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Research Article

BIOTRANSFORMATION OF HESPERIDINE TO HESPERITINE BY *CUNNINGHAMELLA ELEGANS*

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ABSTRACT

The potential of various fungi to biotransform the flavonoid hesperidine was studied to get its active metabolite hesperitin. Eight fungi were screened for the ability to metabolize hesperidine in a manner comparable to humans with a view to develop alternative systems to study human drug metabolism and also to produce active metabolites in large quantities. In the present study, the ability of eight fungi was investigated to predict the pathway of hesperidine metabolism and identification of microbial metabolite along with its structural confirmation. Different fungal cultures were incubated with hesperidine using shaker incubator and samples were analysed by HPLC, metabolite structure was confirmed by LCMS, IR, NMR studies. Of the different fungi screened, *Cunninghamella elegans* showed an extra metabolite peak, at 3.39min. in HPLC compared to its controls, which was confirmed as hesperitine an active metabolite of hesperidine by LCMS(m/z-301.3), IR and NMR. It was found that the *Cunninghamella elegans* converted hesperidine to hesperitin by hydrolysis, which showed the similarity with the human metabolism of hesperidine. It can be concluded that hesperitine an active metabolite of hesperidine was formed by a fungus *Cunninghamella elegans*.

Keywords: Fungi, HPLC, LCMS, NMR.

INTRODUCTION

The approval and usage of drug in human subjects require extensive studies to establish its safety and efficacy. The important factor in the evaluation of safety and efficacy of any drug is the knowledge about drug metabolism[1]. The understanding of drug metabolism plays an important role in the development of new drug entities which can be further evaluated for pharmacological/toxicological activities. Metabolism is really a series of enzymatic transformation leads to chemical alteration of a drug that is, conversion of drug to more polar and hydrophilic metabolites to excrete easily from the body[2] compared to lipophilic substances. The formation of metabolite and its role in the body before excretion is also important to understand drugs safety and toxicity. The use of microorganisms as models of mammalian metabolism is well documented[3-6]. Many scientists have also published several reviews and updates on this topic[7,8]. Synthesis of metabolites in laboratories is tedious and costly process so micro organisms can be used as metabolic factories to produce metabolites. As integral constituents of the diet, flavonoids may exert a wide range of beneficial effects on human health, including protection against cardiovascular disease and certain forms of cancer. Recent studies have shown diverse physiological and pharmacological activities of these natural compounds. Thus in the present study a flavonoid hesperidine was selected to get its active metabolite easily and cheaply. Hesperidine is a flavonoid, present as glycoside in citrus fruits[9]. When ingested, it gets converted into an active metabolite hesperitin by hydolysis and further on conjugation with glucuronides and sulfates, produces hesperitin-7-O-β-D-glucuronide, hesperitin-3'-O-β-D-glucuronide, hesperitin-7-O-sulfate and hesperitin-3'-O- sulfate etc . The present study investigated the ability of different fungi to predict the pathway of hesperidine metabolism[10-13] and to get hesperitine.

MATERIALS AND METHODS

Microorganisms

Aspergillus terreus (NCIM 657), Aspergillus flavus (MTCC 1783), Aspergillus ochraceus (NCIM 1140), Cunninghamella elegans (NCIM 689), Cunninghamella blakesleeana (MTCC 3729), Cunninghamella echinulata (MTCC 4279), Rhizopus stolonifer (NCIM 880), Gliocladium roseum (NCIM 1064). The microbial cultures were obtained from National Chemical Laboratory (NCL), Pune and Microbial Type Culture Collection (MTCC), Chandigarh, India.

Chemicals

Hesperidine was obtained from Sigma, Mumbai, India. Solvents used were Methanol, Water (HPLC grade) and glacial acetic acid obtained from Merck, Mumbai, India. Culture media components, carbon and nitrogen sources obtained from Merck, Qualigens, SD fine chemicals, Mumbai, India.

