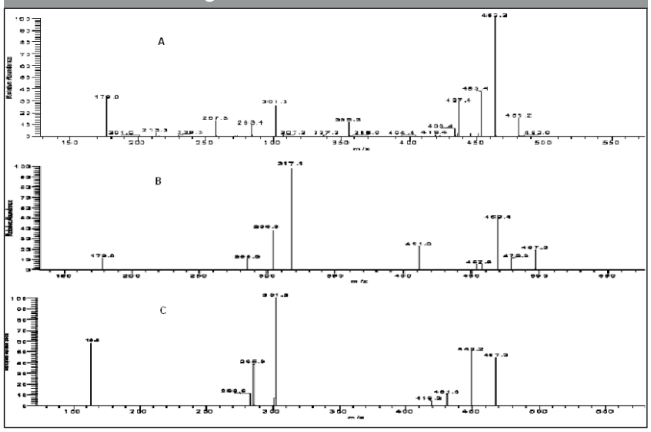


Table 1: Metabolites of silibinin produced by fungal organisms

S. No	Culture	M1 (3.1min)	M2 (7.9min)
1	Absidia coerulea MTCC 1335	-	-
2	Absidia glauca MTCC 982	-	-
3	Aspergillus flavipes NCIM 1209	-	-
4	Aspergillus flavus NCIM 554	-	-
5	Aspergillus flavus NCIM 557	-	-
6	Aspergillus niger NCIM 1006	-	7.2
7	Aspergillus niger NCIM 589	-	7.2
8	Aspergillus niger NCIM 620	-	-
9	Aspergillus ochraceous NCIM 1140	-	-
10	Aspergillus parasiticus NCIM 898	-	-
11	Beauvaria bassiana NCIM 1216	-	-
12	Cunninghamella blakesleana NCIM 687	-	-
13	Cunninghamella blakesleana NCIM 688	5.9	-
14	Cunninghamella echinulata NCIM 691	11.2	17.8
15	Cunninghamella echinulata NCIM 693	8.3	11.6
16	Cunninghamella elegans NCIM 689	3.2	6.4
17	Cunninghamella elegans NCIM 690	4.2	7.9
18	Cunninghamella sp . NCIM 1184	-	-
19	Curvularia lunata NCIM 716	5.8	7.5
20	Fusarium oxysporum NCIM 1008	-	-
21	Mucor plumbeus NCIM 984	10.6	-
22	Mucor rouxii MTCC 386	7.5	-
23	Pencillium brevicompactum MTCC 549	-	-
24	Pencillium chrysogenum NCIM 733	-	-
25	Pencillium chrysogenum NCIM 738	-	-
26	Rhizopus arrhizus NCIM 997	-	-
27	Rhizopus stolonifer NCIM 880	4.1	-
28	Thamnostylum piriforme NCIM 974	-	-
29	Trichothecium roseum NCIM 1147	-	-

*Values indicate the percentage of metabolites ' area in HPLC analysis. The value is the percentage of a metabolite with respect to the total area of drug and all the metabolites formed

Temperature was found to have profound effect on transformation of Silibinin. Temperature of 25-35^{0}C was most favorable for transformation of Silibinin (Fig. 9.). Maximum biotransformation was observed at 27 ^{0}C (32 %) and formation of M1 and M2 was significant (14% and 18%).

Effect of dextrose concentration on the biotransformation of Silibinin using Cunninghamella echinulata NCIM 691 is shown in Fig. 10. Dextrose concentrations from 1.5 to 2.5% were appreciable in the biotransformation of Silibinin and the maximum respone was observed at 2.0% solution with a metabolic rate of 31%. Metabolite M1 could not be detected in the medium without dextrose. On the other hand, its formation was maximum (12%) at 2.0% dextrose concentration, which decreased with further increase in

Fig. 3. Structures of LC MS-MS fragments of Silibinin.

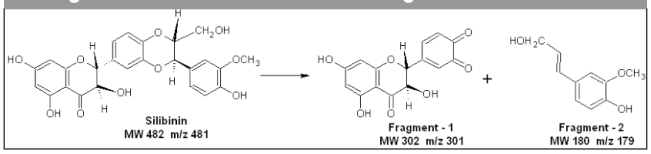


Fig. 4: Structures of LC MS-MS fragments of metabolite M1.

