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Quantitative determination of 1-Deoxynojirimycin in different Mulberry Varieties of India

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Abstract
Mulberry is the chief food plant for silkworm further mulberry is also having many active pharmacokinetics principles. DNJ is one of the active pharmacokinetic principles with potent capability of inhibiting hyperglycemia. In our study, we screened some of the important mulberry varieties of south India to identify the DNJ content. Derivatized samples of each variety of mulberry leaves were analyzed by High Performance Liquid Chromatography (HPLC) with photodiode array detector. Derivatization of each sample was done by the 9-fluorenylmethyl succinimidyl carbonate. DNJ concentration varied between 0.68-2.72 mg/gm in different mulberry varieties (K-2, S-13, S-34, and V-1). The order of 1-deoxynojirimycin concentration in different verities was K-2 > S-13 > S-34 > V-1. Though V-1 variety was developed by breeding methods it did not contains more content of DNJ when compared to the K-2 which was developed by natural selection. As K-2 variety exhibited highest content of 1-deoxynojirimycin it can be exploited for extracting 1-deoxynojirimycin commercially for pharmaceutical purpose.

Keywords: Mulberry, K-2, V-1, DNJ, and HPLC

1. Introduction
Mulberry is a fast growing deciduous plant which grows from sea level to high altitude in various soil types. There are many numbers of species, varieties and improved cultivars throughout the world. The varieties such as K-2, V-1, S-13 and S-34 are popular cultivars in India particularly in south India under rainfed and irrigated conditions. K-2 and V-1 varieties are cultivated in Karnataka, Andhra Pradesh and Tamilnadu under irrigated conditions. These varieties were developed at CSRTI, Mysore and origin of K-2 was selection from natural variability and V-1 was a Hybrid from S30 x Ber C776. S-13 and S-34 varieties were cultivated in Karnataka, Andhra Pradesh and Tamilnadu under Rainfed conditions which were developed at CSRTI, Mysore and the origin was from selection of polycross (mixed pollen) progeny[1]. Different parts of mulberry are known for its pharmaceutical properties. Researchers found that various phytochemical extracts of mulberry are having pharmacokinetic active principles and they are used to cure different human diseases such as diabetes, obesity, inflammatory, neurological and allergic diseases. Mulberry leaves contain many bioactive compounds like as Moranolin (DNJ), moran, flavones, 2-arylbenzofuran, carotenoids, γ -aminobutyric acid, Polyphenols which play a significant role in Hypoglycemic activity, Anticancer action, Antioxidants action, Anti-inflammatory actions [2]. There are many polyhydroxylated alkaloids which were extracted like as piperidine, pyrrolidine, indolozidine[3]. Due to structural similarity to sugars, most of the polyhydroxylated play as antihyperglycemic which is inhibitory activity against glycosidase [4, 5].

1-Deoxynojirimycin 1, is one of the alkaloid iminosugar (Fig.1) which is found in mulberry leaves and some bacterial strains. Many Asian countries like China, Japan and Korea practiced to take tea of mulberry leaves used as antidiabetic. DNJ is a bioactive compound which suppresses the high blood glucose levels that prevent the diabetes mellitus. 1-Deoxynojirimycin or azasugar is a natural product which is firstly reported in 1976 in the root bark of Morus species [6]. Many sugar compounds contains nitrogen in their ring structure and isolated from mulberry, DNJ and many of its derivatives like as Fagomine (1, 2-dideoxynojirimycin), Iso-fagomine, 4-dideoxy-1, 4-imino-D-arabinitol, 1, 4-dideoxy-1, 4-imino-D-ribitol and act as glucosidase inhibitors [7]. DNJ and its derivatives were isolated from many plants and microbes but its concentration was highest in mulberry plants when compared to others [8-14].