Cultures

All the cultures (fungi) were maintained on the respective agar slants at 40C and transferred for every 6 months to maintain viability. Potato dextrose broth (Potato chips, 20 gm / 100 ml (steamed for 30min); dextrose, 2 gm ; yeast extract, 10 mg ; distilled water up to 100 ml; final pH 5.6), was used for *C.elegans,*C.*echinulata A.terreus, A.ochraceus, A.flavus, Gliocladium roseum and Rhizopus stolonifer*; Oat meal flakes, 3gm/100ml (steamed for 30min) for *C.blakesleeana*. The respective media was prepared and autoclaved in individual Erlenmeyer flask at 121^{0}C at 15 lb/sq. in for 30min. before incubation.

Microbial biotransformation

The fermentation was carried out in 250ml Erlenmeyer flasks containing 50ml media labelled as drug control, culture control and sample. For each biotransformation study two controls and one sample was used. The study included the drug control to which drug solution was added and incubated without organism. Culture control consisted of the medium inoculated under identical conditions with only loop full of respective fungus culture to find whether hesperidine would be chemically transformed during incubation period and to exclude any 2^0 metabolites generated by fungi. The two controls were run simultaneously with sample flask that consisted of both drug and loop full of culture. All the flasks were incubated on the orbital shaker for 24-48hrs, operated at 120rpm at 37^{0}C for biotransformation study. Extraction Procedure The incubated media was heated on a water bath at 45^{0}C for 30min and centrifuged at 3000rpm for 10min at 37^{0}C. Clear supernatant liquid was collected into boiling tubes, cotton plugged closely and stored in refrigerator. 20µl aliquot of the solution was injected into the HPLC for analysis.

Analysis

High Pressure Liquid Chromatography

High pressure liquid chromatography(HPLC) analysis was carried out using a HPLC system (Waters, USA) consisted of Waters 515 solvent delivery module and Waters 2489 UV-detector and a Wakosil II SC-18 rs-100ª, 5µl, 4.6 × 250mm stainless steel column. Sensitivity was set at 0.001 a.u.f.s. Mobile phase consisted of methanol: water (0.07% glacial acetic acid) in the ratio of 33:67 was used at a flow rate of 1ml/min. Elution was monitored using UV-detector set at 270nm. The metabolite peak was observed in the sample of *Cunninghamella elegans* in HPLC analysis was collected

from elute. It was evaporated to dryness under vacuum and the residue was sent for analysis of MS, IR and 1HNMR studies.

Mass Spectrometry

The sample of *Cunninghamella elegans* culture showed a metabolite peak at retention time of 3.39 min. in HPLC compared to that of their controls. This metabolite was collected from elute and was reconstituted with mobile phase for analysis by liquid chromatography mass spectrometry operating in the electron spray ionization (ESI) mode. Model used was Agilent 1100 Series LC/MSD. LC coupled to a mass spectrometer operating in the electron spray ionization (ESI) mode. Detector: Ion trap detector, Positive mode, Range: 50-700, Spray Voltage: 3.5 kV, Capillary temperature: 325°C, Nebulizer gas pressure: 40 psi. A mass spectrum was collected to obtain the (M-1) of drug and metabolite.

IR Spectrometry

The metabolite residue collected from HPLC elute of sample culture extracts of *Cunninghamella elegans* and pure hesperidine were subjected to IR analysis using BRUKER ALPHA-E.

^{1}HNMR Spectrometry

The metabolite structure was further confirmed by 1HNMR spectra by using BRUKER AVANCE 400 MHz. Dimethylsulfoxide and methanol were employed as solvents to analyze 1HNMR spectra of hesperidine and its metabolite.

RESULTS

HPLC

The results of HPLC analysis of hesperidine and its metabolite in different culture extracts are given in Table 1. The peak at retention times of 2.7min. represented the solvent peak and peaks at 1.6, 1.8min. represented various culture content peaks where as peak at 12.8min. corresponds to hesperidine. An extra peak at 3.39min. observed in sample of *Cunninghamella elegans* represented the formation of metabolite as shown in Fig.1. Further the structure of the metabolite was confirmed by LCMS, IR and 1HNMR studies.