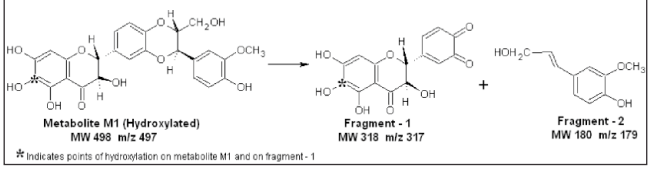


Fig. 5: Structures of LC MS-MS fragments of metabolite M2.

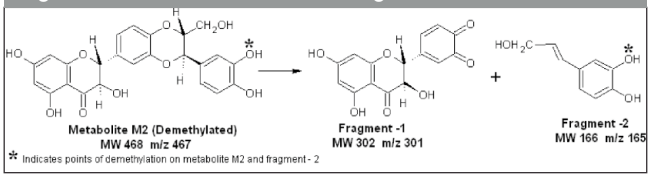
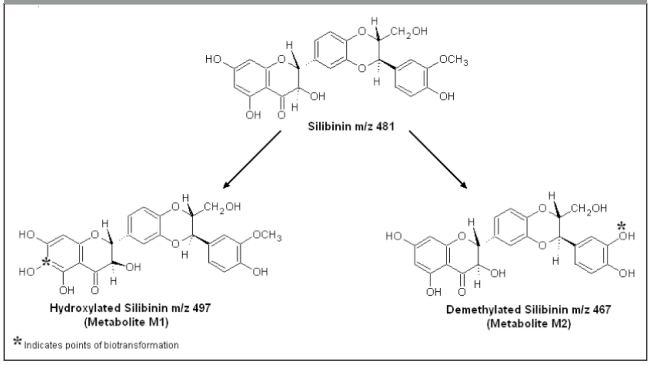


Fig. 6: Proposed biotransformation pathway of Silibinin.



concentration of dextrose. The formation of metabolite M2 was maximum (19%) in medium containing 2.0% dextrose, which also decreased with increase in the concentration of dextrose.

Concentration of Silibinin (Substrate) had significant influence on its biotransformation by *Cunninghamella echinulata* NCIM691. The amounts of metabolites formed varied with the amount of Silibinin added to the dextrose broth (Fig. 11.). The concentration of 0.02 % w/v biotransformation was maximum (30%), all two metabolites produced to a maximum extent M1 (12%) and M2 (18%).

DISCUSSION

The production of metabolites by microbial cultures may be due to the presence of enzymes expressed naturally or induced by the drug or media component(s). All the metabolites produced were eluted before the drug in reverse phase HPLC indicating that they are polar than Silibinin. The

Fig. 7: Effect of days of incubation on biotransformation of Silibinin and formation of metabolites M1 and M2.

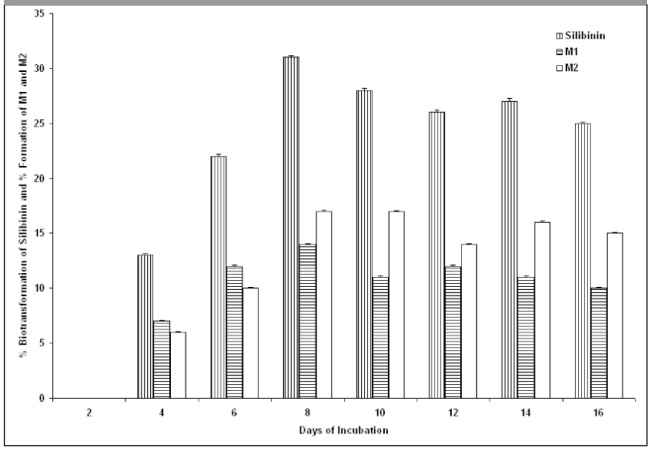


Fig. 8: Effect of pH of media on biotransformation of Silibinin and formation of metabolites M1 and M2.