1-DNJ is a piperidine alkaloid which is known for its potent α-glycosidase inhibition[4]. Many methods were developed by scientists to isolate 1-deoxynαojirimycin from *Morus alba*L. leaves. DNJ compound primarily known as moranoline was isolated from the root bark of the mulberry [6]. In the determination of polyhydroxylated alkaloids, trimethylsilyl (TMS) derivatization was used and followed by GC-MS to determine structural information of compounds. However for silylation removal of water from samples is essential and it is the main disadvantage of this method. Hence Liquid Chromatography was used as an alternative [15]. DNJ lacks chromophore in its molecule and is, therefore, difficult to detect by High Pressure Liquid Chromatography. Derivatization of sample is needed for sample analysis. For the analysis of DNJ from mulberry, 9-Fluorenylmethyl chloroformate (FMOC-Cl) was used for derivatization of the sample [9]. DNJ is an amine group containing compound. Primary and secondary amines react with 9-fluorenylmethyl chloroformate (FMOC-Cl) under mild conditions and make stable DNJ derivatives. FMOC moiety was added with dealkylation in the case of tertiary amines [16]. Derivatization is a process that is used for analysis of N-containing compounds and lacks a chromophore such as amino acids, peptides and other compounds [17-20]. In this process we carried out selective derivatization with FMOC-Cl and used reversed phase High Pressure Liquid Chromatography (HPLC) with UV/Fluorescence detectors. In 2003 a rapid and reliable method was developed for determination of 1-deoxynorijimycin from mulberry leaves for the stabilize of DNJ-FMOC by reducing the pH and also established a most adaptable quantitative compound extraction method for DNJ from the *Morus alba*leaves [9]. Hence the authors aimed to quantify the important compound 1-deoxynojirimycin in different Indian mulberry varieties namely K-2, V-1, S-13 and S-34.

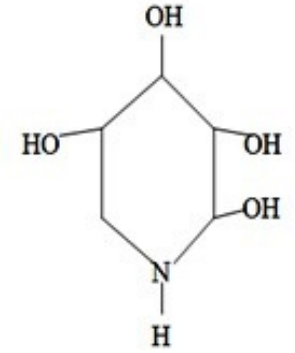

Fig 1: 1-Deoxynojirimycin

2. Material and methods

Leaves of Mulberry varieties (Fig.2) such as K-2, V-1, S-13 and S-34 were obtained from CSTRI, Mysore and Department of Ecology and environmental sciences, Pondicherry University, Puducherry, India and the leaves were cleaned and dried and prepared in powdered form. Hundred milligram powder of each mulberry variety was added to 10 ml aqueous 0.05 M HCl and kept in Ultrasonicator for 30 min and centrifuged at 10000 rpm for 20 min. The supernatants were filtered and then diluted and the supernatants were used for derivatization with FMOC-OSu (9-fluorenylmethyl succinimidyl carbonate) [9].