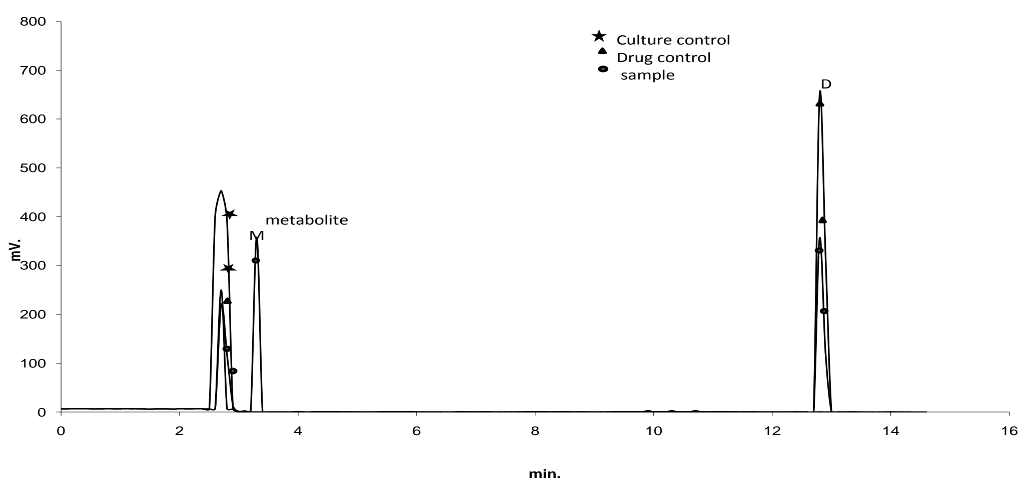

Fig. 1: HPLC chromatogram of hesperidine from culture extracts of *cunninghamella elegans*

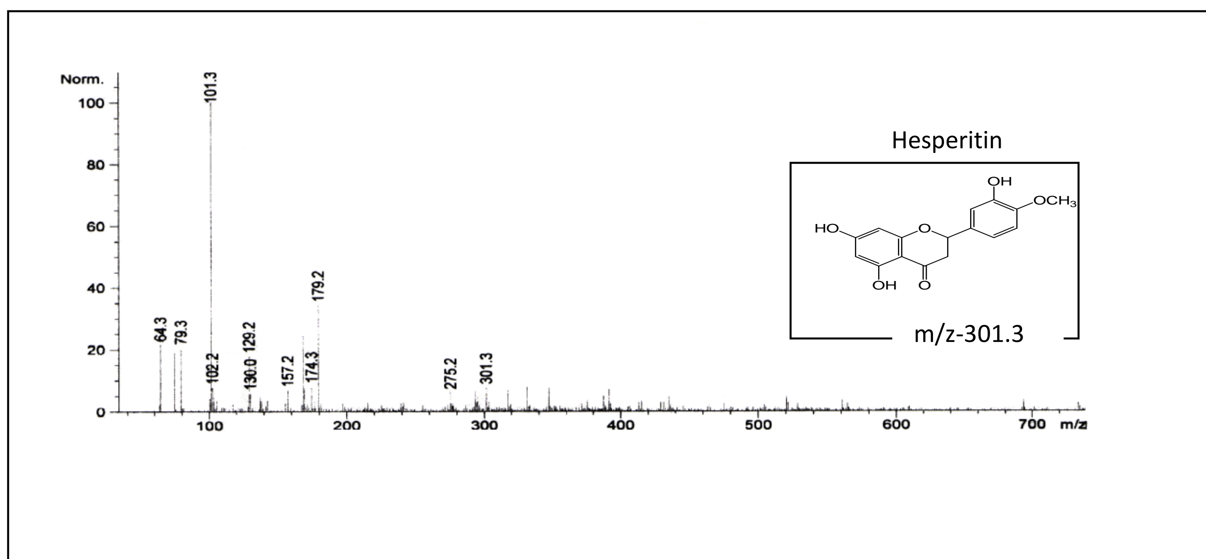

Fig.2: Mass Spectrum of Hesperidine Metabolite Produced By *Cunninghamella Elegans*