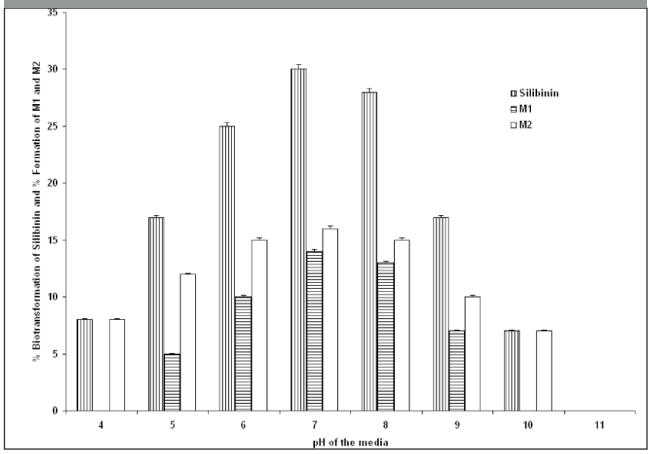


Fig. 9: Effect of temperature on biotransformation of Silibinin and formation of metabolites M1 and M2.

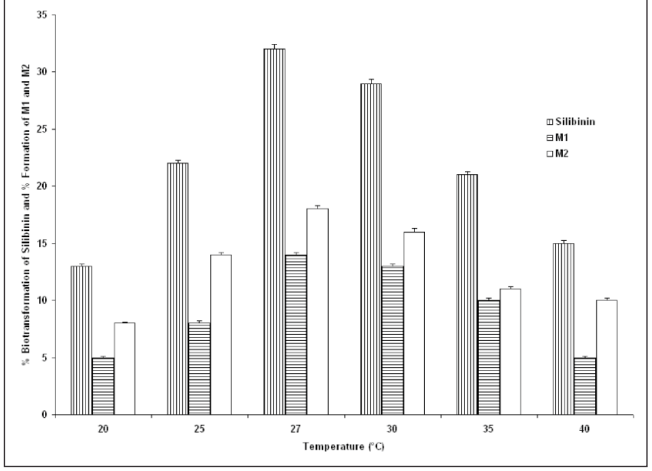


Fig. 10: Effect of dextrose concentration on biotransformation of Silibinin and formation of metabolites M1 and M2.

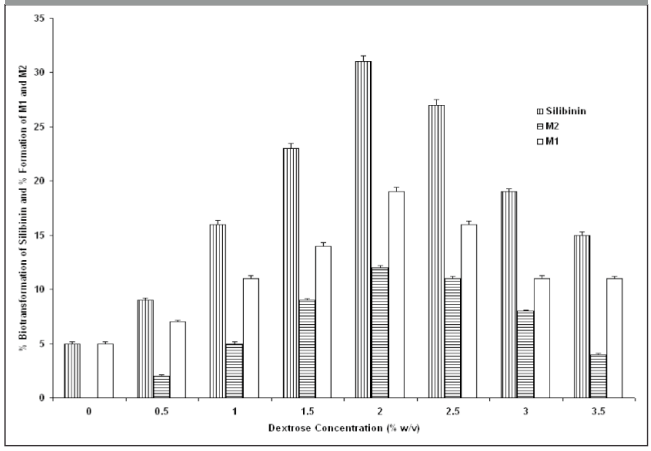
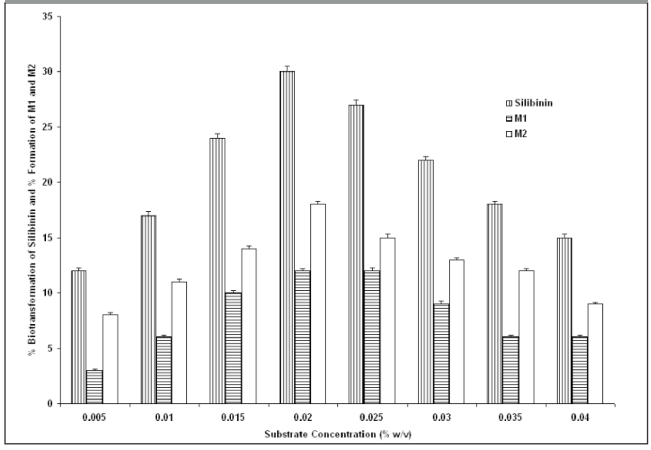


Fig. 11: Effect of substrate (Silibinin) concentration on biotransformation of Silibinin and formation of metabolites M1 and M2.