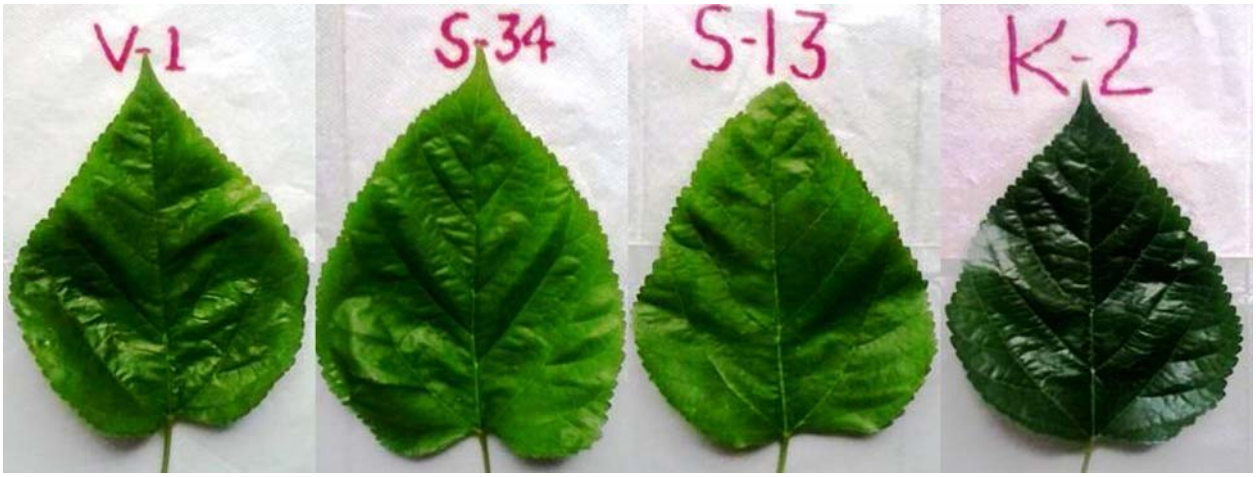

Fig 2: Leaves of Mulberry varieties V-1, S-34, S-13 and K-2

The chemical used were DNJ, FMOC-OSu (Sigma Aldrich) and acetonitrile, acetic acid and methanol (HPLC grade). The water used throughout the experiments was Milli Q water. Instruments and equipment used were HPLC system, Photo diode detector, C-18 detector, Vortex mixing instrument, High speed centrifuge, Ultrasonicator, Nylon syringe filter (0.22 um). High performance liquid chromatography conditions were Mobile phase- Acetonitrile: Aq. 0.1% acetic acid, 50:50 (v/v), Flow rate 1.0 ml/min, column temperature 25 °C and Photo diode detector. Excitation and Emission wavelengths were 254 nm and 322 nm respectively. Stock solution was prepared in HPLC grade water and stored at 4 °C.

2.1 Derivatization of standard and sample extract solution

10 µL of standard and sample extract were taken. Each sample was added with 10 µL of 0.5 sodium borate buffer (pH 8.5) in a microtube. After that 20 µL of FMOC-OSu acetonitrile solution was added, and vortexed for 25 seconds, then kept in water bath for 30 min at 25 °C. Then, 10 µL of 0.1 M Glycine was mixed to stop the reaction and the mixture was diluted with 950 µL of 0.1% aqueous acetic acid to stabilize DNJ-FMOC and the solution was filtered through 0.22 µm nylon

syringe filter. Finally, 10 µL of sample was injected into the HPLC system [9]. Stock solution (10 ppm) of DNJ was diluted with HPLC grade water and to provide a series of working concentrations of 0.50 ppm, 1.0 ppm and 2.0 ppm for constructing the calibration curve. The blank and standards of derivatized samples were analyzed under chromatographic conditions. Calculation method of HPLC was done by standard method [21] and statistical analysis was performed among the obtained DNJ content value by the Duncan's multiples range test [22]. The significance was calculated at 5% level ($P< 0.05$).

3. Results and Discussion

The results of standard solutions were shown in Fig.3 and Fig.4 and Fig.5. Fig.3 was the blank derivatized sample which was showing two peaks, Fig.4 standard derivatized sample of DNJ which was showing three peaks and Fig.5 was showing calibration curve for DNJ standard.

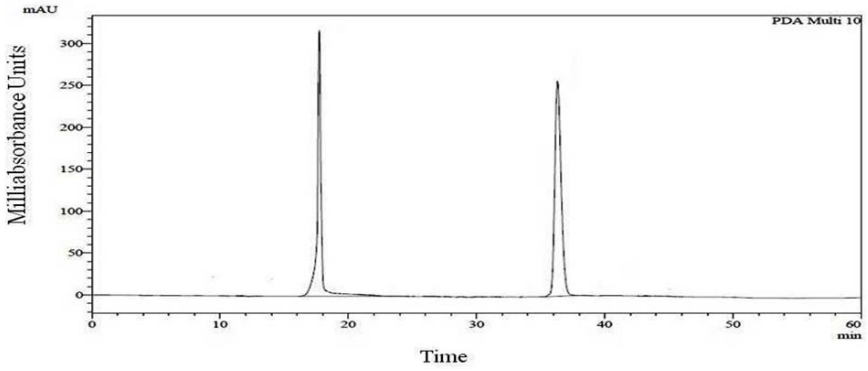

Fig 3: Chromatogram of blank (with Glycine and FMOC)