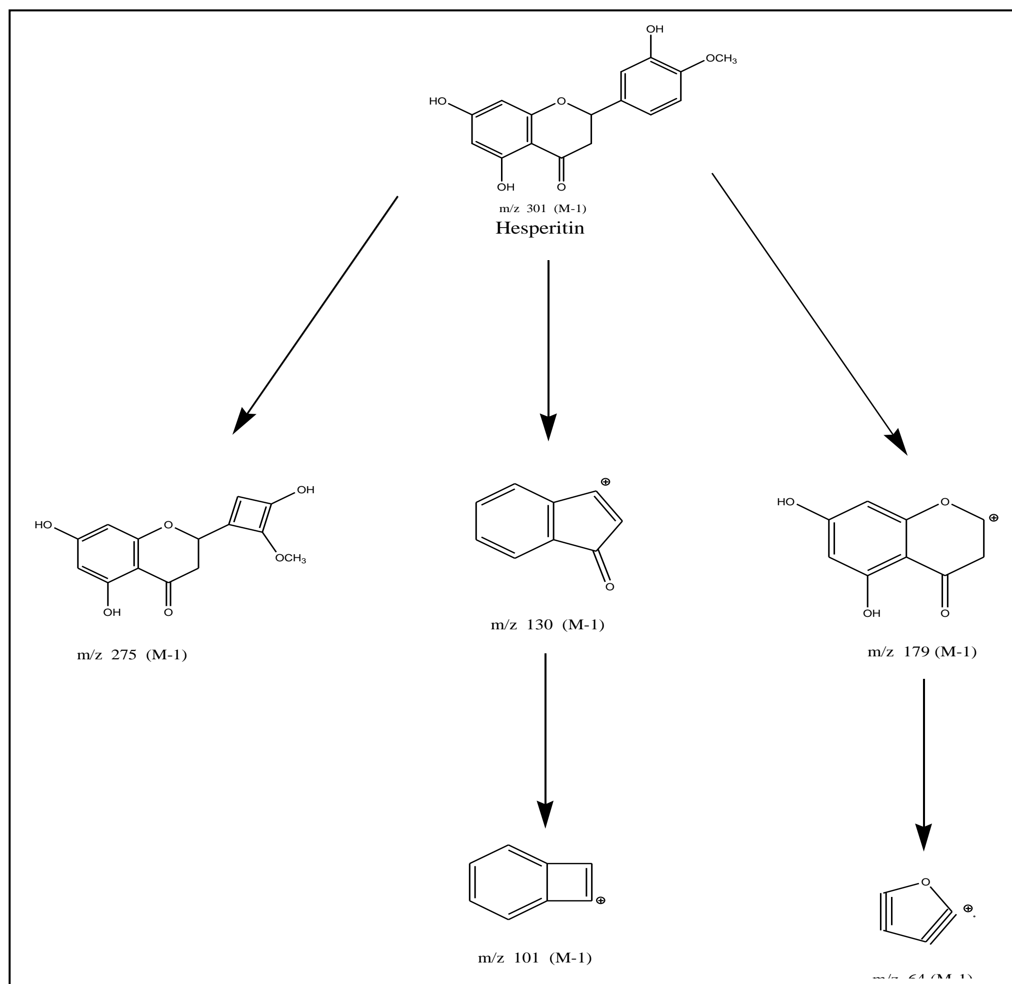

Fig. 3: Mass Fragmentation Pattern of Hesperidine Metabolite Produced By *Cunninghamella Elegans.*