metabolite peaks were identified by HPLC basing on the similarity in UV spectra in the diode array detector. All these new peaks had shown similar UV spectral pattern (from 190 to 370 nm) with that of Silibinin indicating that the drug has undergone only minor structural changes in the process of metabolism by microorganisms. This observation reveals that there was no loss of aromaticity, ring fission or ring fusion since any of these changes would be expected to lead to a high alteration of the metabolites' UV spectra. The UV λ_{max} of the metabolites were within a narrow range with that of Silibinin which indicates that their extinction coefficient at 288nm was not significantly different from that of the parent drug. The Fungal organism *Cunninghamella elegans* was able to N-demethylate malachite green, where the metabolites showed a slight decrease (618 to 608 nm) in absorption maxima (visible λ_{max}) than the substrate malachite green[12].

A large variation was observed in the quantitative metabolite

production among various cultures. Maximum biotransformation was seen with *Cunninghamella echinulata* NCIM 691 (29 %) and minimum with *Rhizopus stolonifer* NCIM 880 (4.2 %) this may be attributed to many reasons viz., expression of low levels of enzymes required for this reaction, substrate/product inhibited biotransformation, non-optimal conditions (media and incubation) for biotransformation[8]. The structure elucidation of the metabolites was carried out from the fragmentation pattern obtained in LC-MS-MS analysis. LC-MS-MS has been widely used in the characterization of metabolites found in microbial metabolism studies[13].

M1 gave the deprotonated molecular ion at m/z 497 that is 16 higher than the Silibinin. This suggests that M1 might be hydroxylation product of Silibinin. The ionization spectrum of the m/z 497 showed product ions at m/z 317 and 179 which suggest that hydroxylation occurred on fragment I of Silibinin. Basing on this mass fragmentation and HPLC retention time, M1 was assumed as a hydroxyl metabolite of Silibinin. This metabolite was reported in mammals [6,7]. Aromatic hydroxylation by the fungi *Cunninghamella bainieri*, *Cunninghamella echinulata* and *Aspergillus niger* [1,14] suggest that aromatic hydroxylation as in the case of hydroxylated Silibinin fragment m/z 317, occur with several organisms. Since this metabolite formation occurs by direct hydroxylation of the aromatic ring of chroman nucleus of Silibinin no specific pathway is involved in this reaction. The fragmentation pattern, the structures of the fragments and the most probable point of attack (represented with an asterick) are shown in Fig. 4.

M2 gave the deprotonated molecular ion at m/z 467 that is 14 lesser than the parent drug. The ionization spectrum of the m/z 467 showed product ions at m/z 301 and 165. Basing on this mass fragmentation and HPLC retention time, M2 was assumed as a demethylated metabolite of Silibinin where the demethylation took place on the methoxy group of Silibinin. Appearance of the fragment with m/z 165 indicates demethylation took place on the 2nd fragment which consists of methoxy group. This metabolite was reported in mammals[6,7]. Several reports suggest that O-demethylation takes place with several organisms. *Cunninghamella echinulata* NRRL 1384 was able to demethylate a thebaine derivative to give the intermediates for the synthesis of buprenorphine[15] and *Streptomyces griseus* was able to demethylate codeine[16]. The fragmentation pattern and the structures of the fragments are shown in Fig. 5. The most probable point of demethylation is the 19th position (represented with asterick in Fig. 5) of Silibinin which has a methoxy group. Since this metabolite formation occurs by direct demethylation of methoxy group of the aromatic ring of Silibinin no specific pathway is involved in this reaction. The proposed biotransformation pathway of Silibinin is shown in Fig. 6. Some of the cultures employed in the present study did not produce any metabolites for Silibinin. This may be attributed to the absence of substrate specific enzymes required for biotransformation. It may also be due to the substrate not reaching the site of biotransformation, i.e., lack of membrane transport processes involved to get the substrate into the site where the enzymes are known to be located.