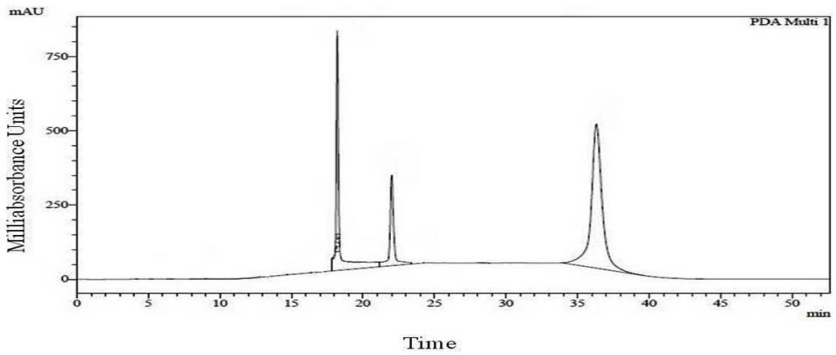

Fig 4: Chromatogram of DNJ standard (with Glycine and FMOC)

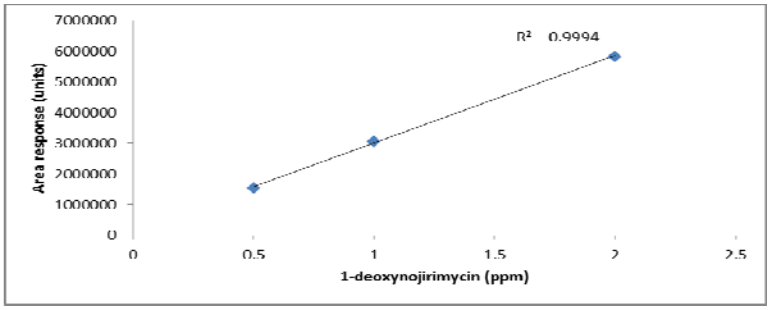

Fig 5: Standard curve for 1-deoxynojirimycin

3.1 1-DNJ determination in samples

Derivatized extract of mulberry leaves of each sample was injected into the HPLC system for the analysis of the DNJ content. Chromatogram of each samples were shown in Fig.6, Fig.7, Fig.8 and Fig.9 and results were shown in Table.1 and Fig.10

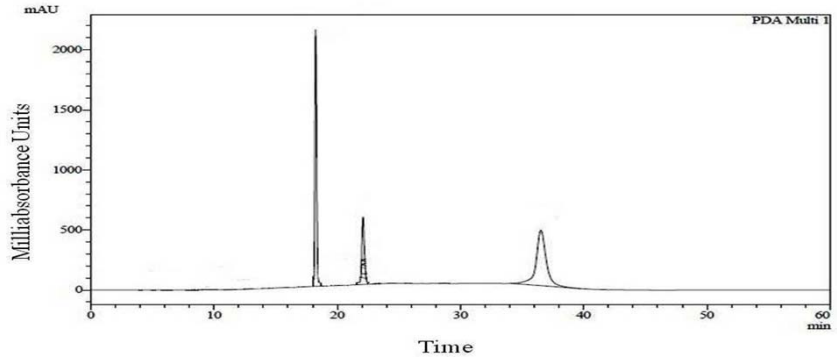

Fig 6: Chromatogram of DNJ of K2 mulberry leaves (with Glycine and FMOC)