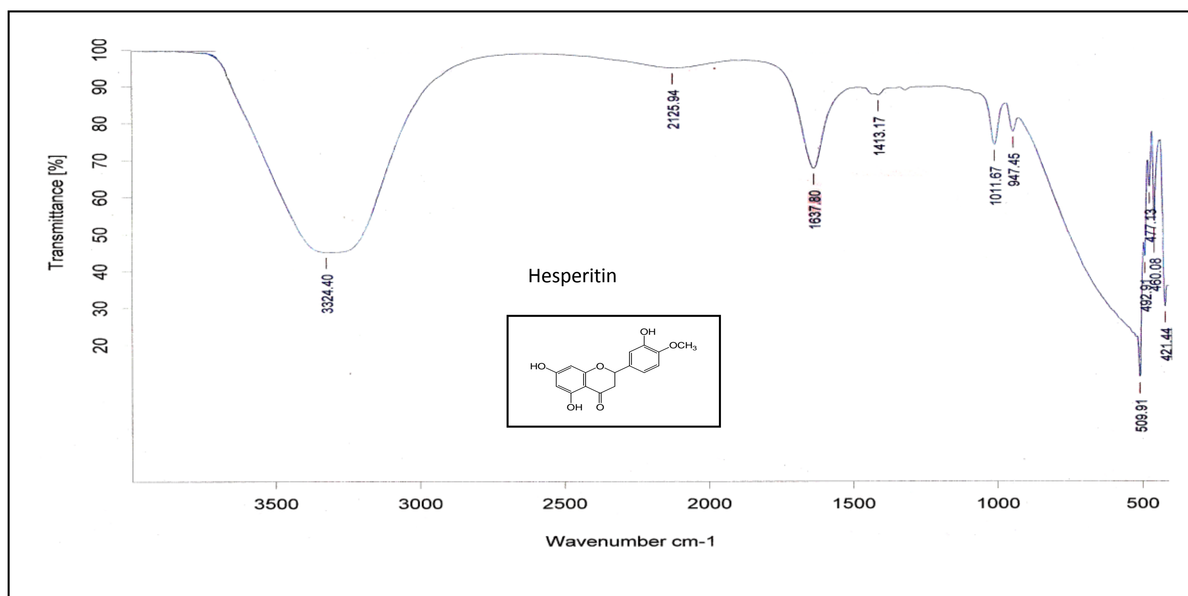

Fig.4: IR Spectrum of Hesperidine Metabolite Produced By *Cunninghamella Elegans*

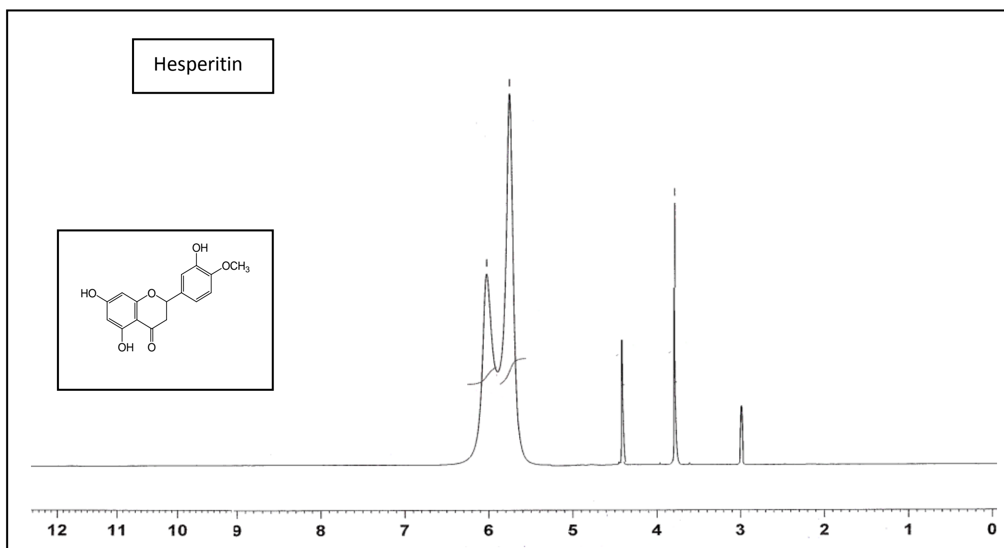

Fig.5: ^{1}HNMR Spectrum of Pure Hesperidine

Table 1: HPLC data of hespiridine and its metabolite from microbial culture extracts.