The varying concentrations of the metabolites in the medium during different days of incubation period may be attributed to the reversible reactions of enzymes involved. The substrate concentration decreased until 8th day after which the concentration remained almost constant, indicating no further biotransformation, in consequence the transformation rate decreased. This fact can be a result of the low levels of nutrients in the medium and enzymes inhibition could also have occurred due to the formation of new compounds in the medium [17]. Enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly situated on their surface. The charges on these groups will vary, according to their acid dissociation constants, with the pH of their environment. This will affect the net charge of the enzymes and the distribution of charge on their exterior surfaces, in addition to the reactivity of the catalytically active groups. Taken together, the changes in charges with pH affect the activity, structural stability and solubility of the enzyme. Even small changes in pH may cause significant changes in the ability of a given enzyme to catalyze certain reactions. In a similar manner to the effect on enzymes, the charge and charge distribution on the substrate (S), product and co-enzymes will also be affected by pH. Extremes of pH will, however, cause a time and temperature dependence, with irreversible denaturation. pH of the media have profound effect on the biotransformation[18]. The differences in the extent of biotransformation at different pH may be due to either varying expression of enzymes or ease of assimilation of drug due to its uniform distribution/precipitation in the medium.

In the case of enzymatic reactions, many enzymes are adversely affected by high temperature. The reaction rate increases with temperature to a maximum level, then abruptly declines with further increase of temperature. As the temperature increases, molecular motion increases resulting in more molecular collisions. If, however, the temperature rises above a certain point, the heat will denature the enzyme, causing the protein to loose its three-dimensional functional shape due to denaturation of hydrogen bonds. Cold temperature, on the other hand, slows down enzyme activity

by decreasing molecular motion. At the same time growth of the microorganisms is also influenced by incubation temperature[9]. The differences in the extent of biotransformation at different temperatures may be due to increased or decreased activity of enzymes and growth of organism at different temperatures. Hydroxylation of 10-dexoartemisinin to 15-hydroxy-10-dexoxoartemisinin by *Aspergillus niger* was recorded at 28^0C[19].

Biotransformation of L-arginine to L-citrulline by *P.putida* was influenced by the concentration of glucose[20]. The differences in the extent of biotransformation at different concentration of dextrose may be either due to increased or decreased biomass (growth) of the organism or differences in the production of enzymes at increased/decreased biomass. Higher concentrations of dextrose are inhibiting the metabolite production. This indicates that higher concentrations of carbon source may be helpful in the growth of the organism but produce low levels of enzymes.

The differences in the extent of biotransformation at different concentrations of Silibinin may be due to either increased or decreased biomass (growth) of the organism or differences in the production of enzymes at increased/decreased biomass and the effects may be attributed to the enzyme saturation and K_m values. Biotransformation of ebastine is influenced by the concentration of substrate and 0.02% concentration of ebastine was optimum for its biotransformation by *C.blakesleeana*[21].

Of all the fungi studied *Cunninghamella echinulata* NCIM 691 showed maximum biotransformation of Silibinin. The metabolites formed are hydroxy and demethylated derivatives of Silibinin which are already reported by mammalian metabolism. As the metabolites formed by microbial cultures are similar to the metabolites produced by mammals, this tool of biotransformation using microbial cultures can be employed for predicting the mammalian metabolites in early stages of drug discovery and development.

CONCLUSION

Of all the fungi studied *Cunninghamella echinulata* NCIM 691 showed maximum biotransformation of Silibinin. The metabolites formed are hydroxy and demethylated derivatives of Silibinin which are already reported by mammalian metabolism. As the metabolites formed by microbial cultures are similar to the metabolites produced by mammals, this tool of biotransformation using microbial cultures can be employed for predicting the mammalian metabolites in early stages of drug discovery and development.

ACKNOWLEDGEMENTS

We would like to thank Dr. Mullangi Ramesh, Dr. Reddy Labs for providing LC MS-MS work done.