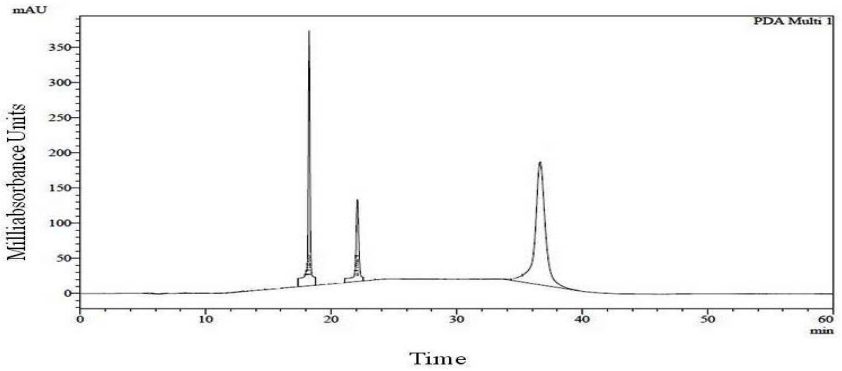

Fig 7: Chromatogram of DNJ of V1 mulberry leaves (with Glycine and FMOC)

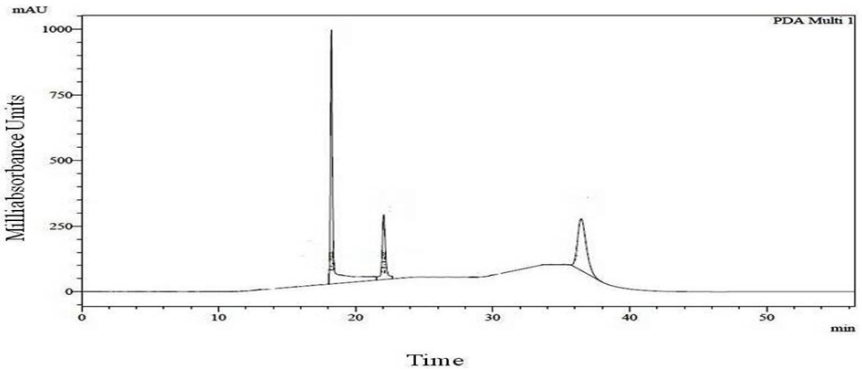

Fig 8: Chromatogram of DNJ of S13 mulberry leaves (with Glycine and FMOC)

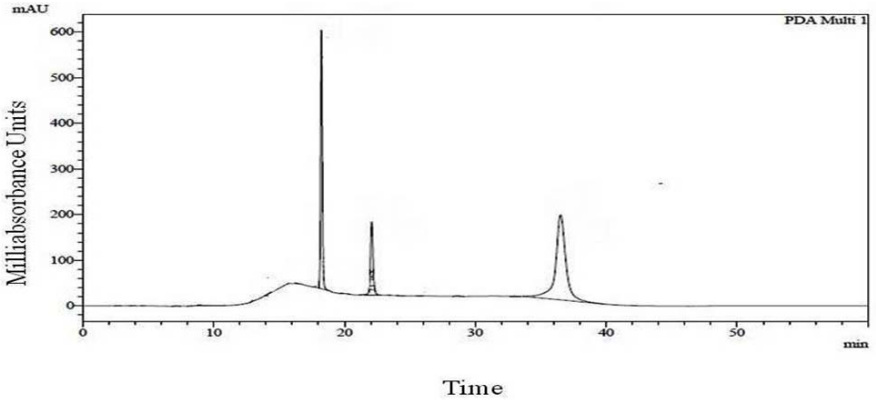

Fig 9: Chromatogram of DNJ of S34 mulberry leaves (with Glycine and FMOC)

The DNJ content of mulberry leaves of four varieties was shown in Table.1 and Fig.10. The variety with highest DNJ concentration was found in Kanva-2 (2.72 mg/gm) whereas the lowest content was found in V-1 (0.68mg/gm) variety.