Name of the organism	Retention time (min.)			
	Blank I	Blank II	Pure hesperidine	Sample
Aspergillus terreus	-	1.6	-	1.6
	2.7	2.7	2.7	2.7
	12.8	-	12.8	12.8
Aspergillus flavus	-	1.6	-	1.6
	2.8	2.8	2.7	2.8
	12.8	-	12.8	12.8
Aspergillus ochraceus	-	1.6	-	1.6
	2.7	2.7	2.7	2.7
	12.8	-	12.8	12.8
Cunninghamella elegans	-	1.6	-	1.6
	2.7	2.7	2.7	2.7
	-	-	-	**3.39***
	12.8	-	12.8	12.8
Cunninghamella echinulata	-	1.6	-	1.6
	2.8	2.8	2.8	2.8
	12.8	-	12.8	12.8
Cunninghamella blakesleeana	2.7	2.7	2.7	2.7
	12.8	-	12.8	12.8
Gliocadiuum roseum	2.7	2.7	2.7	2.7
	12.8	-	12.8	12.8
Rhizopus stolonifer	2.7	2.7	2.7	2.7
	12.8	-	12.8	12.8

Liquid chromatography / Mass spectrometry (LCMS)

Metabolite eluted at retention time of 3.39min.in HPLC which was found only with the sample of *Cunnighamella elegans* was isolated and analysed by LCMS . The mass spectrum of pure hesperidine exhibited a molecular ion peak at m/z 609.3 (M-1) and is supported by a fragment ion peak at m/z 301.2 which is formed by cleavage of glycoside link in hesperidine. The mass spectrum of metabolite of hesperidine showed a molecular ion peak at m/z 301.3 (M-1) as in Fig.2, which is represented as hesperitin an active metabolite formed by hydrolysis of hesperidine, as in mammalian metabolic pathway. The structure of metabolite is supported by fragment ion peaks at m/z 275, 179, 130, 101, 64 as shown in Fig.2. Its Mass fragmentation pattern is shown in Fig.3. Its structure was further supported by IR and 1HNMR spectra.

Infrared Spectrometry (IR)

The metabolite hesperitine structure was confirmed by Phenyl –OH group in the band region of 3324.4 cm-1. carbonyl group with band at 1637.8 cm-1 and 1011.67 cm-1 represented C-O stretching in hesperitine. The IR spectrum of hesperidine metabolite was shown in Fig.4.

Proton Nuclear Magnetic Resonance Spectrometry (1HNMR):

The 1HNMR spectrum of hesperitine showed 3 protons, appeared as singlet due to –OCH3 group at δ 3.9. Methylene protons observed at δ 2.9, –OH group protons are represented at δ 4.6 Multiplet in the region of δ 6.4, is the indicative of aromatic protons conformed the structure of hesperitine as shown in the Fig.5.

DISCUSSION

In the present study, eight fungi were screened for their potential to metabolize hesperidine, a flavonoid to active metabolite hesperitine. *Cunninghamella elegans* showed an ability to convert hesperidine into hesperitin. In human hesperidine, a glycoside is converted to aglycon hesperitin which is an active metabolite, further may be converted to its respective conjugate metabolites. Based on the above results i.e., from HPLC, LCMS, IR and NMR, it was found that the *Cunninghamella elegans* converted hesperidine to hesperitin by hydrolysis, which showed the similarity with the human metabolism of hesperidine. This was supported by the degradation of the glycoside rutin by *Butyrivibro sp.* to its aglycon[9], microbial hydrolysis of solamargine to solasodine, a nitrogen analogue of diosgenin[14]. Hydrolysis of an antitumor agent Cristanol by *Cunninghamella*

elegans was also reported by Hufford et al.,[15]. From the present study it was concluded that the fungus *Cunninghamella elegans* has ability to metabolise hesperidine a flavonoid to its active metabolite hesperitine. Hesperidine and hesperitine exert a wide range of beneficial effects on human health, including protection against cardiovascular disease and certain forms of cancer. Conversion of hesperidine to hesperitine is easier with microbes than chemical processes in laboratory as Srisailam et al., 2003, 2010[16,17] and Xie et al., 2005[18] stated the microbial systems can produce (active) metabolites in large scale. So present study may enlight the production of active metabolite easily by microbes.

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