REFERENCES

1. Ferris JP, Fasco MJ, Stylianopoulou FL, Jerina DM, Daly JW, Jeffrey AM (1973) monooxygenase activity in *Cunninghamella bainieri*: Evidence for a fungal system similar to liver microsomes. Arc Biochem Biophy. 156(1): 97-103.
2. Smith RV and Rosazza JP (1975). Microbial models of mammalian metabolism. J Pharm Sci. 64(11): 1737-1758.
3. Dehmlow C, Erhard J, de Groot H (1996) Inhibition of Kupffer cell functions as an explanation for the hepatoprotective properties of Silibinin. Hepatology. 23(4): 749-754.
4. Dhanalakshmi S, Singh RP, Agarwal C, Agarwal R (2002) Silibinin inhibits constitutive and TNFalpha-induced activation of NF-kappaB and sensitizes human prostate carcinoma DU145 cells to TNFalpha-induced apoptosis. Oncogene. 21(11): 1759-1767.
5. Enjalbert F, Rapior S, Nouguier-Soule J, Guillon S, Amouroux N, Cabot C (2002) Treatment of amatoxin poisoning: 20-year retrospective analysis. J Toxicol Clin Toxicol. 40(6): 715-757.
6. Morazzoni P, Montalbetti A, Malandrino S Pifferi G (1993) Comparative pharmacokinetics of silipide and silymarin in rats. Eur J Drug Metab Pharmacokinet. 18(3): 289-297.
7. Zuber R, Modriansky M, Dvorak Z, Rohovsky P, Ulrichova J, Simanek V, Anzenbacher P (2002). Effect of silybin and its congeners on human liver microsomal cytochrome P450 activities. Phytother Res 16(7): 632-638.
8. Venisetty RK, Ciddi V (2003) Application of microbial biotransformation for the new drug discovery using natural drugs as substrates. Curr Pharm biotechnol. 4(3): 123-140.
9. Azerad R (1999) In Advances in Biochemical Engineering/Biotechnology – Microbial models for drug metabolism, (Scheper., Ed.), Vol 63. Springer Verlag, Berlin Hiedelberg. pp. 169-218.
10. Venisetty RK, Keshetty S, Ciddi V (2004). Optimization of surfactants and polymers to alter the morphology of matt forming fungal cultures, an improved way fungal

drug metabolism studies. Poster presented in 64th International Pharmaceutical Federation Congress in September 2004 held at New Orleans, USA.

11. Raj Kumar V, Srisilam K, Veeresham C (2004). Isocratic HPLC methods for the determination of silybin and its diastereomers. Oral presentation in 56th Indian Pharmaceutical Congress in December 2004 held at Kolkata, India.
12. Cha CJ, Doerge DR, Cerniglia CE (2001) Biotransformation of malachite green by the fungus *Cunninghamella elegans*. Appl Environ Microbiol 67(9): 4358-4360.
13. Huang H, Yang X, Li Q, Sun L, Zong D (2006) Biotransformation of tolbutamide to 4′-hydroxytolbutamide by the fungus *Cunninghamella blakesleeana*. Appl Microbiol Biotechnol. 72(3): 486-491.
14. Pasutto FM, Singh NN, Jamali F, Coutts RT Abuzar S (1987) Microbiological systems in organic synthesis: preparation of racemic prenalterol utilizing *Cunninghamella echinulata*. J Pharm Sci. 76(2): 177-179.
15. Abel AM, Carnell AJ, Davis JA, Paylor M (2003) The synthesis of buprenorphine intermediates by regioselective microbial *N*- and *O*-demethylation reactions using *Cunninghamella echinulata* NRRL 1384. Enzyme and Microbial Technology. 33(5): 743-748.
16. Kunz DA, Reddy GS, Vatvars A (1985) Identification of transformation products arising from bacterial oxidation of codeine by *Streptomyces griseus*. Appl Environ Microbiol. 50(4): 831–836.
17. Harshad R. V , Mohan R.H (2003) Biotransformation of (L)-citronellal to (L)-citronellol by free and immobilized *Rhodotorula minuta*. Electronic J Biotechnol. 6(2): Issue of August 15.
18. Silberbach M, Maier B, Zimmermann M, Buchs J (2003) Glucose oxidation by *Gluconobacter oxydans*; characterization in shaking-flasks, scale-up and optimization of the pH profile. Appl Microbiol Biotechnol. 62: 92-98.
19. Parshikov IA, Muralieedharan KM, Miriyala B, Avery MA, Williamson JS (2004) Hydroxylation of 10-deoxoartemisinin by *Cunninghamella Elegans*. J Nat Prod. 67: 1595-1597.
20. Kakimoto T, Shibatani T, Nishimura N, Chibata I (1971) Enzymatic production of L-citrulline by *Pseudomonas putida*. Appl Microbiol. 22(6): 992-999.
21. Schwartz H, Liebieg-Weber A, Hochstatter J, Bottcher H (1996) Microbial oxidation of ebastine. Appl Microbiol Biotechnol. 44(6): 731–735.

****