Table 1: DNJ determination in different varieties of mulberry

S. No.	Name of varieties	Retention time	Area	Height	Area %	Height%	DNJ (mg/gm)
1	V-1	22.088	1963699	116678	9.501	12.808	0.68[a] ±0.05
2	S-34	22.073	2240418	160685	12.119	16.389	0.72[b] ±0.19 (+5.88)
3	S-13	22.044	4179044	247384	8.133	11.862	1.35[c]±0.04 (+98.52)
4	K-2	22.091	8548625	561712	12.288	14.958	2.78[d]±0.07 (+308.8)

*****Each value is a mean of six replicates estimations, Percent decrease/increase over is given in parenthesis. Means with in a column followed by the same letter not symmetrical different (P>0.05) from each other according to Duncan's multiple range tests.

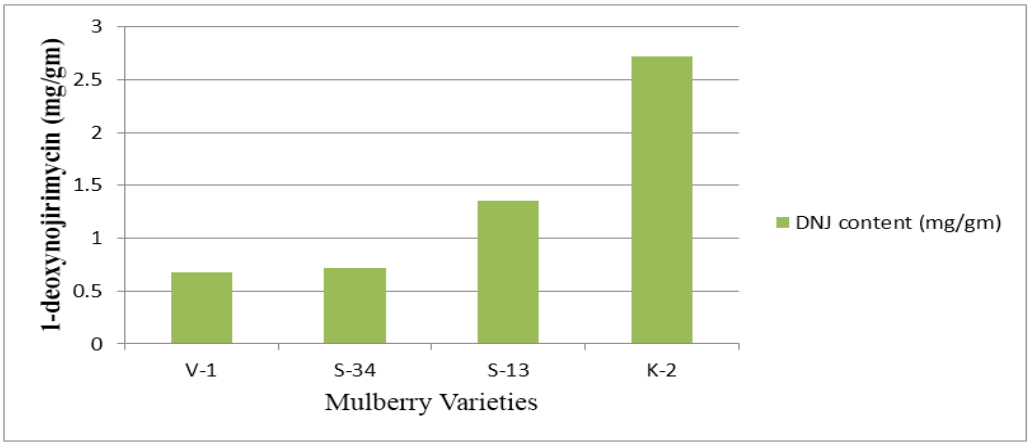

Fig 10: 1-Deoxynojirimycin content of mulberry leaves in four varieties

In our study, the DNJ content in different mulberry varieties varied from 0.68-2.78 mg/gm, which approximately coincided with the observed values by other researchers from different varieties and in different countries [10-14]. The DNJ content observed in different Chinese mulberry leaves was ranging from 1.57 to 3.48 mg/gm [12]. Some researchers also observed that leaf position in a branch is a factor of DNJ concentration [23]. Further it was suggested that the sequence of DNJ content was in the order of shoots> young leaves> mature leaves [23]. Other researchers found wide range of variation in mulberry plant organs and the sequence of concentration was observed as Branch Phloem> Leaves > Branch Xylem and the DNJ concentration was ranging from 0.40-4.9 mg/gm with the help of HPLC-PDA [24]. In our study we also used HPLC-PDA for the determination of DNJ from Indian mulberry varieties and observed the DNJ content. DNJ content which we observed was more or less similar to the earlier results obtained from Chinese varieties. However the results exhibited less content of DNJ in Indian varieties when compared to the Chinese varieties. We obtained significant variation in DNJ concentration among all Indian varieties. The order of DNJ content in different Indian mulberry varieties was K-2 > S-13 > S-34> V-1 and the concentration of DNJ was 2.72 mg/gm >1.35 mg/gm > 0.72 mg/gm > 0.68 mg/gm of dry leaves. DNJ concentration varied among the different varieties and exhibited significant difference among different varieties. From the above discussion it can be concluded that though V-1 variety was developed by breeding methods it did not contained more content of DNJ when compared to the K-2 which was developed by natural selection. It is also recommended that K-2 mulberry leaf can be used for extraction of DNJ at commercial level.

4. Conclusions

We quantified the 1-deoxynojirimycin with the help of HPLC-PDA in the leaves of four important mulberry varieties. The level of DNJ in the leaves was higher in the K-2 variety than other leaves of mulberry varieties.

5. Acknowledgements

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